Enhanced Resistance of Bone Marrow Transplanted Mice to Bacterial Infection Induced by Recombinant Granulocyte-Macrophage Colony-Stimulating Factor

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The in vivo effect of recombinant murine granulocyte-macrophage colony stimulating factor (rGM-CSF) on the resistance of mice to bacterial infection and on the number and function of neutrophils was studied in lethally irradiated mice transplanted with syngeneic bone marrow cells. Bone marrow transplanted (BMT) mice were injected intraperitoneally with 150 ng rGM-CSF or buffer solution (diluent) twice daily for 18 consecutive days. Total neutrophil recovery from the peripheral blood and the number of neutrophils mobilized into the peritoneal cavity were accelerated in rGM-CSF--treated recipients. Peritoneal neutrophils isolated from mice treated with rGM-CSF exhibited primed superoxide generation (O$_2^-$) after in vitro stimulation with suboptimal concentrations of phorbol myristate acetate (PMA), as compared with control mice (treated with diluent). No additional increase in O$_2^-$ production occurred upon in vitro incubation of these cells with rGM-CSF. The protective activity of rGM-CSF was examined in mice injected with Salmonella typhimurium. There was a 44- and 9-fold increase in the number of S. typhimurium at 96 hours postinfection in the spleen and liver, respectively, of control mice, as compared with rGM-CSF--treated mice, after a single injection of the bacteria (3 x $10^7$ per mouse). All the untreated control mice died within 14 days postinoculation ($1 x 10^7$ bacteria per mouse), whereas 35% of the mice treated with rGM-CSF remained alive for more than 30 days postinfection. These findings suggest the possible use of granulopoiesis and enhanced functional activity of phagocytic cells is induced by rGM-CSF and is responsible for enhanced resistance of BMT mice to bacterial infection.

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MATERIALS AND METHODS

Mice. Inbred male BALB/c mice, 6 to 8 weeks old, were used throughout this study. All mice were kept in conventional animal facilities.

Reagents. The murine rGM-CSF used in these studies was a generous gift from Dr. J. J. Mermod (Glaxo, Geneva, Switzerland). The production and purification of this CSF synthesized by E. coli has been described previously. Ferricytochrome C (type III) and superoxide dismutase (SOD; type I, 3000 U/mg protein) were obtained from Sigma Chemical Co, St Louis, MO. PMA was purchased from Consolidated Midland Corp, Brewster, NY. Iscove's modified Dulbecco's medium (IMDM) was from GIBCO, Grand Island, NY. Fetal calf serum (FCS) was purchased from Hyclone Lab, Logan, UT.

Irradiation, BMT, and injection schedule of rGM-CSF. Host mice were placed in plastic retainers and subjected to 9.5 Gy from a $^{60}$Co source at a dose rate of 94 Gy/min.

Donor mice were sacrificed and marrow cells were flushed from femur and tibiae with IMDM into sterile polystyrene tubes, and then dispersed into single cells by repeated aspiration through a
22-gauge needle. Viable nucleated cells (1 x 10^6) in 0.3 mL saline pyrogen-free were injected into the tail vein of irradiated syngeneic recipients 2 hours after irradiation.

Mice were injected intraperitoneally (IP) with 0.2 mL of diluent or saline containing 150 ng rGM-CSF twice daily at 8:00 am and 5:00 pm. The first injection was performed 24 hours after BMT. Mice were injected with rGM-CSF for 18 consecutive days.

Hematologic evaluations. At various times after BMT (as indicated in "Results") peripheral blood obtained from the retroorbital veins of the mice was collected in heparinized (44.7 μL) capillaries and counted by a Coulter counter (model S+; Coulter Electronics, Hialeah, FL). A differential cell count (100 cells counted) was taken on a Wright-Giemsa-stained cytocentrifuge preparation from four mice per group in each of three experiments.

Assay of peritoneal neutrophils. On day 17 post-BMT, control or rGM-CSF-treated mice were injected IP with 2 mL calcium caseinate (0.2% wt/vol in a solution of 0.168 mol/L NaCl, pH 7.2) followed by a second injection 15 hours later. Mice were sacrificed 3 hours after the second injection, and peritoneal cells were collected after injection of 5 mL of isotonic saline. Exudates containing a large number of contaminating erythrocytes were discarded. The peritoneal exudate cells were washed three times with phosphate-buffered saline (PBS) at 4°C. The cells were found to comprise greater than 85% neutrophils as estimated by differential counts of stained cells.

