RECOMBINANT HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR

Effects on Hematopoiesis in Normal and Cyclophosphamide-treated Primates

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Human granulocyte colony-stimulating factor (G-CSF),[1] produced by the bladder carcinoma cell line 5637, has been purified to apparent homogeneity (1) and molecularly cloned (2). G-CSF purified from both the 5637 cell line and *Escherichia coli* support the growth of predominantly neutrophil colonies in a colony-forming unit of granulocytes/macrophages (CFU-GM) assay, and in the presence of accessory cells, support the growth of early erythroid progenitors burst-forming unit of erythrocytes (BFU-E) and pluripotential progenitors (CFU-GEMM) (granulocytes, erythrocytes, monocytes, and macrophages) (1–3). G-CSF is also capable of promoting the differentiation of some myeloid leukemic cell lines (e.g., HL-60, WEHI-3B-D$^+$) and fresh myeloid leukemic cells (1–3), and has been reported to enhance the chemotactic peptide binding on peripheral blood neutrophils (3). In addition, G-CSF can significantly increase the ability of neutrophils to kill tumor targets in vitro through antibody-dependent cellular cytotoxicity (ADCC) (4). Initial in vivo results with recombinant human G-CSF (rhG-CSF) in hamsters indicate a specific action on the neutrophil lineage with increases of three- to sixfold in peripheral blood neutrophils (5). We report here the in vivo effects of human rhG-CSF in normal and cyclophosphamide (CY)-treated cynomolgus monkeys (cynomolgus macaque, *Macaque fasicularis*).

Materials and Methods

Animals. Primates were obtained from Hazelton Research Animals, Inc., Reston, VA. Animals used in this study were maintained according to National Institutes of Health guidelines for the use and care of laboratory animals, and protocols approved by the Memorial Sloan-Kettering Cancer Center Institutional animal use and care committee. For administration of rhG-CSF, or bone marrow or blood sampling, the animals were anesthetized with ketamine hydrochloride (Parke-Davis, Morris Plains, NJ).

G-CSF Preparation. rhG-CSF expressed in *E. coli* was purified as described previously

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[1] *Abbreviations used in this paper:* ANC, absolute neutrophil count; CSF, colony-stimulating factor; CY, cyclophosphamide; GEMM, granulocyte/erythrocyte/monocyte/macrophage; GM, granulocyte/macrophage; NBT, nitroblue tetrazolium; WBC, white blood cell.

(2, 5). The homogeneous protein had a concentration of 0.5 mg/ml in 10 mM sodium acetate buffer, pH 4.1. Before injections, rhG-CSF was diluted to the appropriate concentration in a final volume of 250 μl in saline containing 0.1% heat-inactivated autologous serum. Endotoxin levels were determined by the Limulus amebocyte lysate assay (Whittaker M. A. Bioproducts, Walkersville, MD) and were undetectable. The CSF activity of the protein (10^8 U/mg) was determined as described (5). The rhG-CSF was administered subcutaneously into alternate thighs.

Neutrophil Function Assays. Peripheral blood neutrophils were separated from heparinized blood by 3% gelatin sedimentation followed by fractionation over discontinuous Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) gradients as described (6), and neutrophils were recovered from the 75% layer. The cell concentration was adjusted to 10^6 cells/ml.

Chemotaxis was measured using a modification of the method of Boyden (7). Briefly, blind well chambers were filled with zymosan (Sigma Chemical Co., St. Louis, MO) in HBSS, covered with 5-μm-pore membrane filter (Millipore Corp., Bedford, MA) and the top chamber filled with 2×10^5 neutrophils in HBSS containing 2% BSA (Sigma Chemical Co.). After incubation (3 h, 37°C), the filters were fixed, stained with hematoxylin, and cleared in xylene. The number of neutrophils crossing the filter (lower surface) were counted using a light microscope (×400).

Nitroblue tetrazolium (NBT) reduction was tested according to a method described by Gifford (8). Briefly, neutrophils were separated from a single drop of blood by their propensity to stick to glass, and were exposed to NBT and counterstained with nuclear fast red. Percentage of cells containing intracellular blue formazan particles were counted using a light microscope.

