Granulocyte colony-stimulating factor (G-CSF) is one member of a family of hematopoietic growth factors that are required for proliferation and differentiation of hematopoietic progenitor cells. A complementary DNA encoding human G-CSF was cloned and expressed in Escherichia coli or Chinese hamster ovary (CHO) cells, and large-scale production of biologically active recombinant human G-CSF (rhG-CSF) was established. Administration of rhG-CSF increased the peripheral blood (PB) neutrophil count and reduced periods of neutropenia in animals, and patients with solid tumors after the treatment with cytotoxic drugs. These observations support the clinical usefulness of rhG-CSF in patients receiving cytotoxic chemotherapy when assessed on the basis of absolute neutrophil count in PB.

We recently reported that rhG-CSF is a potent activator of human mature neutrophils and enhances in vitro the release of superoxide (O$_{2}^{-}$) stimulated by the chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (FMLP). We also showed that rhG-CSF restores the O$_{2}^{-}$-releasing capacity in vitro in neutrophils from patients with myelodysplastic syndrome, in which the O$_{2}^{-}$-releasing capacity is markedly impaired. We investigated the effect of rhG-CSF administration on neutrophil functions in patients with malignant lymphoma receiving cytotoxic chemotherapy. Results show that rhG-CSF rapidly activates human neutrophils both in vivo and in vitro. After administration of rhG-CSF, both FMLP-induced O$_{2}^{-}$ release and expression of C3bi-receptors were markedly increased; these effects were sustained for at least 24 hours after a single 30-minute intravenous (IV) administration of rhG-CSF.

**MATERIALS AND METHODS**

**Patients.** Seven adult patients with malignant lymphoma were studied. Their characteristics are shown in Table I. All patients had a good performance status. Three patients received previous chemotherapy. Three patients had bone marrow (BM) involvement; one of them was in a leukemic state. Informed consent was obtained from all patients.

Administration of rhG-CSF. Highly purified rhG-CSF produced by E. coli was supplied by Kirin Brewery (Tokyo, Japan) in collaboration with Amgen Biologicals (Thousand Oaks, CA). Specific activity of purified rhG-CSF (an apparent molecular weight [mol wt] of 18,800) was approximately 1 x 10$^{8}$ U/mg protein when assayed by colony formation of human BM cells. Endotoxin contamination of purified protein was <1 ng/mg protein. rhG-CSF (50 to 800 μg/m$^2$ body surface) was diluted in 100 mL 5% glucose in water, and was infused IV in 30 minutes once daily. The treatment consisted of daily administration of rhG-CSF for 14 days, beginning 72 hours after chemotherapy with cytotoxic drugs. The same preparation of rhG-CSF was also used for in vitro experiments with human neutrophils. The cytotoxic drugs used for treatment of malignant lymphoma are shown in Table 1. Each patient received two consecutive courses of the same cytotoxic drugs with an interval of 4 to 5 weeks. Five patients received rhG-CSF after the first course of chemotherapy, and two patients (patients 3 and 5) received rhG-CSF after the second course.

Preparation of neutrophils. PB was obtained from the patients immediately before, during (at 6.5 hours and on day 2 or 3), and after (day 15) treatment with rhG-CSF. Neutrophils were prepared as described previously with dextran sedimentation and centrifuga-

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tochrome at Japan) equipped with thermostatted cuvette holder, Tokyo, K., Tokyo, Japan) and suspended for reduction for concentration. The release of O₂ was calculated from cytochrome described previously. The cell suspension in HBSS was added to a reaction with Conray-Ficoll. Contaminated erythrocytes in neutrophil fractions were removed by hypotonic lysis. Neutrophil fractions were suspended in Hank's balanced salt solution (HBSS) (Nissui Seiyaku, Tokyo, Japan) and contained more than 96% neutrophils (band-form and segmented neutrophils).

Determination of O₂⁻ release. O₂⁻ was assayed spectrophotometrically by superoxide-dismutase inhibitable reduction of ferricytochrome c, and continuous assay was performed in a Hitachi 557 spectrophotometer (a double-wavelength spectrophotometer, Hitachi, Tokyo, Japan) equipped with thermostatted cuvette holder as described previously. The cell suspension in HBSS was added to a 1-mL cuvette containing 80 μmol/L ferricytochrome c with or without rhG-CSF (25 ng/mL) to obtain a final volume of 0.9975 to 0.995 mL. Final cell concentration was 5 × 10⁶ cells/mL. The reaction mixture in a cuvette was preincubated for 10 minutes at 37°C. The cuvette was placed in a thermostatted cuvette holder (37°C) of a spectrophotometer, and reduction of ferricytochrome c was measured at 550 nm with a reference wavelength at 540 nm. FMLP (10⁻⁷ mol/L) or phorbol myristate acetate (PMA, 100 ng/mL) was added to the reaction mixture in a cuvette to obtain a final volume of 1 mL while the time course of cytochrome c reduction (the absorbance change at 550 – 540 nm) was followed on the recorder. The release of O₂⁻ was calculated from cytochrome c reduced for 5 minutes after addition of FMLP and from the initial linear portion of the cytochrome c reduction for PMA.

