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Effect of organic acids, trypsin inhibitors and dietary protein on the pharmacological activity of recombinant human granulocyte colony-stimulating factor (rhG-CSF) in rats

Yoshiteru Ushirogawa, Yoshinori Nakahigashi, Akiko Kiriyama and Kanji Takada

Department of Pharmaceutics and Pharmacokinetics, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607 (Japan)

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Summary

The effects of organic acids, trypsin inhibitors and dietary protein on the oral availability of recombinant human granulocyte colony-stimulating factor (rhG-CSF) was investigated in rats by measuring the blood total leucocyte (BTL) counts. As organic acids, tartaric, succinic and citric acids were used at 2.30–7.68 mg/kg. Two trypsin inhibitors, soybean trypsin inhibitor (SBTI, 0.8–3.2 mg/kg) and chicken egg white trypsin inhibitor (CETI, 1.92 mg/kg), together with ovalbumin (0.80 mg/kg) as a representative of dietary proteins were employed with co-administration of citric acid (4.61 mg/kg). After intraduodenal administration of several kinds of test rhG-CSF solutions to rats, blood samples were collected for 48 h and the BTL counts were determined. The results are expressed as relative increase in BTL counts as compared to that before administration of the dose. The areas under the curves (AUC; % BTL increase × h) obtained for test solutions containing tartaric, succinic and citric acids were 2.1, 1.1 and 5.1 times greater than that of the control experiment, where rhG-CSF solution buffered with phosphate buffer (pH 6.1) was used. On administration of SBTI (3.20 mg/kg) and CETI (1.92 mg/kg) in combination with citric acid (4.61 mg/kg), the AUC values were determined to be 6.5 and 5.1 times greater than that of the control experiment, respectively. Co-administration of ovalbumin (0.80 mg/kg) with citric acid (4.61 mg/kg) also increased the AUC 6.3-fold as compared to the control. However, a low dose of SBTI (0.80 mg/kg) co-administered with citric acid did not exert a synergistic action.

Introduction

Several colony-stimulating factors have been shown to generate granulocytes from immature hematopoietic progenitor cells (Sherr et al., 1989; Sherr, 1990). Among them, granulocyte colony-stimulating factor (G-CSF) is a hematopoietic glycoprotein controlling the proliferation of granulocytes and macrophages (Nicola, 1987). The gene encoding human G-CSF was isolated and molecularly cloned. Human G-CSF has been expressed as recombinant protein (Nagata et al., 1986; Souza et al., 1986). Recombinant human

Correspondence: K. Takada, Department of Pharmaceutics and Pharmacokinetics, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, Japan.

G-CSF (rhG-CSF) is also capable of supporting the formation of granulocyte colonies from committed precursor cells (Souza et al., 1986; Cohen et al., 1987). In addition, in patients with various cancers, rhG-CSF has been applied in order to accelerate hematological recovery after high-dose chemotherapy with and without autologous bone marrow rescue, and has been found to result in marked shortening of the duration of neutropenia (Bronchud et al., 1987; Teshima et al., 1989). Thus, rhG-CSF dramatically affects the systemic level of eosinophils.

The clinical use of rhG-CSF is limited to the i.v. route. However, our previous report showed that intraduodenally administered rhG-CSF is available, as judged from the increase in total blood leucocyte counts, in rats where pharmaceutical additives such as a surface-active agent and an organic acid were used (Takada et al., 1989). However, the dose of rhG-CSF in this study was considerably high, 300–600 μg/kg. In the case of administration of rhG-CSF into the gastrointestinal (GI) tract, a considerable amount of rhG-CSF encounters hydrolysis in the GI tract by digestive enzymes before absorption into enterocytes. Our previous study suggested that the rate of hydrolysis of rhG-CSF in the GI tract is significantly decreased on addition of organic acids and when in the presence of inhibitors such as trypsin inhibitors and dietary proteins in competition with hydrolytic enzymes (Takada et al., 1991). Furthermore, a number of papers have suggested that orally administered peptides or proteins such as insulin (Kidron et al., 1982) and vasoactive peptide (Saffran et al., 1988) exhibit pharmacological activities during in vivo experiments on rats. However, the components used to increase the oral availability of insulin and vasoactive peptide are not permitted for general use as pharmaceutical additives.