Results and Discussion

Effects of rhG-CSF in Healthy Monkeys. The effects of rhG-CSF on hematopoiesis in vivo were determined initially in two monkeys receiving 10 μg/kg/d divided in two daily subcutaneous injections for 2–4 wk. The white blood cells (WBC) count and absolute neutrophil count (ANC) of a rhG-CSF-treated monkey and a control buffer–treated monkey are shown in Fig. 1*A*. The WBC count in the treated monkey increased dramatically 24 h after initiation of treatment, and by day 6 reached a plateau between 4.2×10^4 and 5.6×10^4 cells/mm^3, with an ANC of between 3.1×10^4 and 4.2×10^4 cells/mm^3. This WBC level was maintained throughout the remaining 3 wk of treatment. There was no significant change in the WBC or ANC of the control animal during the same treatment period (Fig. 1*A*). The second animal treated at 10 μg/kg/d for 14 d showed a WBC increase from 9×10^3 cells/mm^3 to a level between 4.86×10^4 and 5.01×10^4 cells/mm^3 (Fig. 1*B*). The pattern of response to rhG-CSF in both monkeys receiving 10 μg/kg/d was very similar (Fig. 1, *A* and *B*). To assess the dose response to rhG-CSF, the second monkey subsequently received a dose of 100 μg/kg/d for an additional 2 wk (Fig. 1*B*). At 100 μg/kg/d, the WBC count ranged between 8.0×10^4 and 1.20×10^5 cells/mm^3. In addition, a third monkey was treated at 1 μg/kg/d for 15 d and exhibited a WBC plateau ranging from 1.8×10^4 to 2.9×10^4 cells/mm^3 (Fig. 1*B*). These data indicate a dose-dependent increase in WBC and ANC. In both monkeys the WBC count returned to normal levels 3 d after rhG-CSF treatment (data not shown).

Analysis of the absolute numbers of peripheral blood neutrophils, lymphocytes, monocytes, erythrocytes, eosinophils, reticulocytes, and platelets was performed every other day. The increase in WBC was predominantly due to the increase of absolute numbers of neutrophils (Fig. 1*A*). The absolute numbers of eosinophils

