Absorption of Recombinant Human Granulocyte Colony-Stimulating Factor (rhG-CSF) from Rat Nasal Mucosa

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Nasal absorption of recombinant human granulocyte colony-stimulating factor (rhG-CSF) was examined in the rat. The relative bioavailability of rhG-CSF for subcutaneous administration was ~2%, as evaluated from the immunologically active rhG-CSF concentration in rat plasma and the area under the curve (AUC) of the plasma rhG-CSF concentration versus time for 8 hr. Pharmacological availability relative to subcutaneous administration was determined from the increase in total blood leukocyte numbers. The pharmacological availability was 5–10%, determined from the AUC for the increased ratio of total leukocyte numbers versus time for 48 hr; it was slightly dependent on the pH and the osmotic pressure of the dosing solution. Accordingly, the plasma concentration of rhG-CSF did not always reflect its pharmacological effects. Relative bioavailability and pharmacological availability were increased about 23 times and 3 times, respectively, by polyoxyethylene 9-lauryl ether (Laureth-9), but no increase in availability occurred with sodium glycocholate. The increase in total leukocyte numbers was maintained during multiple rhG-CSF dosing, and the addition of Laureth-9 further increased the pharmacological effects of this agent. This study indicates that nasal administration of rhG-CSF is an effective parenteral administration route.

KEY WORDS: granulocyte colony-stimulating factor (rhG-CSF); recombinant human granulocyte-CSF; nasal absorption in rats; bioavailability; leukocyte number.

INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF)[5] is a hematogenic factor that stimulates granulocyte precursor cells (CFU-GM) in the bone marrow and specifically promotes their differentiation and proliferation into granulocytes. Human G-CSF (hG-CSF) has recently been isolated and purified from a human squamous cell carcinoma line, CHU-2 (1). This molecule is a hydrophilic glycoprotein (MW 20,000), which is composed of a disialo (p*I* 5.5) and a monosialo (p*I* 5.8) group. Recombinant hG-CSF (rhG-CSF) has been produced with a high purity and in large quantities from Chinese hamster ovary cells (2,3). The amino acid conformation and sugar structure of this rhG-CSF are confirmed to be identical to those of natural hG-CSF (4,5).

rhG-CSF is administered intravenously and subcutaneously by daily multiple dosing for the treatment of neutropenia during tumor chemotherapy, as well as for bone marrow transplantation therapy. Hence, the use of more convenient routes of administration would be preferable.

The oral bioavailability of protein drugs is low, because of low membrane transport and high enzymatic degradation in the gastrointestinal (GI) tract. Thus, nasal, buccal, rectal, vaginal, ocular, and pulmonary administration represent alternative routes with lower peptide-degrading enzyme activity. Nasal administration of protein drugs is especially promising, since this route involves easy administration, low enzyme activity, and rapid appearance of the drug in the vascular circulation (6). Nasal absorption of rhG-CSF has been reported to be effective in Sendai virus and herpes simplex virus infections (7) but with no quantitative absorption data.

In this study, we examined the relationship between the relative bioavailability and the pharmacological availability of rhG-CSF following intranasal administration to evaluate whether the nasal route was therapeutically effective. We also studied the enhancement of the nasal absorption of rhG-CSF by absorption enhancers.

MATERIALS AND METHODS

Chemicals

The drugs and absorption enhancers used in this study and their sources were as follows: sodium glycocholate from Tokyo Kasei Co. Ltd., Tokyo; polyoxyethylene 9-lauryl ether (Laureth-9) from Nikko Chemicals Co. Ltd., Tokyo; sodium pentobarbital (Nembutal injection, 50 mg/mL) from Sankyo Co. Ltd., Tokyo; and sodium phenobarbital (Phenobal, 0.1 g/mL) from Dainippon Pharmaceutical Co. Ltd., Osaka, Japan. A bulk solution of rhG-CSF (phosphate buffer solution; 440 μg/mL; sp act, 10^8 U/mg protein) was supplied by Chugai Pharmaceutical Co. Ltd., Tokyo. Other reagents were of analytical grade or better.

