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In vivo stimulation of granulopoiesis by recombinant human granulocyte colony-stimulating factor

(bone marrow/colony-forming cells/leukopenia)

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ABSTRACT **Osmotic pumps containing *Escherichia coli*-derived recombinant human granulocyte colony-stimulating factor (rhG-CSF) were attached to indwelling jugular vein catheters and implanted subcutaneously into Golden Syrian hamsters. Within 3 days, peripheral granulocyte counts had increased >10-fold with a concomitant 4-fold increase in total leukocytes. Microscopic examination of Wright–Giemsa-stained blood smears from rhG-CSF hamsters showed that only the neutrophil subpopulation of granulocytes had increased. No significant changes in lymphocyte or monocyte counts were observed during the course of continuous rhG-CSF treatment. After subcutaneous injection at rhG-CSF doses of up to 10 μg·kg^{-1}·day^{-1} only granulocyte counts were affected. However, at higher dose levels, a transient thrombocytopenia was noted. Erythrocyte and lymphocyte/monocyte counts remained unaffected by rhG-CSF over the entire dose range (0.3–300 μg·kg^{-1}·day^{-1}) studied. Total leukocyte counts increased 3-fold within 12 hr after a single s.c. injection of rhG-CSF. This early effect was associated with an increase in the total number of colony-forming cells and the percent of active cycling cells in the marrow. A sustained elevation of peripheral leukocyte and marrow progenitor counts was observed following seven daily s.c. injections of rhG-CSF. The ability of rhG-CSF to increase the production and release of granulocytes from the marrow may underlie the beneficial effect it produced on the restoration of peripheral leukocyte counts in hamsters made leukopenic by treatment with 5-fluorouracil.**

Colony-stimulating factors are a class of growth factors necessary for the survival, proliferation, and differentiation of hematopoietic progenitor cells *in vitro* (1, 2). Four different classes of murine colony-stimulating factors (CSFs) have been identified that affect macrophage, bipotential granulocyte/macrophage, granulocyte, and multipotential (multi-colony-stimulating factor or interleukin 3) progenitors. Similarly, human CSFs that act on granulocyte and macrophage progenitors have been identified (3, 4).

All the CSFs are glycoproteins that stimulate hematopoietic progenitors at picomolar concentrations. However, they differ with respect to the cell lineage primarily affected and the biological responses they produce. For example, human granulocyte-CSF (G-CSF) isolated from human bladder carcinoma line 5637 (5), cloned, and expressed in *Escherichia coli* (6) not only supports the growth and differentiation of normal human granulocyte progenitors, but also induces differentiation of both human and murine myelomonocytic leukemia cells. The cloning of the cDNA for murine G-CSF has demonstrated a 70% sequence homology between the human and murine factors (7). Human G-CSF has also been isolated from human squamous cell carcinoma line CHU-2, and the gene has been cloned and expressed in monkey COS cells (8). In contrast to human G-CSF, human granulocyte/macrophage colony-stimulating factor, which enhances the proliferation of normal human progenitors, neither stimulates normal murine hematopoietic cells nor induces the differentiation of murine myelomonocytic WEHI-3B(D+) cells (9).

The availability of large quantities of recombinant human G-CSF (rhG-CSF) has made it possible to study its activity *in vivo*. In this paper we report on the *in vivo* action of rhG-CSF in Golden Syrian hamsters and demonstrate an elevation of peripheral blood neutrophils and bone marrow progenitors.

MATERIALS AND METHODS

rhG-CSF. *E. coli*-derived rhG-CSF was purified as described (6) and had a specific activity of 10^8 units/mg when assayed in a granulocyte/macrophage colony-forming unit assay. ^{35}S-labeled rhG-CSF (specific activity, 5 μCi/μg; 1 Ci = 37 GBq) was prepared from *E. coli* grown in the presence of L-[^{35}S]cysteine and L-[^{35}S]methionine. Endotoxin contamination was <1 ng/mg of pure protein as determined by the limulus amebocyte lysate assay.