In this report, the effects of organic acids, dietary trypsin inhibitors and dietary protein on the pharmacodynamics of rhG-CSF have been investigated by performing in vivo experiments on rats after the intraduodenal administration of several kinds of test rhG-CSF solutions and the efficiency of these compounds as pharmaceutical additives has been confirmed.

Materials and Methods

A solution of rhG-CSF (250 μg/ml) was obtained from Kirin Brewery Co., Ltd (Tokyo, Japan). Anhydrous tartaric, succinic and citric acids were supplied by Wako Pure Chemical Co. Ltd (Osaka, Japan). Crude soybean trypsin inhibitor (SBTI; type II-S) and partially purified chicken egg white trypsin inhibitor (CETI; type II-O) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Ovalbumin was obtained from Wako Pure Chemical Co. All other reagents were commercial products of reagent grade.

Preparation of test solution

The rhG-CSF test solutions containing tartaric and succinic acids (both at 150 mM) were prepared by dissolving 11.25 mg of tartaric acid and 8.86 mg of succinic acid in 0.5 ml of rhG-CSF solution, respectively. Test solutions of rhG-CSF containing 75, 150 and 250 mM citric acid were prepared by dissolving 7.2, 14.4 and 24.0 mg, respectively, of citric acid in 0.5 ml of rhG-CSF solution. The control rhG-CSF test solution was prepared by addition of 1 ml of phosphate buffer to 0.5 ml of the rhG-CSF solution. The pH of the resulting test solution was 6.1. The test solution of rhG-CSF containing SBTI was prepared by dissolving 14.4 mg of citric acid and 2.5 or 10.0 mg of SBTI in 0.5 ml of rhG-CSF solution. The rhG-CSF test solution containing CETI was also prepared by dissolving 14.4 mg of citric acid and 6.0 mg of CETI in 0.5 ml of rhG-CSF solution. The test solution of rhG-CSF containing ovalbumin was prepared by addition of 14.4 mg of citric acid and 2.5 mg of ovalbumin to 0.5 ml of the rhG-CSF solution and leaving the resulting mixture to stand in a refrigerator at 4°C for a period of 24 h. The concentration of rhG-CSF in each test solution was 250 μg/ml.

Animal study

Three male Wistar rats (SLC, Hamamatsu, Japan), weighing 300–400 g, were used in each experimental group. Under anesthesia induced by an intraperitoneal injection of sodium pentobarbital (45 mg/kg), midline incision was performed. Test drug solution was administered to rat duo-

denum using a microsyringe through a pore on the gut near the pylorus. The dose of rhG-CSF was 40 μg/kg for all groups. Group I rats correspond to the control experimental group and received 480 μl/kg of control rhG-CSF test solution. The other groups of rats received test solutions containing organic acids, trypsin inhibitors or dietary protein. Two groups, namely, nos II and III were treated with 160 μl/kg of test rhG-CSF solution containing tartaric acid (3.60 mg/kg) and succinic acid (2.84 mg/kg), respectively. Group IV comprised nine rats on which the effect of adding different amounts of citric acid were investigated. Rats belonging to group IV-1–IV-3 were given the test rhG-CSF solution containing various amounts of citric acid, i.e., 2.30, 4.61 and 7.68 mg/kg, respectively. Group V-1 and V-2 rats received 160 μl/kg of test rhG-CSF solution containing citric acid (4.61 mg/kg) and different amounts of SBTI (0.80 and 3.20 mg/kg, respectively). Group V-3 rats were treated with the same test rhG-CSF solution in the middle region of the small intestine, where the respective doses of SBTI and citric acid were 3.20 and 4.61 mg/kg. Group VI rats received the test rhG-CSF solution containing citric acid (4.61 mg/kg) and CETI (1.92 mg/kg). Group VII rats were given 160 μl/kg of test rhG-CSF solution containing citric acid (4.61 mg/kg) and ovalbumin (0.80 mg/kg). Another group of three rats were used as a sham-operated group and received saline (160 μl/kg).