Preparation of rhG-CSF Solution for Intravenous, Subcutaneous, and Intranasal Administration

The pH and osmotic pressure of the original rhG-CSF bulk solution were about 8 and 174 mOsm/kg, respectively. These conditions were adjusted to pH 3–8 with 1 *N* HCl and to 174–1148 mOsm/kg with NaCl. Tween 20 was added to the solutions to make a final concentration of 0.01% (w/v), and the final rhG-CSF concentration was 250 μg/mL. For the comparison of administration routes, a rhG-CSF solution of pH 6.5 and 285 mOsm/kg was used. The effects of pH on rhG-CSF absorption were examined at pH 3, 4, 5, 6.5, and 8. The effects of osmotic pressure on rhG-CSF absorption were examined at 174, 285, 580, and 1148 mOsm/kg.

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[5] *Abbreviations used:* rhG-CSF, recombinant human granulocyte colony-stimulating factor; AUC, area under curve of plasma rhG-CSF concentration versus time or area under curve of increase ratio of total leukocyte numbers versus time; Laureth-9, polyoxyethylene 9-lauryl ether.

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The enhancing effects of 1% Laureth-9 and sodium glycocholate on nasal rhG-CSF absorption were examined at pH 6.5.

Animal Experiments for Pharmacokinetic Study

Male SD rats (8 weeks old, 250–300 g) were used (Clea Japan, Inc., Tokyo). Their femoral artery and femoral vein were cannulated with polyethylene tubing (PE-50, Clay Adams, NJ). The surgery for nasal absorption followed the method of Hirai *et al.* (8) and was carried out when animals were anesthetized with an intraperitoneal combination of sodium phenobarbital (100 mg/kg) and sodium pentobarbital (50 mg/kg). Following the intranasal administration of rhG-CSF, the rats were restrained on their backs during the absorption experiment.

Intravenous and subcutaneous administrations were performed through the femoral vein cannula and into the back skin, respectively, without anesthesia. In all experiments, doses of 10 μg/kg were used for intravenous and subcutaneous administration and doses of 100 μg/kg were used for intranasal administration.

Blood samples were collected periodically from the femoral artery for 24 hr after intravenous and subcutaneous administration, but only 8 hr after intranasal administration because of the surgery condition according to Hirai *et al.* (8).

Animal Experiment for Pharmacodynamic Study

The ratio of the increase in total leukocyte numbers following intranasal administration to the increases following intravenous and subcutaneous administration was examined to determine pharmacological availability. For all administration and blood collection, ether anesthesia was used. Intravenous and subcutaneous administrations were performed through the tail vein and the back skin of the rat, respectively. Administration into the nasal cavity of the rat was performed by micropipette. About 50 μL of blood was collected periodically from the tail vein for 48 hr following intravenous, subcutaneous, and intranasal drug administration at 9 AM. Twenty microliters of the collected samples was supplied for the determination of leukocytes.

Multiple Intranasal and Subcutaneous Dosing Experiments

rhG-CSF was administered at fixed doses (100 μg/kg intranasally and 10 μg/kg subcutaneously) at constant intervals (once a day, at 9 AM) for 6 days. As a negative control, saline solution was administered intranasally.

The enhancing effect of Laureth-9 was examined by monitoring results after a rhG-CSF solution containing 1% of this enhancer was administered intranasally three times, at 2-day intervals, at 9 AM. Total leukocyte numbers were determined at 8 hr before and after each administration.

Assay

The plasma concentration of rhG-CSF was determined by enzyme immunoassay (9). Total leukocyte numbers were counted with a Microcellcounter (Sysmex F-500, Toa Medical Electronics, Japan).

Data Analysis

The area under the curve (AUC) of the plasma rhG-CSF concentration was calculated by the trapezoidal rule, and the bioavailability was determined by the ratio of corrected AUC for dose. For pharmacological effect, the ratio of the increase in total leukocyte numbers after rhG-CSF administration from the numbers before administration (initial numbers) to the latter numbers was used, since the total leukocyte numbers have generally large intersubject variation. The pharmacological availability was determined by the ratio of AUC of the pharmacological effect vs time.