Animals and Procedures. Male Golden Syrian hamsters (100–120 g) were obtained from Simonsen Laboratories (Gilroy, CA) and housed for at least 5 days prior to experimentation. Continuous intravenous infusion of pyrogen-free control buffer solution (37 mM NaCl/20 mM NaOAc, pH 5.4) or rhG-CSF was initiated by s.c. implantation of an Alzet osmotic pump (model 2001; Alza, Palo Alto, CA) attached to an indwelling right jugular vein catheter in hamsters anesthetized with sodium pentobarbital. The pumps were calibrated to deliver either 1.5 μl of buffer solution per hr or 1.5 μg of rhG-CSF per hr for up to 7 days. At the intervals indicated in the figures, blood samples were obtained by cardiac puncture and analyzed as described below. Similarly, dose–response relationships on hematological parameters were studied by injecting graded doses of rhG-CSF subcutaneously at 0800–1000 hr and again at 1600–1700 hr for 3 consecutive days. At 24 hr after the last injection, blood samples were obtained by cardiac puncture.

Hematocrits and platelet counts were quantitated in centrifuged blood samples with a QBCV hematology system (Clay Adams). Total leukocyte counts were determined with a Coulter counter model ZM (Coulter). Differential leukocyte counts were determined manually on Wright–Giemsa-stained blood smears.

Granulocyte/Macrophage Colony-Forming Unit Assay. Colony-forming cells (CFC) were assayed on bone marrow cells from each femur following centrifugation in Ficoll/Hypaque

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Abbreviations: Ara-Cyt, 1-β-D-arabinofuranosylcytosine; CFC, colony-forming cells; CSF, colony stimulating factor; G-CSF, granulocyte colony-stimulating factor; 5-FUra, 5-fluorouracil; r, recombinant; h, human.

(Pharmacia) and depletion of the adherent cell fraction by incubation with McCoy's medium in tissue culture dishes. Separated cells were incubated further with or without 1-β-D-arabinofuranosylcytosine (Ara-Cyt) at 100 μg/ml for 1 hr at 37°C, washed three times in medium, and then plated at a density of 10^5 cells per ml of 0.3% agar that included McCoy's 5A medium, 10% (vol/vol) heat-inactivated fetal calf serum, and 1000 units of rhG-CSF (10 ng) as described (6).

Drug-Induced Neutropenia. Hamsters were given a single i.p. injection of 5-fluorouracil (5-FUra; 125 mg/kg) dissolved in pyrogen-free 0.9% NaCl solution adjusted to pH 11.5 with 0.5 M KOH. Beginning 24 hr thereafter, they were injected daily s.c. with either control buffer solution or rhG-CSF at 10 μg/kg. At daily intervals, blood samples were taken from groups of four hamsters each, and total leukocyte counts were determined as described above.

Plasma Clearance of rhG-CSF. A group of five hamsters was each injected with 1.3 μCi of ^{35}S-labeled rhG-CSF through an indwelling jugular vein catheter. At intervals between 5 min and 4 hr, blood samples were obtained from an indwelling left carotid artery catheter, and plasma was prepared. Trichloroacetic acid-precipitable radioactivity in 0.1-ml aliquots was determined. All results were normalized to the radioactivity present in the initial 5-min sample.

RESULTS

Specificity of rhG-CSF. The ability of rhG-CSF to increase granulocyte counts *in vivo* was demonstrated in hamsters during the course of continuous i.v. infusion using implanted osmotic pumps. As shown in Fig. 1, the total number of leukocytes increased 4-fold within 3 days. The increased leukocyte counts (no. per ml) was entirely accounted for by a 10-fold elevation in the number of granulocytes; lymphocyte/monocyte levels were unaffected by rhG-CSF. Granulocyte counts continued to increase for an additional 4 days and then returned to control levels following depletion of rhG-CSF in the pumps.

Dose–Response. The effects of graded doses of rhG-CSF administered s.c. on the peripheral blood counts are illustrated in Fig. 2. Granulocyte counts were significantly elevated at a dose level as low as 10 $\mu g \cdot kg^{-1} \cdot day^{-1}$ and increased further in a dose-dependent manner thereafter. In contrast, rhG-CSF had no effect on lymphocyte/monocyte counts or the hematocrit over the entire dose range studied. Although platelet counts were unchanged at dose levels up to 10 $\mu g \cdot kg^{-1} \cdot day^{-1}$, a dose-dependent decrease was observed at higher dose levels.