Before drug administration, 0.1 ml of blank blood sample was removed by puncturing the tail artery. After drug administration, the pore made in the gut was closed with a drop of tissue cement (Aron Alpha®, Sankyo Co. Ltd, Tokyo). Single blood samples (100 μl) were taken by arterial puncture of the tail after drug administration at 6, 9, 15, 18, 24, 32 and 48 h. The BTL counts were determined manually on gentian violet-stained blood smear: briefly, 0.9 ml of Turk solution was added to 100 μl of the blood sample, followed by staining of the leucocytes. BTL counts were evaluated under an ultramicroscope. The BTL count is expressed as a relative value, deter-

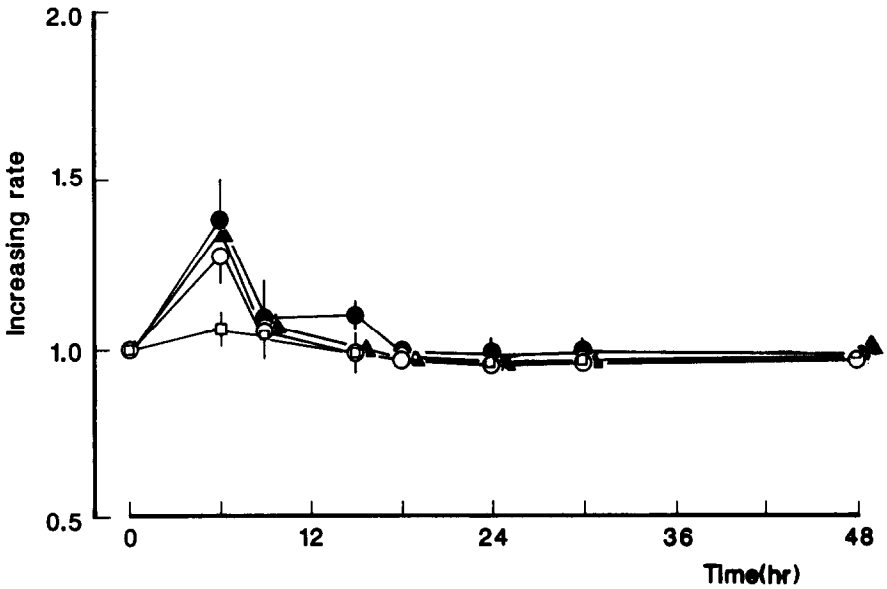

Fig. 1. Effect of organic acids as additives on blood total leucocyte dynamics after administration of rhG-CSF solution (40 μg/kg). (□) Sham-operated rats; (○) rhG-CSF solution, pH 6.1 (control, group I); (●) rhG-CSF solution + tartaric acid (group II); (▲) rhG-CSF solution + succinic acid (group III). Each point represents three individual determinations, and is expressed as the mean ± S.E.

mined by dividing the BTL count at each time point after drug administration by the respective control value, namely, the pre-dosing BTL count.

Statistics

Statistical differences were assumed to be reproducible when $p < 0.05$ (two-sided t-test).

Results

The BTL dynamics after intraduodenal injection of two kinds of test rhG-CSF solution containing organic acid in groups II and III rats are represented in Fig. 1 along with that of the control and sham-operated groups. Group I (i.e., the control group) received rhG-CSF test solution (pH 6.1). In group I rats, the maximum increase in BTL count was 127% and was observed at 6 h after administration. In rats of groups II and III, the peak BTL count appeared at 6 h and the maximum increases amounted to 138 and 134%, respectively.