Statistical Analysis

Levels of statistical significance were assessed with Student's t test. Significant differences were judged as P values less than 0.05.

RESULTS

The time courses of the plasma concentration of rhG-CSF following intravenous (dose, 10 μg/kg), subcutaneous (dose, 10 μg/kg), and intranasal (dose, 100 μg/kg) administration are compared in Fig. 1. In the intravenous and subcutaneous dose range of 10–200 μg/kg, the AUC of plasma rhG-CSF concentration increased linearly with dose (data not shown). The plasma concentration of rhG-CSF following intranasal administration attained the peak level (about 3 ng/mL) at about 4 hr and gradually decreased thereafter. The AUCs (ng hr mL^{-1}) from 0 to 8 hr were 196.5 ± 37.5 (SE), 92.5 ± 17.3, and 18.0 ± 7.8 for intravenous, subcutaneous, and intranasal administration, respectively.

Accordingly, the bioavailabilities of rhG-CSF after intranasal administration were 0.92 ± 0.40 and 1.95 ± 0.85% for intravenous and subcutaneous administration, respectively.

The increase ratio of the total leukocyte numbers after

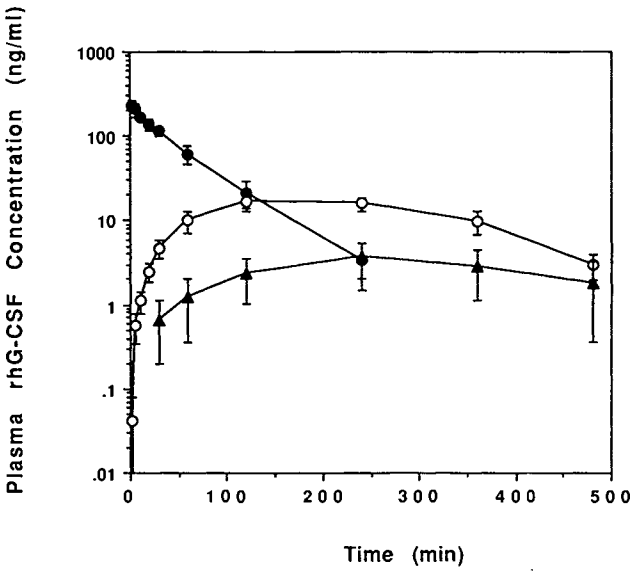

Fig. 1. Plasma concentration of rhG-CSF following intravenous (dose, 10 μg/kg; ●), subcutaneous (dose, 10 μg/kg; ○), and intranasal (dose, 100 μg/kg; ▲) administration. Values represent the mean ± SE of more than five rats.

rhG-CSF administration was used as the pharmacological effect as stated under Data Analysis. In the intravenous and subcutaneous dose range of 10–200 μg/kg, the AUC of the effect vs time curve for both administrations increased linearly (data not shown). The time courses of increase ratio of the total leukocyte numbers after rhG-CSF by three administration routes are shown in Fig. 2. The increase ratios are not significantly different at the observation times between intravenous and subcutaneous administration. The ratio increased to about 150% of the initial numbers at 4 hr and attained peak values (about 170–180% of the initial numbers) by 12 hr.

Following intranasal rhG-CSF administration, peak values (about 150%) of the initial numbers) were observed at about 4–8 hr. These results, shown in Fig. 2, were obtained with a dosing solution of pH 6.5. The increase in total leukocyte numbers was also measured after intranasal administration of rhG-CSF solutions at pH 3, 4, 5, and 8 (Table I). The AUC values were compared with those obtained after subcutaneous administration (Table I). Also in Table I, the pharmacological effects are shown by ΔAUC_{0-48}, which is the difference in the AUC_{0-48} between subcutaneous or intranasal administration and the control. The pharmacological availability following intranasal administration is shown as the ratio of ΔAUC_{0-48} following intranasal administration to that following subcutaneous administration, corrected for the dose.