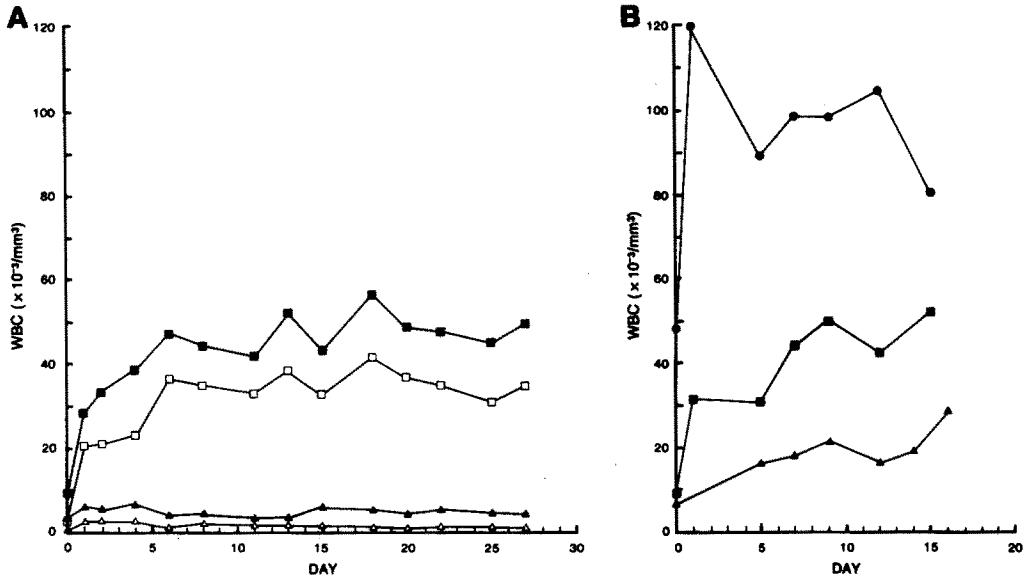

FIGURE 1. Time course of peripheral blood WBC and ANC of healthy cynomolgus monkeys treated with rhG-CSF and a control monkey receiving only buffer. (*A*) WBC level (■) and ANC (□) of a healthy monkey treated with 10 μg/kg/d rhG-CSF subcutaneously in two daily doses. The WBC level (▲) and ANC (△) of the control monkey is also shown in *A*. (*B*) Effects of different concentrations of rhG-CSF: (▲), 1 μg/kg/d; (■), 10 μg/kg/d; and (●), 100 μg/kg/d. Note that the monkey receiving 10 μg/kg/d was subsequently treated with 100 μg/kg/d, and therefore day 0 of 100 μg/kg/d corresponds to day 16 of 10 μg/kg/d.

and monocytes did not change significantly. There was a dose-dependent increase in the absolute number of lymphocytes (1.5–2.5-fold) in monkeys receiving 10 μg/kg/d or 100 μg/kg/d. The majority (~80%) of peripheral blood lymphocytes were found to be T cells, as assessed by reactivity with the anti-human T cell antibody T11. No significant changes in erythrocyte, reticulocyte, or platelet counts or hemoglobin concentrations were noted.

The monkey receiving 10 μg/kg/d for 28 d and the control monkey were sacrificed on day 29. Before sacrifice, peripheral blood smears were made and stained (Giemsa) for differential cell counts (Fig. 2*A*). Neutrophils predominated in the blood smear from the rhG-CSF-treated monkey. Immediately after sacrifice, sternal bone marrow specimens were taken for histologic examination, serially dehydrated, and embedded in plastic for thin sectioning. Sections of the bone marrow from the treated monkey demonstrated hypercellularity and a predominance of neutrophil precursors (Fig. 2*B*). The numbers of myeloblasts, promyelocytes, myelocytes, and bands were increased 5–10-fold over those in the specimen from the control monkey.

Extramedullary Hematopoiesis. The spleen of the treated monkey was not increased in size or weight, but showed prominent white follicles on the subcapsular and cut surfaces. Histologically, the internodular areas were filled with mature granulocytes and foci of extramedullary myelopoiesis, demonstrating myeloblasts and myelocytes. Generally lymph nodes were increased twofold in size due to lymphoid hypercellularity, and occasional foci of extramedullary