The effect of the amount of citric acid added on the BTL dynamics of rhG-CSF is shown in Fig. 2. The peak BTL count was significantly increased in group IV-2 and IV-3 rats which received rhG-CSF test solution containing citric acid (4.61 and 7.68 mg/kg) as compared to the groups (nos II and III) receiving the other organic acids. The increases were observed to amount to 170 and 175%, respectively. Although the effect of citric acid appeared at high doses (4.61 and 7.68 mg/kg) no significant difference in the BTL increases was found between the two doses. However, citric acid was observed to have a weaker effect on lowering of the dose to 2.30 mg/kg.

Fig. 3 depicts the results obtained when test solutions containing both citric acid (4.61 mg/kg) and SBTI (0.80 and 3.20 mg/kg) were administered to group V-1 and V-2 rats. In the case of the test solution containing SBTI (0.80 mg/kg; group V-1), the increase in the rate of BTL was less than that of the control group. However, on increasing the dose of SBTI to 3.20 mg/kg, the maximum increase in BTL was determined to be 165%. Furthermore, the duration of the increase in BTL was prolonged to 24 h. This result suggests that intraduodenally administered rhG-CSF

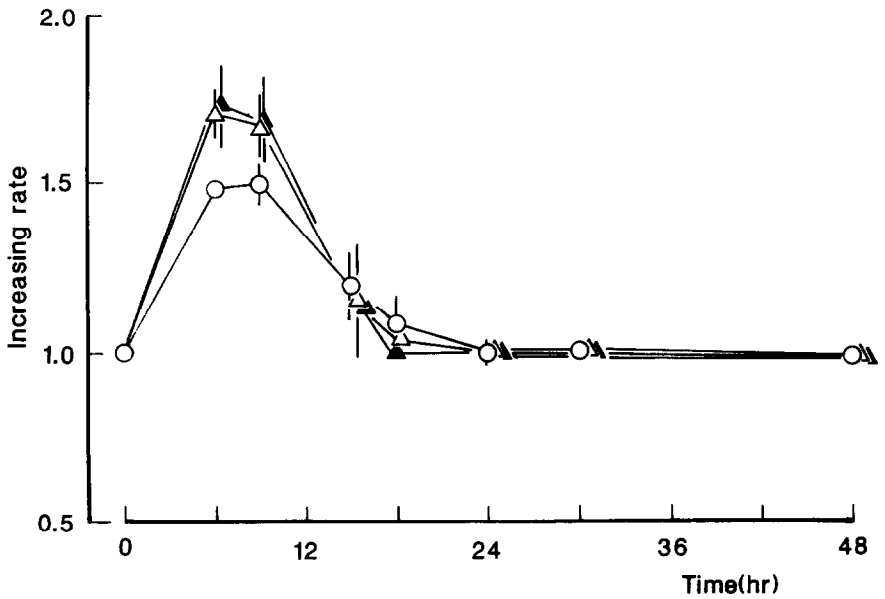

Fig. 2. Effect of concentration of citric acid on blood total leucocyte dynamics after administration of rhG-CSF solution (40 μg/kg). [Citric acid]: (○) 2.30 mg/kg (group IV-1); (△) 4.61 mg/kg (group IV-2); (▲) 7.68 mg/ml (group IV-3). Each point represents three individual determinations, and is expressed as the mean ± S.E.