The pharmacological availability at all pH values was 5–10% for 48 hr. Regarding the effects of the osmotic pressure of the dosing solution on total leukocyte numbers, there were slight increases from the control value in the AUC_{0-48} of the pharmacological effects under hypotonic (174 mOsm/kg) and isotonic (285 mOsm/kg) conditions (Fig. 3).

Table I. AUC from 0 to 48 hr (AUC_{0-48}), Differences in AUC (ΔAUC_{0-48}) Between Subcutaneous (SC) (Dose, 10 μg/kg) or Intranasal (IN) (Dose, 100 μg/kg) Administration and the Control, and Pharmacological Availability with Intranasal to Subcutaneous Administration

	AUC_{0-48} (% hr)	ΔAUC_{0-48} (% hr)[a]	Availability (%)[b]
IN control[c]	4369	—	—
SC (pH 6.5)	6164	1795	100
IN (pH 3)	6111	1742	9.7
IN (pH 4)	5219	850	4.7
IN (pH 5)	5797	1428	8.0
IN (pH 6.5)	5728	1359	7.6
IN (pH 8)	5712	1343	7.5

[a] ΔAUC_{0-48} = AUC $(SC)_{0-48}$ − AUC (IN control)$_{0-48}$.
[b] Availability = [ΔAUC $(IN)_{0-48}$/dose (IN)]/[ΔAUC $(SC)_{0-48}$/dose (SC)].
[c] Saline solution was administered intranasally.

The effects of 1% Laureth-9 and 1% sodium glycocholate on nasal rhG-CSF absorption are shown in Figs. 4 and 5. In the presence of Laureth-9, the peak level of the pharmacological effect was 1.5-fold that without the enhancer (Fig. 4).

Glycocholate had no effect on ΔAUC_{0-48} in the group where rhG-CSF was administered without surfactant, but Laureth-9 increased it (Fig. 5) about three times. These Laureth-9 enhancing effects were also found in the plasma concentration of rhG-CSF, shown in Fig. 6; the concentration was increased 10- to 100-fold by Laureth-9. The bioavailabil-

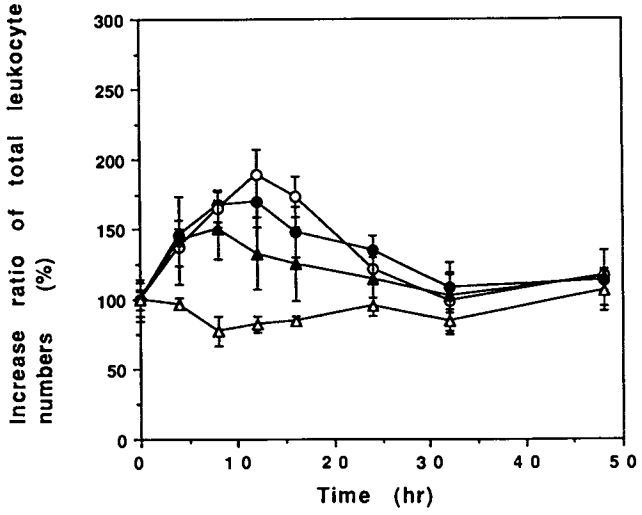

Fig. 2. Leukocyte numbers following intravenous (●), subcutaneous (○), and intranasal (▲) administration of rhG-CSF. In the control (△), saline solution was administered intranasally. Values on the ordinate represent the increase ratios of total leukocyte numbers; 100% indicates the initial value before administration. Doses are the same as those given in the legend to Fig. 1. Values are the mean ± SE of more than four rats.