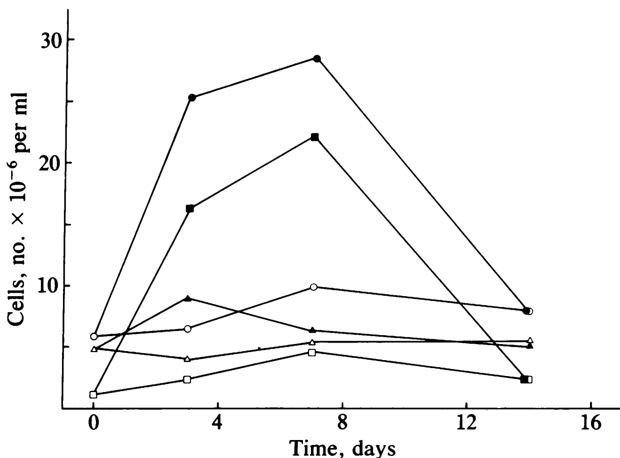

FIG. 1. Changes in leukocyte counts during continuous i.v. infusion of rhG-CSF. Hamsters were implanted on day 0 with osmotic pumps attached to indwelling jugular vein catheters. The pumps delivered either 1.5 μl of control buffer solution per hr (open symbols) or 1.5 μg of rhG-CSF per hr (closed symbols). On the days indicated, blood samples were obtained, and total leukocyte (circles), granulocyte (squares), and lymphocyte/monocyte (triangles) counts were determined. Each point is the mean value for three animals.

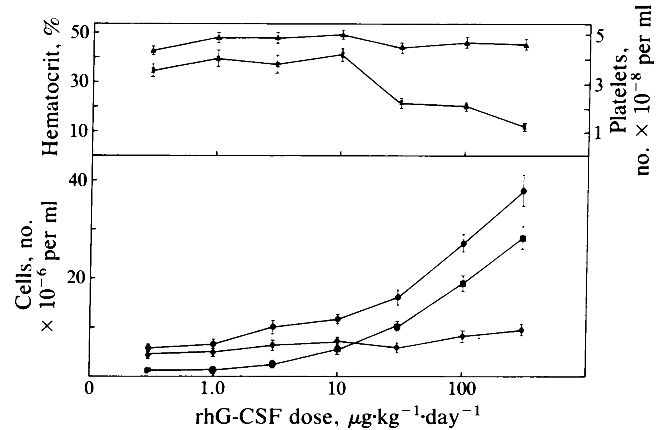

FIG. 2. rhG-CSF dose–response curve. rhG-CSF was administered s.c. in two equally divided doses for 3 consecutive days. At 24 hr after the last dose, blood samples were obtained for determination of hematocrit (▲), platelet (×), total leukocyte (●), granulocyte (■), and lymphocyte/monocyte (◆) counts. Each point is the mean of four animals with SEM indicated by bars.

Time Course of Response. The onset and duration of action of a single s.c. injection of rhG-CSF (100 μg/kg) is shown in Fig. 3. Total leukocyte counts were elevated as early as 2 hr after administration of the factor and reached peak levels within 12 hr. Circulating leukocytes remained elevated for at least 36 hr and then returned to control values.

Bone Marrow Progenitors. Since significant increases in peripheral blood neutrophils were found after a single s.c. injection of rhG-CSF, the effects on bone marrow progenitors were evaluated 12 hr after and 7 days after rhG-CSF administration. The data summarized in Table 1 show that the doubling of the total number of leukocytes in the peripheral blood was accompanied by a 50% increase in bone marrow cellularity. In addition, the number of progenitors in the bone marrow increased as reflected by an increase in the number of CFC. To evaluate the cycling status of the bone marrow progenitors after rhG-CSF treatment, half of the low-density

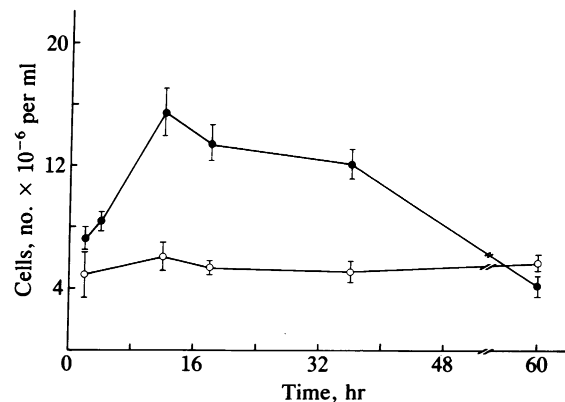

FIG. 3. Time course of rhG-CSF effect. A single 0.1-ml s.c. injection of control buffer solution (○) or rhG-CSF at 100 μg/kg (●) was administered. At the time points indicated after injection, blood samples were obtained, and total leukocyte counts were determined. Each point is the mean of four animals with SEM indicated by bars.