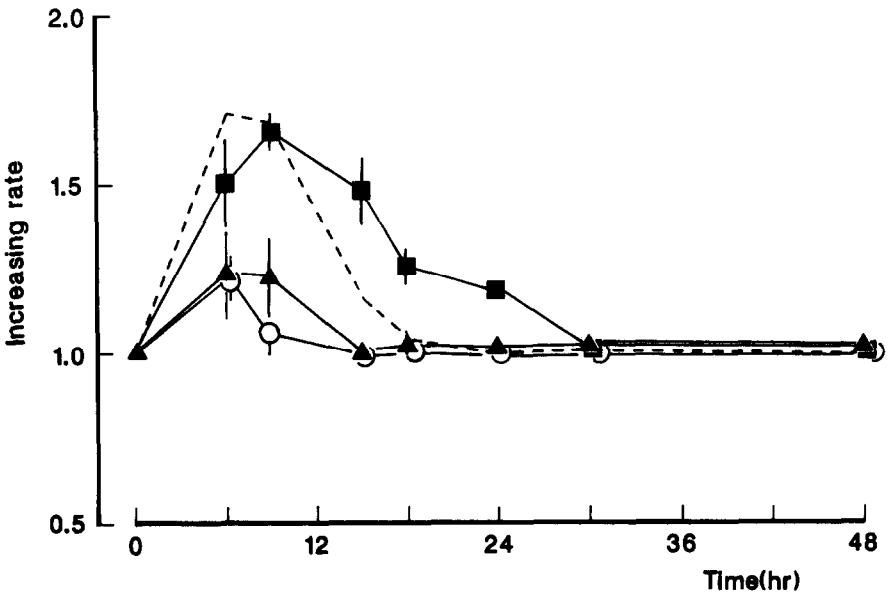

Fig. 3. Effect of concentration of SBTI on blood total leucocyte dynamics after administration of rhG-CSF solution (40 μg/kg). Each of the following test solutions contained citric acid at the same concentration (4.61 mg/kg): (■) + SBTI (3.2 mg/kg) (group V-1); (▲) + SBTI (0.8 mg/kg) (group V-2); (○) + SBTI (0.8 mg/kg) (group V-3: for this group, test solution was administered to the middle region of the small intestine); (- - - -) no SBTI added (group IV-2). Each point represents three individual determinations, and is expressed as the mean ± S.E.

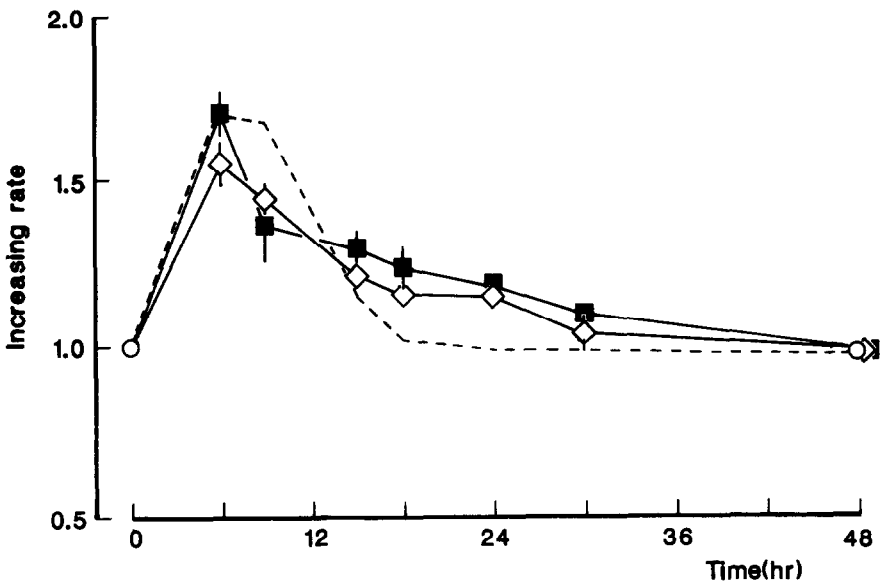

Fig. 4. Effect of ovalbumin and CETI on blood total leucocyte dynamics after administration of rhG-CSF solution (40 μg/kg). Each of the following test solutions contained the same concentration of citric acid (4.61 mg/kg): (■) + CETI (1.92 mg/kg) (group VI); (◇) + ovalbumin (0.8 mg/kg) (group VII); (- - - -) no addition (group IV-2). Each point represents three individual determinations, and is expressed as the mean ± S.E.