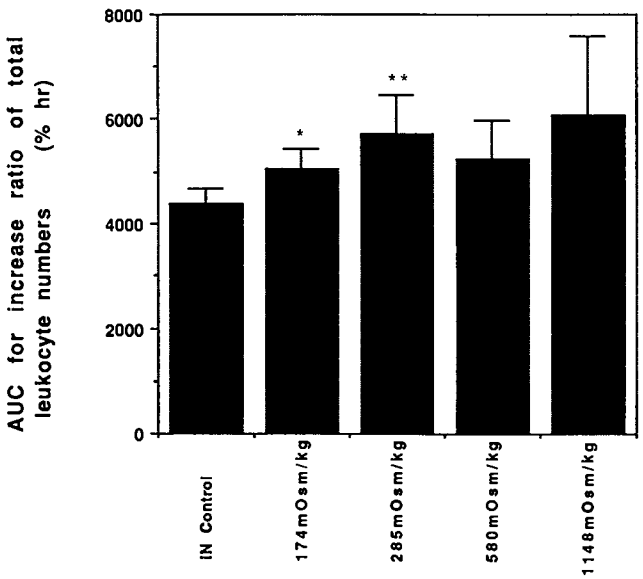

Fig. 3. Effects of osmotic pressure of dosing solution on AUC from 0 to 48 hr (AUC_{0-48}) on the increase ratio of total leukocyte numbers versus time following intranasal administration of rhG-CSF. Doses are the same as those given in the legend to Fig. 1. Values of the columns are the mean ± SE of more than four rats. (**) $P < 0.01$ vs IN control; (*) $P < 0.05$ vs IN control.

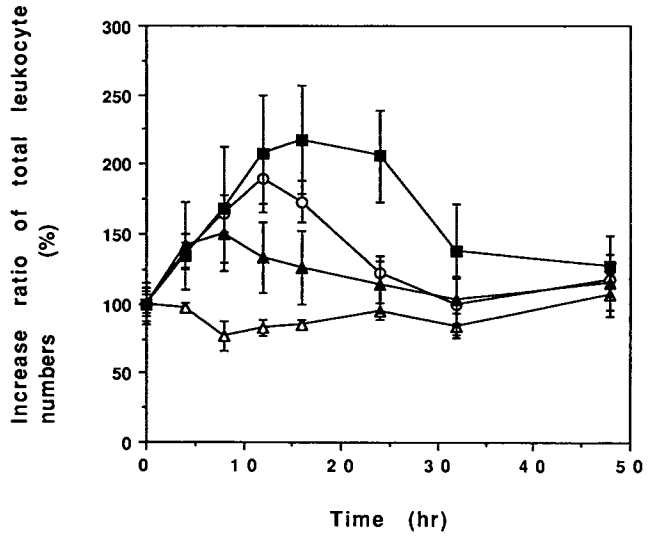

Fig. 4. Leukocyte numbers following subcutaneous (dose, 10 μg/kg; ○) and intranasal (dose, 100 μg/kg; ▲) administration, and intranasal administration with 1% Laureth-9 (dose, 100 μg/kg; ■), of rhG-CSF. In the control (△), saline solution was administered intranasally. Data for subcutaneous administration, intranasal administration without enhancer, and the control are the same as those shown in Fig. 2. Values are the mean ± SE of four rats for intranasal administration with Laureth-9.

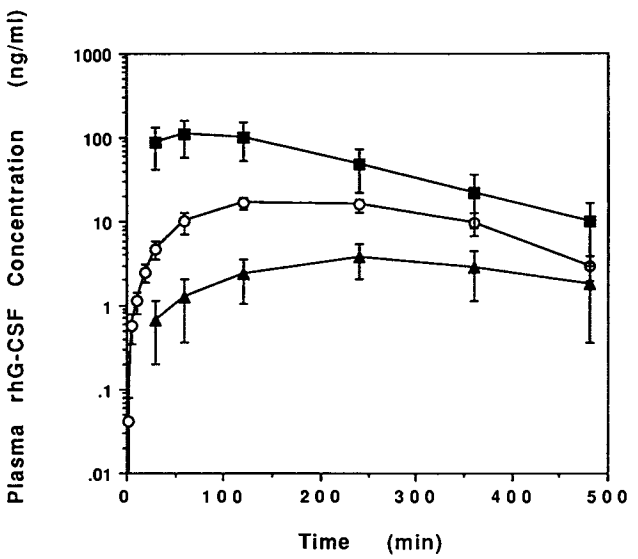

Fig. 6. Plasma concentration of rhG-CSF following subcutaneous administration (dose, 10 μg/kg; ○), intranasal administration without enhancer (dose, 100 μg/kg; ▲), and intranasal administration with 1% Laureth-9 (dose, 100 μg/kg; ■). Data for subcutaneous and intranasal administration are the same as those shown in Fig. 1. Values for intranasal administration with Laureth-9 are the mean ± SE for five rats.