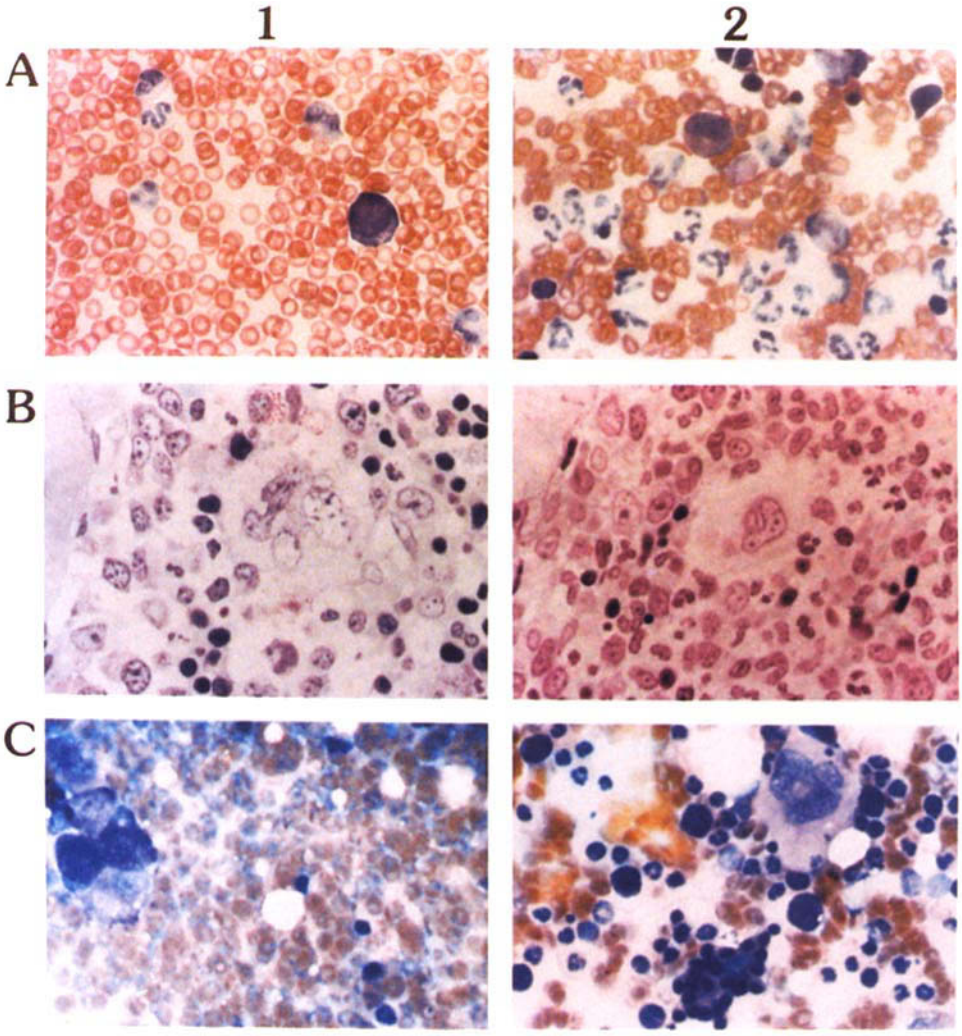

FIGURE 2. Effect of rhG-CSF on normal and CY-treated monkeys. Peripheral blood smears, bone marrow smears and bone marrow biopsy specimens were prepared and stained according to the May-Grunwald-Giemsa technique. (*A*) Peripheral blood smears; (*1*) from a control monkey, demonstrating normal numbers and distribution of cells; and (*2*) from a monkey treated with 10 μg/kg/d of rhG-CSF for 28 d, showing a significant neutrophilia. × 400. (*B*) Marrow biopsies showing normal proportions of erythroid and other myeloid elements from an untreated monkey (*1*) and predominance of granulocytes and their precursors in an rhG-CSF treated monkey (*2*). × 500. (*C*) Monkey bone marrow aspirates taken 2 wk after treatment with CY. Hypocellularity is present in the specimen from the monkey that received only CY (*1*), while the bone marrow smear from a similarly treated monkey, also given rhG-CSF starting on the third day after the second dose of CY, shows normal cellularity, with all elements present and a predominance of granulocytes and their precursors (*2*). × 400.

myelopoiesis (bands, myelocytes, promyelocytes, and myeloblasts) were observed. The thymus was moderately enlarged, and rare foci of myelopoiesis were noted. No other organs (e.g., liver, kidney, etc.) demonstrated evidence of hematopoietic activity. No toxicity was seen by clinical or laboratory examination.

TABLE I
Peripheral Blood Neutrophil Functions of rhG-CSF-treated Monkeys

In vivo treatment	n	Chemotaxis*		NBT reduction‡
		With zymosan	Without zymosan	
		cells/field		%
rhG-CSF	2	126 ± 14	7.6 ± 4.8	73 ± 14
None	4	109 ± 39	16 ± 9.0	35 ± 5.0

Peripheral blood neutrophil functions of control and rhG-CSF (≥10 μg/kg/d) treated monkeys. Peripheral blood neutrophils were isolated from monkeys on the 21st day of treatment, and separated as described (6).

* Chemotaxis is measured as the number of neutrophils (mean of five random fields) crossing a 5-μm filter using zymosan as the chemoattractant. The results are shown as mean ± SD, with or without zymosan as the chemoattractant.