TABLE 1

Comparison of biological activities among rhG-CSF solutions

Parameter	rhG-CSF test solution							
	Control	Tartaric acid (3.60 mg/kg)	Succinic acid (2.8 mg/kg)	Citric acid (4.61 mg/kg)	Citric acid (4.61 mg/kg) + SBTI (0.8 mg/kg)	Citric acid (4.61 mg/kg) + SBTI (3.2 mg/kg)	Citric acid (4.61 mg/kg) + CETI (1.92 mg/kg)	Citric acid (4.61 mg/kg) + ovalbumin (0.8 mg/kg)
AUC [a]	148 ± 52 [b]	293 ± 141 [b]	170 ± 13 [b]	755 ± 117 [b]	270 ± 54 [b]	972 ± 114 [b]	754 ± 71 [b]	937 ± 134 [b]
Relative rate of increase (%) [b]	100	197	115	510	182	657	509	633

[a] AUC was determined to represent the total pharmacological activities of the test solutions and was calculated by multiplying the % increase in BTL count with time (h). Each value represents the mean ± S.E.

[b] Indicates a statistically significant difference from the value for the control experiment, determined by Student's *t*-test ($p < 0.05$).

is more readily available on addition of SBTI (3.20 mg/kg). On the other hand, when test solution containing citric acid (4.61 mg/kg) and SBTI (3.20 mg/kg) was administered into regions midway along the small intestine, about 20 cm distal from the duodenum, the increase in BTL was small. This result suggests that rhG-CSF administered in such a manner is less readily available than when administered into the upper regions (duodenum) of rat small intestine.

The effects of CETI (group VI) and ovalbumin (group VII) on the pharmacodynamics of rhG-CSF administered into rat duodenum as solutions are represented in Fig. 4. In both cases, the maximum increase in BTL appeared at 6 h following administration and the duration of the increase was prolonged to 24 h.

In order to evaluate the total pharmacological activities of the test solutions investigated, the area under the curve (AUC; % BTL increase × h) was calculated, resulting in the data listed in Table 1. The first row in Table 1 shows the authentic AUC value for each test solution. The second row was determined by dividing the AUC value of each test solution by that of the control solution (observed in group I rats). These values therefore provide a relative index for the pharmacological activity of test solutions. The effects of tartaric acid (group II) and succinic acid (group III) on the increase in AUC were not significantly different from that of the control study (group I). The test solution containing citric acid (4.61 mg/kg; group IV-2) exhibited a 5.1-fold increase in AUC. Furthermore, the AUC increased to a 6.5- or 6.3-fold greater value compared to that observed in the control group on the addition of SBTI (3.2 mg/kg) or ovalbumin (0.8 mg/kg).

Discussion

Our previous report (Takada et al., 1991) suggested that protective action was exerted by both citric and tartaric acids on the hydrolysis of rhG-CSF during the study of in vitro stability. However, citric acid gave rise to a markedly greater activity of rhG-CSF as compared to tartaric acid in the present in vivo pharmacodynamic study. This difference is probably ascribable to the absorption-enhancing effect of citric acid. Indeed, citric acid has been reported to have an absorption-enhancing effect on both heparin (Sue et al., 1976) and LHRH (Okada et al., 1982). In our study, the effect of citric acid decreased at 2.30 mg/kg, although it was almost identical to those observed at 4.61 and 7.68 mg/kg. According to the data of Cho et al. (1989), using an epithelial monolayer cell transport system, the absorption enhancing effect of citric acid appeared at 0.1 mM. Even when 50 μl of the test solution containing 2.30 mg/kg citric acid (namely, 75 mM) was administered into rat duodenum after 100-fold dilution with GI fluid, absorption was still observed to be enhanced by citric acid. It may therefore be stated that the reduction in the effect of citric acid at 2.30 mg/kg is not due to the enhancement of absorption but, rather, to the decrease in the pH shift produced by citric acid. SBTI demonstrated the strongest degree of protection against hydrolysis of rhG-CSF in our in vitro experiment (Takada et al., 1991).