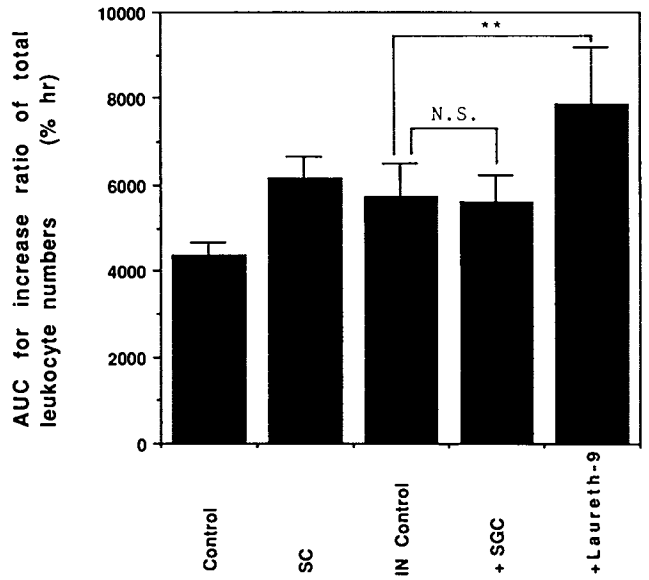

Fig. 5. Effects of 1% sodium glycocholate (SGC) and 1% Laureth-9 in the dosing solution on AUC from 0 to 48 hr (AUC_{0-48}) on the increase in total leukocyte numbers following intranasal (IN) administration of rhG-CSF. In the intranasal control (IN control), saline solution was administered intranasally. Data for subcutaneous administration, intranasal administration without enhancer, and control are the same as those shown in Fig. 2. Doses are the same as those shown in the legend to Fig. 1. Values of the columns are the mean ± SE of more than four rats. (**) $P < 0.01$ vs IN control; NS, not significant.

ities were increased to 21.1 ± 13.7 and 44.7 ± 29.0% by Laureth-9 for intravenous and subcutaneous administration, respectively.

Figure 7 shows changes in the total leukocyte numbers following multiple subcutaneous and intranasal once-daily administration of rhG-CSF for 6 days. Both administration routes led to increases in leukocyte numbers and these were

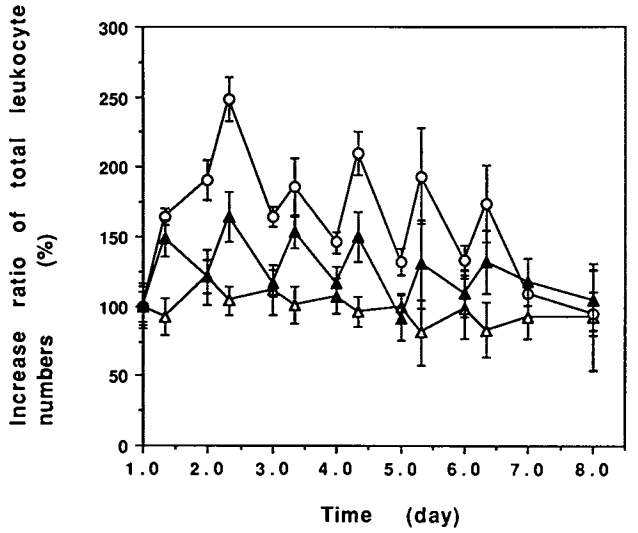

Fig. 7. Leukocyte numbers following multiple subcutaneous (dose, 10 μg/kg; ○) and intranasal (dose, 100 μg/kg; ▲) administration of rhG-CSF once a day for 6 days. In the control (△), saline solution was administered intranasally. Data are the mean ± SE of more than four rats.