Determination of plasma concentration of G-CSF. The plasma concentration of G-CSF was determined by a radioimmunoassay (RIA) using polyclonal rabbit anti-rhG-CSF antibody, ¹²⁵I-labeled rhG-CSF, and goat anti-serum to rabbit IgG (N. Takanashi, manuscript in preparation). The sensitivity of the assay used was 0.25 ng/mL, and a linear dose response was observed through 25 ng/mL in human plasma. Plasma samples were collected at indicated time points (0, 0.5, 1.0, 1.5, 2.5, 4.5, 8.5, and 12.5 hours) after rhG-CSF administration and assayed in parallel with appropriate standards for G-CSF quantitation. Experimental variability in this assay routinely averaged 5%.

Determination of C3bi-receptor expression. Heparinized blood was obtained at the indicated times (0, 0.25, 0.5, 1, 2, and 24 hours) after rhG-CSF administration, and mixed with anti-MoAB monoclonal antibody (MoAB) directly conjugated with fluorescein isothiocyanate (FITC). The cells were incubated for 30 minutes at 4°C and analyzed by cytofluometry in an Ortho Spectrum III (Ortho Diagnostic Systems, Westwood, MA) as described previously.

Reagents. Cytochrome c type III, FMLP, PMA, and superoxide dismutase were purchased from Sigma Chemical (St Louis, MO), Conray was purchased from Mallinkrodt (St Louis, MO), and Ficol was purchased from Pharmacia Fine Chemicals, (Piscataway, NJ).

Statistical analysis. Student's t test and the paired t test were used to determine statistical significance.

RESULTS

Effect of rhG-CSF administration on absolute neutrophil count. The total white blood cell (WBC) count in the PB was increased after 30-minute rhG-CSF administration, and WBCs increased in response to rhG-CSF were predominantly neutrophils. Figure 1 shows the changes of the absolute neutrophil count in the PB during 24 hours after initial administration of various doses (50 to 800 μg/m²) of rhG-CSF. The absolute neutrophil count was increased in all patients within 4.5 hours and reached a maximum by 6.5 to 12.5 hours after rhG-CSF administration. Although a high dose of rhG-CSF (800 μg/m²) induced a dramatic increase in the absolute neutrophil count (patient 7), a lower dose of rhG-CSF (50 μg/m²) also induced a remarkable increase in the absolute neutrophil count (patient 1). There was no definite relationship between the dosage of rhG-CSF administered and an increase in the absolute neutrophil count, presumably owing to the preceding cytotoxic chemotherapy (Fig 1). Administration of rhG-CSF not only induced a rapid increase in the absolute neutrophil count but also ameliorated the decrease in the neutrophil count after the cytotoxic chemotherapy in all patients (Fig 2). The period of neutropenia (<1,000 or <500/μL) was significantly (P < .05) reduced by rhG-CSF treatment in all patients. The periods of neutropenia with <1,000 and <500/μL in the course with rhG-CSF treatment were 3.6 ± 4.6 and 1.7 ± 2.4 days, respectively, whereas those in the course without rhG-CSF treatment were 7.7 ± 6.6 and 5.4 ± 6.2 days, respectively.
The nadir of neutrophils in the course with rhG-CSF treatment was 1.226 ± 1,381/μL, whereas that in the course without rhG-CSF treatment was 492 ± 722/μL. Treatment with rhG-CSF was well-tolerated, and no patients had any side effects.