SBTI elicits a considerably greater increase in BTL when used in combination with citric acid. In particular, the duration of the increase induced in BTL was observed to be prolonged when using a high concentration of SBTI (3.2 mg/kg). According to the concept of Schneeman et al. (1975), trypsin in the upper small intestine prevents the release of cholecystokinin (CCK) into the blood and SBTI effectively removes trypsin from the intestine through binding to trypsin, thereby releasing CCK and increasing pancreatic enzyme output. Consequently, the addition of a small amount of SBTI induced the secretion of enzyme from the pancreas, and, as a result, the hydrolysis of rhG-CSF in the GI tract was accelerated. However, as CCK may be involved in such a negative feed-back regulatory mechanism, its release into the bloodstream should display saturation behavior (Green et al., 1972; Ihse et al., 1977). Namely, the initial effect of trypsin-bound SBTI would be to cause the rapid release of stored CCK from intestinal cells, accompanied by the induction of a maximum in the extent of enzyme secretion. Exhaustion of CCK stores would occur on continuous removal

of trypsin and the new level of basal secretion would be at the maximum for the rate at which new CCK could be synthesized and released. On the depletion of the entire CCK stores reaching completion, the rate of release of CCK would become dependent on the rate of production in the cells. Since even a slight shortage of trypsin in the duodenum induces saturation of the rate of release of CCK into the bloodstream, inhibition of the remaining trypsin by SBTI should not affect enzyme secretion from the pancreas through the participation of CCK. Therefore, the addition to test solutions of SBTI in excess amounts over that necessary to induce saturation behavior in enzyme secretion from the pancreas should result in the overproduction of pancreatic enzyme being inhibited by SBTI. The above hypothesis is consistent with our results, demonstrating the addition of a high dose of SBTI (3.2 mg/kg) to bring about an increase in BTL counts.

Taking into account the proposal that the site of CCK production is located in the upper regions of the small intestine, a test solution containing citric acid and SBTI was administered to an area midway along the small intestine in order to avoid the secretion of pancreatic enzyme via a mechanism of feed-back regulation. Kidron et al. (1982) observed considerable decreases in blood glucose levels following the ileal administration of a test solution of insulin supplemented with SBTI and sodium deoxycholate. However, no significant pharmacological effect of rhG-CSF was detectable during our examination of the administration of a test solution containing SBTI and citric acid to the middle part of the small intestine. In a preceding study (Takada et al., 1991), we demonstrated the lack of any significant difference in hydrolytic enzyme activity between the upper and middle regions of rat small intestine. Hence, the main site of absorption of rhG-CSF is believed to be located in the upper region of the small intestine of the rat.

In addition, CETI was also investigated in the present study. According to our previously reported data (Takada et al., 1991), the dose of CETI employed (1.92 mg/kg) was equal to SBTI (0.80 mg/kg) in the extent of inhibition of trypsin. CETI also led to a significant increase in BTL counts. We may therefore conclude that the inhibitory effect of CETI on feed-back regulation is weaker than that of SBTI. For dietary proteins such as casein, pancreatic enzyme secretion via CCK has previously been reported to be induced on intraduodenal administration of dietary protein induction of pancreatic enzyme secretion through inhibition of feed-back regulation. Green et al. (1972) reported this effect to be much weaker for casein than for SBTI. Furthermore, Fushiki et al. (1989) determined the extent of such an effect in the case of ovalbumin to be about 50% lower than that for casein. The reason for the observation of the above phenomenon should be attributable to the characteristic binding between inhibitors and dietary protein. Binding of dietary protein to hydrolytic enzyme is only a temporary process, whereas in the case of inhibitors this takes place irreversibly and the resultant complex exists as a crystalline structure (Sweet et al., 1974). Hence, the influence of inhibitors is strong and is believed to be sustained over prolonged periods of time and to lead to considerable acceleration of the process of release of CCK. Ovalbumin is thus considered to have a much wider range of applicability.

In this study of the effects of organic acids, trypsin inhibitors and dietary protein on the pharmacodynamics of rhG-CSF when administered to rat duodenum, we have found that, among the organic acids, citric acid was efficient in eliciting an increase in BTL counts. Furthermore, the results suggest the importance of trypsin inhibitors and dietary protein as pharmaceutical additives during administration in combination with citric acid.

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