Assay of NADPH-dependent O_2^- production. Generation of O_2^- was measured by determining the rate of SOD-inhibitable ferricytochrome C reduction, essentially as described by Pick and Mizel. In brief, peritoneal neutrophils (mobilized from normal mice or BMT mice treated with rGM-CSF or diluent) were suspended in 160 μmol/L solution of ferricytochrome C in Hank’s balanced salt solution (HBSS). The cell suspension (1 x 10^6 cells/mL) was plated in 96-well flat-bottomed tissue culture plates (Falcon, Oxnard, CA), 100 μL per well. The cells were preincubated with 6 ng/mL rGM-CSF or diluent for 2 hours, after which 5 μmol/L PMA was added, and the cells were incubated for various periods of time (30 to 60 minutes) at 37°C. At the end of the incubation, the plates were transferred to a Multiskan apparatus, (Flow Laboratories, McLean, VA), and the absorbance was read at 550 nm against a blank of cytochrome C incubated for the respective time at 37°C in the absence of cells. The amount of O_2^- produced per well was calculated from the extinction coefficient for the absorption of reduced cytochrome C, minus oxidized cytochrome C, as read at 550 nm, by the equation ΔE_{550} = 2.1 x 10^6 (mol/L) x 1 cm^-1. Results were expressed as nanomoles of O_2^- per 10^6 cells per minute. Specificity of cytochrome C reduction was verified by its elimination in the presence of 300 units/mL of SOD.

Assessment of therapeutic activity of rGM-CSF to bacterial infection. The in vivo therapeutic effect of rGM-CSF was assessed in mice infected with S typhimurium on day 9 after BMT. For infection, mice were inoculated IP with 1 x 10^7 or 3 x 10^7 S typhimurium suspended in 0.3 mL of saline. These inocula were prepared by diluting fresh bacteria cultures with saline to an appropriate optical density. The infected mice continued to be treated with rGM-CSF or diluent according to the injection schedule, as described above. Eight mice were sacrificed 48 and 96 hours post-bacterial infection. Spleen and liver of each mouse were homogenized separately in a loose glass homogenizer. Dilutions of the homogenized tissues were seeded in trypticase soy agar (in duplicates). Plates were incubated at 37°C for 24 hours and the number of colonies was determined. The number of bacteria per organ was calculated. Results are expressed as mean ± SE of two experiments containing four mice per group per experiment. The percentage of survivors relative to the total number of infected mice was determined daily.

Statistical analysis. Statistical analyses were performed using the Student's t test and paired t test. Results were considered significant when P was <.05.

RESULTS

Peripheral blood changes. Since the ability to defend against bacterial infections is dependent on the number as well as the activity of polymorphonuclear leukocytes, a study was conducted on the ability of rGM-CSF to restore leukocyte counts in lethally irradiated mice after BMT. Peripheral blood white cell counts were performed on lethally irradiated mice after BMT, and twice daily IP injections of 150 ng rGM-CSF were given for 18 sequential days. As shown in Fig 1, a marked increase in total viable cell counts was observed in rGM-CSF-treated mice on days 8 to 18 post-BMT (at which time the experiments were terminated) as compared with control mice. On day 11 post-BMT, the level of neutrophils in control and rGM-CSF treated mice was 1.12 ± 0.1 versus 2.17 ± 0.2 x 10^9/L, respectively. The 2.5-fold elevation in leukocyte counts observed on day 15 post-BMT resulted from a fourfold elevation in neutrophil levels, the only significant change observed. No significant effect was observed on the number of eosinophils or monocytes in the blood (data not shown).

Effect of rGM-CSF on the number and function of peritoneal neutrophils. BMT mice were injected twice daily with 150 ng rGM-CSF or diluent for 18 days. On day 17 the mice were injected with 2 mL calcium caseinate. Three hours after the second injection (on day 18) the number and function of the peritoneal mobilized neutrophils were determined. The average neutrophil count per mouse was 119.5 x 10^9 and 68.3 x 10^9 for rGM-CSF and control mice, respectively (mean ± SE of four experiments, five mice per group in each experiment).