Table 1. Low-density nonadherent fraction of the total bone marrow cells

Exp.	Sample	WBC × 10^{-6} per ml	Cells, no. × 10^{-7} per femur	CFC, no. per 10^5 cells	% Ara-Cyt-sensitive cells
1	Control	6.5 ± 0.6	1.4 ± 0.1	20.6 ± 1.2	46.6 ± 1.7
	rhG-CSF	11.8 ± 0.6	2.1 ± 0.1	37.9 ± 2.7	64.6 ± 2.0
2	Control	6.7 ± 0.2	1.2 ± 0.1	ND	ND
	rhG-CSF	18.3 ± 2.2	3.0 ± 0.1	ND	ND

In experiment 1, four hamsters were each given a single s.c. injection of rhG-CSF at 10 μg/kg or buffer. Twelve hours later, bone marrow from each animal was isolated and subject to density gradient centrifugation and adherent-cell depletion. In addition, the number of total peripheral blood leukocytes (WBC) were determined. Half of the bone marrow cells were incubated with Ara-Cyt at 1 μg/ml and the other half with media. CFC were evaluated by incubation of 10^5 bone marrow cells in triplicate in an agar-based granulocyte/macrophage colony-forming unit assay containing 1000 units of rhG-CSF. In experiment 2, two groups of five hamsters were each given 7 daily subcutaneous injections of rhG-CSF at 30 μg/kg or buffer. Numbers represent means of four or five animals ± SEM. ND, not done.

nonadherent bone marrow population from treated and control animals was treated with Ara-Cyt at 100 μg/ml for 1 hr before plating in the granulocyte/macrophage colony-forming unit assay. Ara-Cyt selectively inhibits DNA synthesis and is primarily cytotoxic to cells in S phase of the cell cycle (10–12). Therefore, after exposure to Ara-Cyt, evaluation of the number of CFC relative to the number of CFC in control cultures gives an estimate of the percent of cells in active cell cycle. As can be seen in Table 1, the percent of Ara-Cyt-sensitive cells increased after rhG-CSF treatment, indicating an increase in the number of active cycling cells. The data also show that elevated marrow progenitor and peripheral leukocyte counts were sustained following seven daily injections of rhG-CSF.

Recovery from Drug-Induced Leukopenia. One of the potential uses of CSFs is to hasten recovery from chemotherapy-induced leukopenia. To ascertain the feasibility of this approach, the effect of rhG-CSF on peripheral leukocyte counts was measured in hamsters pretreated with 5-FUra. As shown in Fig. 4, leukopenia developed rapidly in hamsters given a single i.p. injection of 5-FUra (125 mg/kg). In animals posttreated with control buffer solution, a nadir leukocyte count 75% below the pretreatment value was observed at 6 days after 5-FUra administration. During the subsequent 1–2 days, leukocyte counts returned to pretreatment levels. In contrast, hamsters given daily s.c. injections of rhG-CSF (10 μg/kg) commencing 24 hr after 5-FUra displayed a nadir in leukocyte counts at day 5 and a return to pretreatment values ≈1.5 days earlier than those given control buffer.

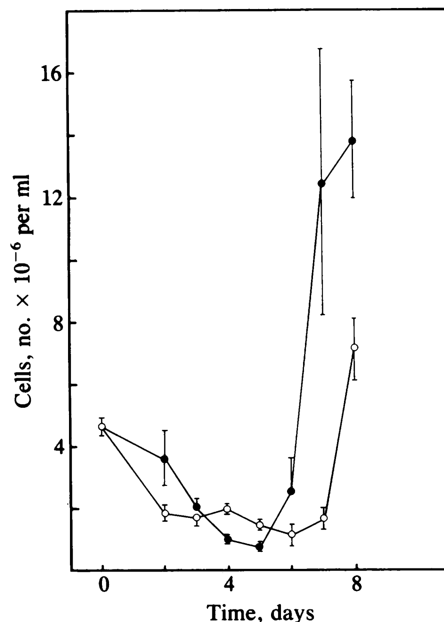

FIG. 4. Recovery from drug-induced leukopenia. Hamsters were injected i.p. with 5-fluorouracil (125 mg/kg) on day 0. Starting 24 hr thereafter, 0.1 ml of control buffer solution (○) or rhG-CSF at 10 μg/kg (●) was injected s.c. each day. At the time points indicated, blood samples were obtained, and total leukocyte counts were determined. Each point is the mean of four animals with SEM indicated by bars.