‡ The percentage of neutrophils (≥100 cells examined) containing intracellular blue formazan particles.

Neutrophil Functions. In view of the dramatic peripheral blood neutrophilia in animals receiving rhG-CSF, we measured the neutrophil activities in vitro on the 21st day of treatment. As shown in Table I, the neutrophils of treated monkeys functioned normally in a standard test for chemotaxis. Neutrophils were then tested for their ability to reduce NBT as measured by the percentage of cells containing intracellular blue formazan particles. ~73% of the neutrophils recovered from treated animals contained intracellular blue formazan particles, whereas only 35% of the cells from the control animal contained such particles (Table I). These results indicate that the expanded pool of neutrophils in treated animals are functionally normal and may have an enhanced ability kill to phagocytized bacteria.

Effects of rhG-CSF in CY-treated Monkeys. To test the effect of rhG-CSF in chemotherapy-induced cytopenia, we treated three monkeys with CY (60 mg/kg/d, two doses). This dosage leads to bone marrow aplasia and peripheral blood pancytopenia by day 4–7 after treatment. Two of the three monkeys also received rhG-CSF (10 μg/kg/d), while the third received only CY. As shown in Fig. 3, one monkey received rhG-CSF for 14 d, starting 72 h after the last dose of CY. By the day after the first day of rhG-CSF, the WBC count in this monkey rose from 2.3×10^3 to 7×10^3, and then fell to 4×10^3 and 3.7×10^3 in the subsequent 2 d. This brief rise in the WBC was most likely due to demargination of neutrophils. A second monkey was treated with rhG-CSF for 6 d before CY, during the CY administration, and for an additional 21 d. This monkey acquired a peripheral WBC level of 5.23×10^4 cells/mm^3 before CY treatment. The first dose of CY did not lead to an expected decrease of the peripheral WBC, but rather was followed by a continued increase to 7.1×10^4 cells/mm^3. The WBC counts declined in all three monkeys to a level under 2.5×10^3 cells/mm^3 by day 4 after CY, and in the rhG-CSF-pretreated monkey, to a brief and transient nadir of 200 WBC by day 6. In both rhG-CSF-treated monkeys, the peripheral WBC increased dramatically by day 6–7 after CY, reaching levels of $\sim 5.0 \times 10^4$ WBC/mm^3 by the 10th day after CY. These WBC levels are similar to those seen