Effect of rhG-CSF administration on neutrophil \( \text{O}_2^- \) release. To assess the in vivo activation of neutrophil function by rhG-CSF, we studied release of \( \text{O}_2^- \) in human neutrophils stimulated by FMLP. PB was obtained immediately before (day 1), during (at 6.5 hours and on day 2 or 3), and after (day 15) treatment with rhG-CSF. As shown in Table 2, enhanced release of \( \text{O}_2^- \) was already detected 6.5 hours after rhG-CSF administration. Neutrophils isolated 6.5 hours after rhG-CSF administration released greater amount of \( \text{O}_2^- \) (1.2- to 2.4-fold, \( P < .02 \)) with FMLP stimulation than neutrophils isolated immediately before rhG-CSF treatment in all patients tested. The representative time courses of \( \text{O}_2^- \) release stimulated by FMLP are shown in Fig 3. The priming effect was sustained for at least 24 hours after a single 30-minute IV administration of rhG-CSF, since neutrophils isolated on day 2 before a second administration of rhG-CSF released a still greater amount of \( \text{O}_2^- \) in response to FMLP than neutrophils isolated before rhG-CSF treatment (Table 2 and Fig 3). The priming effect was sustained during the entire course of rhG-CSF treatment, and FMLP-induced \( \text{O}_2^- \) release in neutrophils isolated on day 15 was 1.6- to 7.8-fold greater than that in neutrophils isolated before rhG-CSF treatment (Table 2). The priming effect was not strictly dependent on the dosage of rhG-CSF administered when assessed on the basis of magnitude of enhancement and \( \text{O}_2^- \) release was not observed (data not shown).

Reduced responsiveness to further in vitro challenge of rhG-CSF in neutrophils isolated after rhG-CSF treatment. Our previous experiments showed that preincuba-

![Graph](image)

**Table 2. In Vivo and In Vitro Effects of rhG-CSF on \( \text{O}_2^- \) Release in Human Neutrophils Stimulated by FMLP**

<table>
<thead>
<tr>
<th>Patient</th>
<th>rhG-CSF</th>
<th>( \text{O}_2^- ) Release (nmol/5 min per 5 x 10^6 Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>In Vivo</td>
<td>Before 6.5 h Day 2(3)* Day 15</td>
</tr>
<tr>
<td>1</td>
<td>−</td>
<td>1.7 ± 0.1 2.0 ± 0.1† 3.5 ± 0.3‡ 3.7 ± 0.8†</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4.3 ± 0.7§ 2.3 ± 0.3 3.8 ± 0.4 7.3 ± 1.2</td>
</tr>
<tr>
<td>2</td>
<td>−</td>
<td>4.1 ± 0.6 ND 4.7 ± 0.3 7.3 ± 0.5§</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6.0 ± 0.5</td>
</tr>
<tr>
<td>3</td>
<td>−</td>
<td>0.4 ± 0.1 ND 1.2 ± 0.1§ 3.1 ± 0.6‡</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.2 ± 0.1§ ND 1.2 ± 0.1§ 3.4 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>−</td>
<td>1.1 ± 0.3 2.6 ± 0.2‡ 5.1 ± 0.6‡ ND</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4.5 ± 0.3§ 3.0 ± 0.5 6.5 ± 0.4‡ ND</td>
</tr>
<tr>
<td>5</td>
<td>−</td>
<td>4.0 ± 0.3 7.0 ± 0.6‡ 7.5 ± 0.6‡ ND</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7.1 ± 0.2§ 6.9 ± 0.9 6.8 ± 1.3 ND</td>
</tr>
<tr>
<td>6</td>
<td>−</td>
<td>1.1 ± 0.2 ND ND 7.1 ± 0.6‡</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.3 ± 0.1§ ND ND 7.4 ± 0.4</td>
</tr>
<tr>
<td>7</td>
<td>−</td>
<td>2.8 ± 0.3 ND 3.1 ± 0.4 4.5 ± 0.5‡</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5.4 ± 1.1</td>
</tr>
</tbody>
</table>

Neutrophils were isolated immediately before, during (at 6.5 hours and on day 2 or 3), and after (day 15) treatment with rhG-CSF, and \( \text{O}_2^- \) release stimulated by FMLP (10^{-7} \text{ mol/L}) was assayed with or without rhG-CSF (25 ng/mL). Data are means ± SD of triplicate determinations.

*Patients 3, 4, and 5 were tested on day 2 and patients 1, 2, and 7 were tested on day 3.

†, †Significantly increased as compared with values obtained before rhG-CSF treatment († \( P < .01 \), † \( P < .02 \)).