Assays were performed on the ability of peritoneal mobilized neutrophils to produce O_2^- in response to suboptimal concentrations of PMA (5 μmol/L). As can be seen in Fig 2,
with diluent for the same period of time (55 ± 0.5 nmol/10⁶ cells/30 minutes versus 25 ± 0.4 nmol/10⁶ cells/30 minutes). No increase in O₂⁻ production occurred upon in vitro incubation of neutrophils isolated from rGM-CSF injected mice, with 6 ng/mL GM-CSF and 5 nmol/L PMA. Preliminary studies have shown that in vitro maximum priming of peritoneal neutrophils for O₂⁻ production in the presence of PMA occurs upon preincubation of the cells with 6 μg/mL rGM-CSF for 2 hours. Therefore, this concentration of rGM-CSF was chosen in the present experiments.

Effect of rGM-CSF on bacterial infection in BMT mice. The protective activity of rGM-CSF was examined in BMT mice infected with various doses of S typhimurium. In these experiments a single IP injection of 1 × 10⁷ or 3 × 10⁷ S typhimurium was given on the ninth day after BMT. rGM-CSF or diluent were injected twice daily according to the schedule.

As can be seen in Table 1, at both challenge doses (1 × 10⁷ and 3 × 10⁷ bacteria), mice receiving rGM-CSF showed enhanced antibacterial resistance. At 48 hours postinfection with 3 × 10⁷ bacteria per mouse, an 11- and 14-fold increase in the number of S typhimurium developed in the spleen and liver, respectively, of control mice as compared with rGM-CSF–treated mice. The protective effect of rGM-CSF could be observed also 96 hours postinfection (with the higher challenge dose), at which time a 44- and 9-fold increase in the number of bacteria developed in the spleen and liver of control versus rGM-CSF–treated mice.

rGM-CSF showed a protective effect against infections induced by S typhimurium (Fig 3). At the lower challenge dose of bacteria, all infected mice died within 14 days postinfection, while 35% of rGM-CSF–treated mice survived over 30 days. With the higher challenge dose, 10% of rGM-CSF–treated mice survived over 30 days, while all control mice died within 8 days postinfection.

**DISCUSSION**

The findings of our study demonstrate that rGM-CSF is a potent inducer of antibacterial resistance in bone marrow transplanted mice. This enhanced resistance was paralleled by increases in the number of circulating neutrophils in the peripheral blood, as well as in the overall pool of neutrophils.
GM-CSF ENHANCES RESISTANCE TO BACTERIAL INFECTIONS

Neutrophils are essential for host defense against microbial infections. A drop in their number or functional activity may lead to severe infections. Recently, GM-CSF has been used to shorten the period of neutropenia after autologous BMT, to promote hematopoietic recovery after cytotoxic chemotherapy in patients with malignancy, and to enhance neutrophil function in acquired immunodeficiency syndrome patients. Our study shows that neutrophils of BMT mice treated in vivo with rGM-CSF are primed for enhanced superoxide release when stimulated with PMA. This finding is in agreement with results reported by Kaplan et al and Sullivan et al in preliminary human clinical trials, and by Welte et al in studies conducted on monkeys.

In primates treated with rGM-CSF, Mayer et al obtained enhanced oxidative metabolism and also a toxic effect on an E. coli strain as assessed in vitro. To the best of our knowledge, ours is the first report on the effect of rGM-CSF on the development of a bacterial infection in BMT mice and on host resistance to microbial infections.

It should be noted that Minami et al and McIntyre et al have recently found that interleukin-1 enhances antibacterial resistance in mice. Also, Cohen et al have reported on resistance of neutropenic hamsters to bacterial infections after their daily dosing with recombinant human granulocyte colony-stimulating factor (G-CSF), while Matsumoto et al found that in neutropenic mice subjected to intraperitoneal infection with bacteria or yeasts, G-CSF exerted a protective effect against systemic infections with these microorganisms.

In vivo studies have indicated that intraperitoneal injection of rGM-CSF into mice results in a rise in peritoneal macrophages, eosinophils, and neutrophils, and that the peritoneal macrophages were larger than resting macrophages, or other leukocytes, and might in part have been due to interactions with other factors or indirect effects, our findings indicate that rGM-CSF could be efficacious in limiting the duration of neutropenia and possibly also in mitigating the risk of the development of bacterial infections associated with bone marrow suppression.

REFERENCES


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