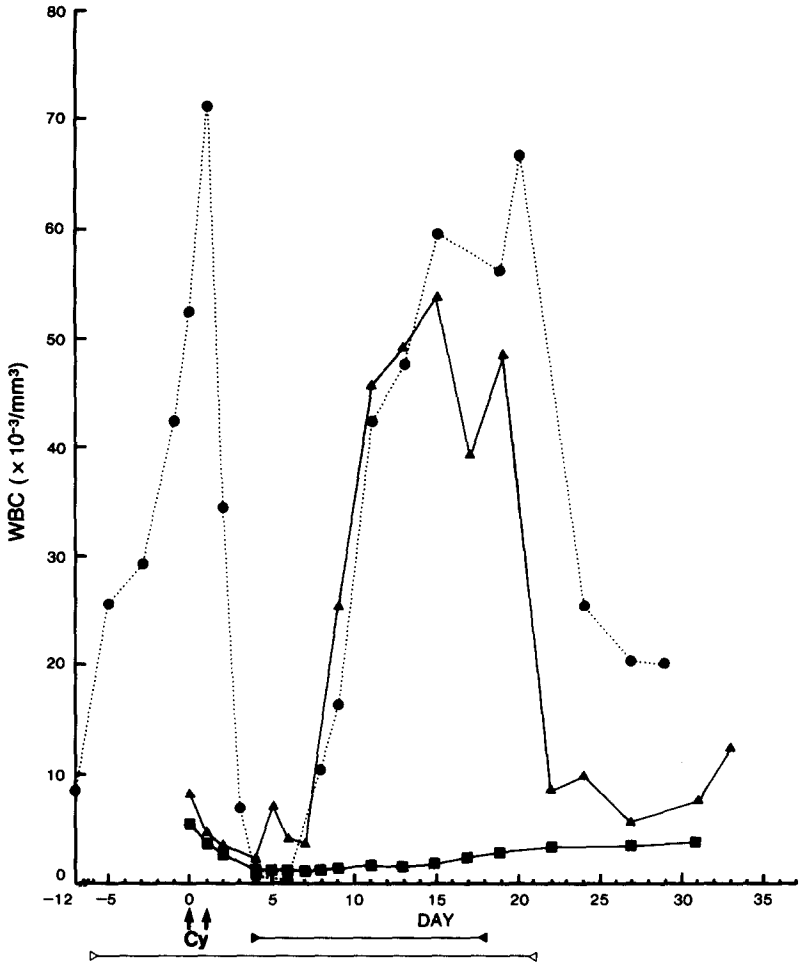

FIGURE 3. Time course of peripheral blood WBC of three monkeys treated with CY (Cytoxan; Bristol Meyers, Syracuse, NY; 60 mg/kg/d, two doses, treatment designated by arrows on days 0 and 1): (■), control monkey; (▲), monkey treated with rhG-CSF starting 72 h after the last dose of CY until day 17 after CY; and (●), monkey pretreated with rhG-CSF starting 6 d before CY and continued treatment to day 20 after CY. The rhG-CSF treatment period is designated for each animal: (▶—), post-CY-treated, and (▷—), pre-CY-treated.

in normal monkeys receiving only rhG-CSF at the same dose. The ANC ranged between 90 and 100% from day 7 to day 20 after CY, while lymphocytes remained <10% and monocytes <2% during the same time period. After cessation of rhG-CSF administration, the WBC count never dropped below normal levels. The percentage of neutrophils also returned to normal values (30–40%). In contrast, the control monkey remained pancytopenic, with WBC levels $<4 \times 10^3$ cells/mm^3, up to 4 wk after CY treatment (Fig. 3). As shown in Fig. 2*C*, the bone marrow aspirate (day 14 after CY) of the rhG-CSF-treated monkey showed evidence of recovery of all cell lineage precursors, whereas the control monkey (CY alone) demonstrated persistent hypocellularity. The time

course of post-CY platelet recovery was similar in all three monkeys (rhG-CSF-treated or control), requiring ~2 wk to return to normal levels.

The early recovery after CY suggests that the availability of this growth factor is one of the critical limiting steps in the proliferation and differentiation of bone marrow progenitor cells after CY treatment. The dramatic recovery of neutrophils in the rhG-CSF-treated monkeys after CY is also indicative of the functional integrity of progenitor cells capable of responding to rhG-CSF. These data suggest, however, that pretreatment with rhG-CSF does not prevent CY-induced cytopenia when given in the regimen described herein.

Summary

We examined the in vivo effects of recombinant human granulocyte colony–stimulating factor (rhG-CSF) in primates (cynomolgus monkeys) treated with subcutaneous doses of rhG-CSF for 14–28 d. A dose-dependent increase in the peripheral white blood cells (WBC) was seen, reaching a plateau after 1 wk of rhG-CSF treatment. The elevation of WBC was due to an increase in the absolute neutrophil count. These results demonstrate that rhG-CSF is a potent granulopoietic growth and differentiation factor in vivo. In cyclophosphamide (CY)-induced myelosuppression, rhG-CSF was able to shorten the time period of WBC recovery in two treated monkeys to 1 wk, as compared to more than 4 wk for the control monkey. Its ability to significantly shorten the period of chemotherapy-induced bone marrow hypoplasia may allow clinicians to increase the frequency or dosage of chemotherapeutic agents. In addition, the increase in absolute numbers of functionally active neutrophils may have a profound effect in the rate and severity of neutropenia-related sepsis. Furthermore, the activities reported here indicate a potential role for rhG-CSF in the treatment of patients with myelodysplastic syndrome, congenital agranulocytosis, radiation-induced myelosuppression, and bone marrow transplantation.

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