§, ||, ‡Significantly increased as compared with values obtained from neutrophils preincubated without rhG-CSF in vitro (§ \( P < .01 \), || \( P < .02 \), ‡ \( P < .05 \)).
tion of human neutrophils with 25 ng/mL rhG-CSF for 10 minutes at 37°C is sufficient for priming the cells in vitro. As shown in Table 2, when neutrophils isolated before rhG-CSF treatment were preincubated in vitro with 25 ng/mL rhG-CSF for 10 minutes at 37°C, significant enhancement (1.5- to 4.1-fold; \( P < 0.02 \)) of \( \Delta \) release stimulated by FMLP was observed in all patients. On the contrary, when neutrophils isolated 6.5 hours after rhG-CSF administration were preincubated in vitro with rhG-CSF, no further enhancement of \( \Delta \) release was observed in any patients tested; i.e., neutrophils were desensitized to rhG-CSF and further priming in vitro did not occur in neutrophils already primed in vivo. When the in vitro priming study was performed on day 2 (or 3) and day 15, a significant priming effect was observed in two of six patients tested on day 2 (or 3) and in two of five patients tested on day 15, respectively. In vitro priming by rhG-CSF on day 15 was observed in two patients receiving lower doses of rhG-CSF (50 and 100 \( \mu \)g/mL, respectively), whereas in vitro priming was not observed in the other patients receiving higher doses of rhG-CSF (>200 \( \mu \)g/mL) (Table 2). Neutrophils isolated 6.5 hours after rhG-CSF administration and primed in vitro released a significantly (\( P < .02 \)) smaller amount of \( \Delta \) with FMLP stimulation than did neutrophils isolated before rhG-CSF treatment and primed in vitro (patients 1 and 4 in Table 2).

The changes in neutrophil functions observed in the present experiments might have been caused by the cytotoxic chemotherapy itself. To test this possibility, neutrophils were isolated from two patients who were not receiving rhG-CSF, and \( \Delta \) release stimulated by FMLP was determined immediately before and 72 hours after cytotoxic chemotherapy. As shown in Table 3, cytotoxic chemotherapy did not cause consistent enhancement of \( \Delta \) release, but rather impaired \( \Delta \) release. Table 3 also shows that the responsiveness of neutrophils to rhG-CSF was not impaired by cytotoxic chemotherapy.

**Pharmacokinetic study.** The plasma concentration of G-CSF was measured by RIA. Before administration of rhG-CSF, G-CSF was not detected in all patients by this assay system, indicating that the plasma concentration of G-CSF was <0.25 ng/mL. Pharmacokinetic study of rhG-CSF performed in three patients (patients 1, 2, and 4) showed a half-life \( (t_1/2) \) of 114 minutes (calculated from the initial slope), in good agreement with the \( t_1/2 \) (110 minutes) obtained by a bioassay. It was noteworthy that administration of as little as 50 \( \mu \)g/mL rhG-CSF (patient 1) gave a plasma concentration of >20 ng/mL (26.8 ng/mL at 30 minutes), which continued for at least 30 minutes, a condition sufficient for maximal priming of human neutrophils in vitro. Administration of 200 \( \mu \)g/mL rhG-CSF (patient 4) gave a plasma concentration of 98.9 ng/mL at 30 minutes and 5.9 ng/mL at 12.5 hours, respectively.

**Effect of rhG-CSF administration on neutrophil C3bi-receptors.** We recently showed that pretreatment of human neutrophils with rhG-CSF in vitro increases expression of C3bi-receptors. We then studied the in vivo effect of rhG-CSF on neutrophil C3bi-receptors. As shown in Fig 4, administration of rhG-CSF also increased expression of C3bi-receptors on human neutrophils, as measured by binding of FITC-labeled MoAb (anti-Mol) to this antigen. The mean fluorescence values obtained with anti-Mol increased after administration of rhG-CSF. The increase in binding of anti-Mol was rapid (evident within 15 minutes) and was sustained for at least 24 hours after a single IV administration of rhG-CSF.

**DISCUSSION**

The present study shows that rhG-CSF administration rapidly (evident within 4.5 hours) increases the absolute neutrophil count in the PB of patients with malignant lymphoma irrespective of BM involvement by lymphoma cells, and an increased level of neutrophil count is still observed 24 hours after a single 30-minute IV administration.
of rhG-CSF. In addition, rhG-CSF administration ameliorated the decrease in neutrophil count after cytotoxic chemotherapy and reduced the period of neutropenia in all patients. These findings are consistent with recent studies performed in patients with nonhematologic malignant tumors\(^6\) and further support the clinical usefulness of rhG-CSF in patients receiving cytotoxic chemotherapy.