Plasma Clearance of rhG-CSF. To better understand the relationship between *in vivo* activity and circulating levels of rhG-CSF, plasma clearance of ^{35}S-labeled rhG-CSF was measured following a single-bolus i.v. injection. As shown in Fig. 5, ^{35}S-labeled rhG-CSF was cleared in a biexponential manner with an estimated distribution half-life of 0.5 hr and an elimination half-life of 3.8 hr.

DISCUSSION

After comparing the *in vitro* sensitivity of bone marrow cells from humans and several rodent species (13), the hamster

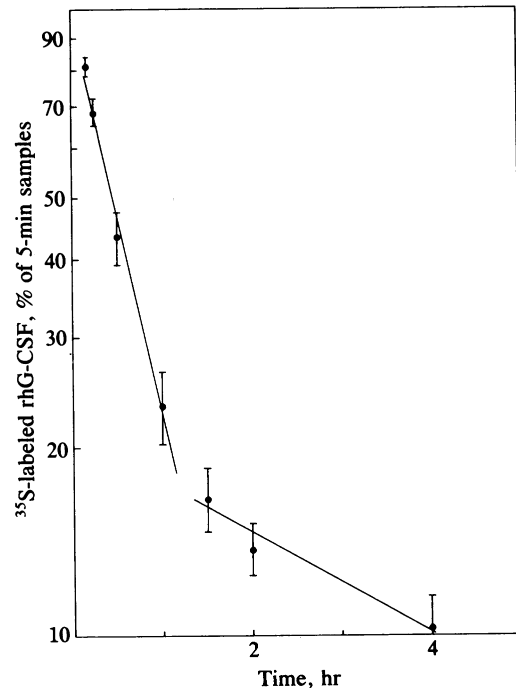

FIG. 5. Plasma clearance of rhG-CSF. Hamsters were injected with 1.3 μCi of ^{35}S-labeled rhG-CSF (specific activity, 5 μCi/μg) through an indwelling right jugular vein catheter. At the intervals shown, blood samples were obtained from an indwelling left carotid artery catheter, and plasma was prepared. Trichloroacetic acid-precipitable radioactivity was determined from 0.1-ml plasma aliquots. Results were normalized to the radioactivity present in the 5-min samples. Each point is the geometric mean ± SEM of five animals.

was chosen as a model to study the *in vivo* activity of rhG-CSF. The results obtained demonstrate that this factor exerts a potent, rapid, and specific effect on the proliferation of granulocyte progenitors. Microscopic examination of Wright–Giemsa-stained blood smears showed that this action was confined to the neutrophil subpopulation of granulocytes.

As shown in Figs. 1 and 2, the increase in circulating leukocytes following rhG-CSF administration could be entirely accounted for by the elevation in granulocyte counts. The number of circulating lymphocytes and monocytes remained unchanged even at dose levels that produced greater than 10-fold increase in granulocyte counts. Similarly, rhG-CSF did not exert an acute effect on the hematocrit (Fig. 2) and did not alter the total erythrocyte count during and following 1 week of continuous i.v. infusion (data not shown). In contrast, daily s.c. injection of rhG-CSF at dose levels >10 $\mu g \cdot kg^{-1} \cdot day^{-1}$ resulted in a dose-dependent decrease in circulating platelets. This may represent an initial toxic effect of the factor in hamsters since a reversal of the thrombocytopenia occurred during the course of continuous i.v. infusion (data not shown). These results demonstrate the specificity of action of rhG-CSF on the neutrophil population in hamsters. A sustained and specific elevation of peripheral neutrophil counts has also been observed in cynomolgus monkeys treated for up to 4 weeks with rhG-CSF (14).