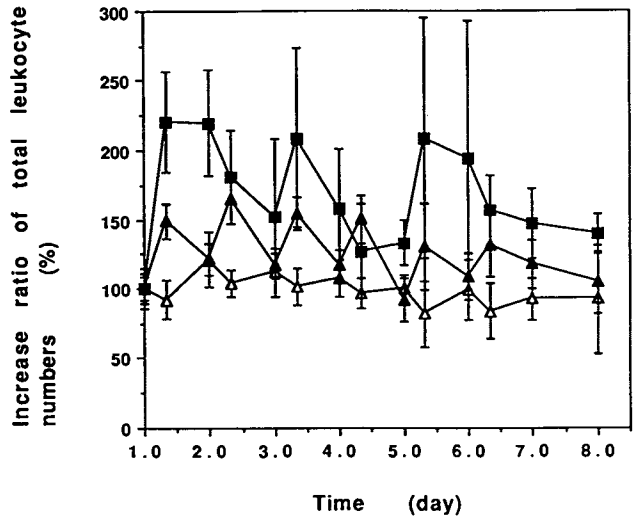

Fig. 8. Leukocyte numbers following multiple intranasal administration of rhG-CSF (100 μg/kg) without Laureth-9 (▲) and with 1% Laureth-9 (■) once every other day for 6 days. Data for intranasal administration of rhG-CSF (▲) and for the control (△) are the same as those shown in Fig. 8. Data for intranasal administration are the means ± SE of more than four rats.

kept in an almost constant pattern. After this rhG-CSF administration was finished, the leukocyte numbers soon returned to the control level.

Multiple intranasal administration with Laureth-9 was performed once every other day for 6 days; this was compared with intranasal administration without enhancer, performed once a day for 6 days (Fig. 8). In the latter case, without Laureth-9, leukocyte numbers always returned to the control level (the minimum level) by the next administration, but the addition of Laureth-9 increased the minimum level to a significantly higher level than the control level and kept it at a steady state.

DISCUSSION

The absolute bioavailability of rhG-CSF for nasal absorption for 8 hr was 0.92 ± 0.40% (SE) and this value was not contradictory to the value of 0.5% obtained from the relationship between absorption and molecular weight, presented by MacMartin *et al.* (10). The relative bioavailability of rhG-CSF following intranasal administration versus subcutaneous administration, for a dosing solution of pH 6.5, determined from the AUC for the plasma concentration for 8 hr, was about 2.0% (Fig. 1). This low bioavailability was one-sixth the pharmacological availability determined from the AUC for the increase in total leukocyte numbers for 48 hr, about 7.6% (Fig. 2, Table I). Accordingly, the plasma concentration of rhG-CSF did not always reflect its pharmacological effects.

Hirai *et al.*, in their examination of the enhancement of nasal insulin absorption by surface active agents (11), found that some bile salts, such as sodium glycocholate, enhanced the membrane permeability of protein drugs and inhibited their decomposition by peptidases. In this study, we found that sodium glycocholate did not enhance nasal rhG-CSF absorption but that Laureth-9 did have enhancing effects (Figs. 4–6). Our results suggest that sodium glycocholate did not effectively inhibit the enzymatic decomposition of rhG-CSF. Enhancing effects of Laureth-9 have also been reported on the absorption of insulin by Hirai *et al.* (11), methyl-human growth hormone by Daugherty *et al.* (12), and PEG 2000 by Donovan *et al.* (13). However, the enhancing mechanism of Laureth-9 may be related to nasal membrane damage (11), and thus the recovery rate of the membrane should be considered when setting up dosing intervals.

Multiple nasal administration of rhG-CSF once a day for 6 days maintained the increased level of leukocyte numbers; after the treatment period, the values returned to the constant control level (Fig. 7). When rhG-CSF was administered together with Laureth-9 in multiple doses once every other day for 6 days, the pharmacological effect was maintained at a higher steady state level than that found during multiple dosing without an enhancer (Fig. 8).

In conclusion, pharmacological effects after nasal dosing occurred rapidly, similar to the pattern observed after intravenous and subcutaneous administration (Fig. 2). Multiple administration maintained the pharmacological effect and coadministration with Laureth-9, at a reduced frequency, resulted in greater pharmacological effects than administration without an enhancer.

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