Another important goal of the present study was to assess the functional aspect of PB neutrophils increased in response to rhG-CSF administration. When neutrophils isolated before rhG-CSF treatment were preincubated with rhG-CSF in vitro, \(O_2^-\) release stimulated by FMLP was enhanced in all patients; ie, neutrophils were rapidly primed in vitro by rhG-CSF.\(^12\) The present experiments showed that neutrophils were also rapidly primed in vivo by rhG-CSF because neutrophils isolated 6.5 hours after rhG-CSF administration released a greater amount of \(O_2^-\) with FMLP stimulation than did neutrophils isolated before rhG-CSF treatment. The enhanced release of \(O_2^-\) stimulated by FMLP was sustained for at least 24 hours after a single 30-minute IV administration of rhG-CSF. The responsiveness to further in vitro challenge of rhG-CSF was lost or reduced in neutrophils isolated after rhG-CSF treatment, indicating that neutrophils already primed in vivo are desensitized to further in vitro challenge of rhG-CSF. These findings are consistent with our recent observation\(^16\) that neutrophils optimally primed in vitro by rhG-CSF do not respond to further addition of rhG-CSF. Furthermore, cytotoxic chemotherapy itself neither caused consistent enhancement of \(O_2^-\) release nor impaired the responsiveness of neutrophils to rhG-CSF. These findings suggest that enhanced release of \(O_2^-\) during the course of rhG-CSF treatment is caused by the specific action of rhG-CSF. Additional factors may contribute to enhancement of \(O_2^-\) release in neutrophils in vivo. No definite relationship between the dosage of rhG-CSF administered and enhancement of \(O_2^-\) release could be explained by the inherent variability of neutrophil responsiveness to FMLP or rhG-CSF and the fact that neutrophil functions are influenced by various substances.\(^17\)

In contrast to the results obtained with FMLP, when PMA was used as a stimulus, no consistent enhancement of \(O_2^-\) release was observed during the course of rhG-CSF treatment. These findings are consistent with our in vitro observations\(^16\) that rhG-CSF enhances \(O_2^-\) release stimulated by receptor-mediated agonists (FMLP and wheat germ agglutinin), but not by agonists that bypass receptors (Ca\(^{2+}\) ionophore ionomycin and PMA). These findings suggest that, like in vitro priming, rhG-CSF administered in vivo modulates the signal transduction pathways linked to FMLP receptors rather than increases the components of the \(O_2^-\)-producing enzyme complexes, such as flavoprotein and cytochrome b\(^{2+}\).\(^18\)

Administration of as little as 50 \(\mu g/m^2\) rhG-CSF caused not only a significant increase in absolute neutrophil count but also a significant activation of neutrophil function. These observations were also supported by pharmacokinetic study of rhG-CSF. Administration of 50 \(\mu g/m^2\) rhG-CSF gave a plasma concentration of >20 ng/mL G-CSF, which continued for at least 30 minutes, a condition sufficient for priming human neutrophils.\(^12\) Administration of higher doses (>200 \(\mu g/m^2\)) rhG-CSF did not cause much greater priming as compared with administration of 50 to 100 \(\mu g/m^2\) rhG-CSF, although the neutrophil count was remarkably increased in patient 7 who received 800 \(\mu g/m^2\) of rhG-CSF. The in vitro priming by rhG-CSF on day 15 was not observed in patients receiving doses of rhG-CSF (>200 \(\mu g/m^2\)). This finding suggests that neutrophils are almost maximally primed in vivo when >200 \(\mu g/m^2\) rhG-CSF is administered. The present experiments also showed that administration of rhG-CSF rapidly (evident within 15 minutes) increased expression of C3bi-receptors, an adhesion-related molecule,\(^27\) on human neutrophils. Both an increased expression of C3bi-receptors and a remarkable increase in neutrophil count may predispose to neutrophil aggregation in the blood vessels, which may cause a serious side effect. The transient decrease in absolute neutrophil count observed within 5 minutes after rhG-CSF administration\(^8\) may be, at least in part, related to an increased expression of C3bi-receptors.\(^21,22\) Indeed, we recently observed that rhG-CSF enhances neutrophil adherence to nylon fiber in vitro with concomitant increase of the expression of C3bi-receptors.\(^16\)

In the present experiments, we showed that rhG-CSF administration not only ameliorates the decrease in absolute neutrophil count after cytotoxic chemotherapy but also activates mature neutrophil functions without accompanying side effects. These findings further support the clinical usefulness of rhG-CSF not only in patients receiving cytotoxic chemotherapy but also in patients whose neutrophil functions are impaired.

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In vivo activation of human neutrophil functions by administration of recombinant human granulocyte colony-stimulating factor in patients with malignant lymphoma

A Ohsaka, S Kitagawa, S Sakamoto, Y Miura, N Takanashi, F Takaku and M Saito