As shown in Fig. 3, leukocyte counts increased rapidly after a single s.c. injection of rhG-CSF (100 μg/kg). Elevation in circulating leukocytes was noted as early as 2 hr after administration and reached a level >3-fold higher than control within 12 hr. Moreover, the stimulatory effect was maintained for more than 36 hr following a single injection of the factor. The long duration of action of rhG-CSF is most likely a reflection of its relatively slow clearance (half-life, 3.8 hr) from the blood (Fig. 5). Although the rate of absorption of rhG-CSF from subcutaneous tissue is unknown, the rise in leukocyte counts observed at 2 hr indicates that absorption is rapid. Consequently, at the dose level used in this study, it is probable that circulating levels of rhG-CSF in excess of the picomolar concentrations needed for *in vitro* activity were maintained over several hours *in vivo*.

As indicated by the data in Table 1, the rapid increase in peripheral leukocytes is associated with a potent stimulating effect of rhG-CSF on bone marrow progenitors. Significant increases in the total number of nonadherent cells per femur, the number of CFC per 10^5 nonadherent cells, and the fraction of proliferating CFC were observed at 12 hr following a single s.c. injection of rhG-CSF (10 μg/kg). While these results do not rule out demargination as a factor contributing to the early increase in circulating leukocytes, they demonstrate that rhG-CSF has a potent capacity to stimulate the production of new granulocytes in the marrow. Moreover, the data in Table 1 show that daily administration of rhG-CSF leads to a sustained elevation of both peripheral leukocyte and bone marrow progenitor counts. These results differ from those reported by Kohsaki *et al.* (15) on the effects of a human urinary extract from aplastic anemia patients on murine granulopoiesis *in vivo*. Although the extract produced an increase in circulating granulocytes in the mouse, this effect was accompanied by a decrease in both marrow cellularity and the number of CFC per femur and by a concomitant increase in CFC per spleen. Similar reciprocal changes in marrow and spleen progenitors were observed by Kindler *et al.* (16) in mice treated with recombinant interleukin 3. Accordingly, the discrepancy in the results may be a consequence of differences in the biological actions of the various CSFs or reflective of differences in hematopoietic control mechanism between hamsters and mice. With regard to the latter possibility, spleen weight remained unchanged in hamsters after seven daily injections of rhG-CSF whereas splenomegaly was observed in mice under similar conditions (unpublished observations).

Although it is conceivable that the *in vivo* effects observed could be caused by endotoxin contamination, two considerations argue against this possibility. First, the absolute level of endotoxin in the preparation was extremely low, i.e., <1 ng/mg. Accordingly, at the dose levels of rhG-CSF used in these studies, only picogram quantities of endotoxin could have been coadministered. Such small amounts would not be expected to affect leukocyte levels. Secondly, we have subcutaneously injected hamsters for 3 days with *E. coli*-derived recombinant interleukin 2 (10 μg/kg) or interferon α (10 μg/kg) and failed to observe any alteration in circulating leukocyte counts. As the endotoxin contamination of both of these preparations was equivalent to that found in rhG-CSF, these results indicate that the *in vivo* effects observed in the present studies were due to rhG-CSF itself and were not caused by endotoxin contamination.

Since rhG-CSF exerted a prompt and specific stimulatory effect on granulopoiesis, it was of interest to determine if it could accelerate the recovery of bone marrow progenitors following cytotoxic chemotherapy. As shown in Fig. 4, peripheral leukocyte counts returned to control values ≈1.5 days earlier in hamsters injected daily with rhG-CSF beginning 24 hr after treatment with 5-FUra. We have also observed an accelerated recovery from cyclophosphamide-induced leukopenia in hamsters treated with rhG-CSF (data not shown). However, a slight delay in the recovery of platelet counts was observed in both 5-FUra- and cyclophosphamide-treated hamsters that were also given rhG-CSF (data not shown). Thus, although circulating CSF activity has been shown to increase after treatment with cytotoxic agents (17), these results indicate that further enhancement of marrow recovery can be achieved with administration exogenous CSFs. The fact that recovery was delayed several days after the initiation of treatment with rhG-CSF suggests that many progenitor cells were killed by 5-FUra. In support of this hypothesis is the fact that progenitors responsive to rhG-CSF *in vitro* are depleted in hamster bone marrow samples obtained 2 days after treatment with 5-FUra (unpublished observations). In any event, these initial results provide a measure of encouragement as to the potential clinical utility of rhG-CSF in the treatment of chemo/radiotherapy-induced neutropenia as well as neutropenia associated with other disease states.

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