The administration of granulocyte-macrophage colony-stimulating factor (GM-CSF) to patients with severe active infections has been questioned because activation of neutrophils may cause tissue injury. To identify the effect of GM-CSF administration on severe sepsis, we examined the survival rate and pathologic changes in vital organs using the rat lethal sepsis model. Rats received 20 μg of recombinant murine GM-CSF (rmGM-CSF) 3 hours after the onset of peritonitis induced by cecal ligation and puncture. After 48 hours, the survival rate did not improve, and earlier deaths than in the control group were observed. In addition, the inhibition of early leukocyte sequestration in the peritoneal cavity was seen in animals treated with GM-CSF. These results suggested that the administration of rmGM-CSF after the onset of sepsis was not beneficial; thus, we concluded that care should be taken in the clinical use of GM-CSF in severe infection.

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MATERIALS AND METHODS

CLP model. Male Wistar rats (Japan Animal, Osaka, Japan) weighing 200 to 250 g were used for all experiments. The procedure of Wichterman et al.18 was adapted to induce septic peritonitis. After overnight fasting, CLP was performed under light ether anesthesia. After a 3-cm midline incision was made, the cecum was ligated with 3-0 silk below the ileocecal valve and then punctured twice with an 18-gauge needle. Physiologic saline (5 mL) was given subcutaneously for resuscitation and animals were then allowed free access to water. The experiment was performed under the ethical guidelines for animal experiments of Osaka University Medical School.

rmGM-CSF treatment. rmGM-CSF was a generous gift from Kirin Brewery Co, Ltd (Tokyo, Japan). Because rmGM-CSF is known to be active in Wistar rats,19 we used this instead of rat or human GM-CSF in the present study. For the experimental dose of rmGM-CSF, preliminary experiments showed that, when normal rats were administered various doses of rmGM-CSF subcutaneously, 20 μg rmGM-CSF showed an apparent increase in the circulating leukocyte count, ie, threefold the normal count within 6 hours which decreased to the initial level within the following 24 hours. The plasma GPT level did not change after administration of rmGM-CSF (20 μg) to normal rats. There were no pathologic findings and, thus, no toxic side effects of rmGM-CSF on vital organs, and so we chose this dose of rmGM-CSF in the present in vivo experiments.

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Rats were subcutaneously injected with rmGM-CSF (20 μg) at a volume of 0.3 mL 3 hours after the induction of CLP because the first occurrence of bacteremia occurred 6 hours after induction in this model. Control rats received the same volume of physiologic saline. GM-CSF was administered 3 hours after CLP to compare the effect of G-CSF administration. In a previous study, G-CSF (15 μg) administration 3 hours after CLP was effective in reducing the mortality, but, when administered 6 hours after CLP, it was not effective. When G-CSF was administered at the same time as or 3 hours before CLP, there was also an improvement in survival, but it was less effective. As the therapeutic efficacy of GM-CSF was investigated, GM-CSF was not administered before or at the same time as induction of CLP. All experiments were conducted by the same person using the same general procedure, and survival experiments were performed blind. Blood samples were taken aseptically from the tail vein at suitable time intervals for hematologic and biochemical examinations. Solutions of rmGM-CSF and physiologic saline were not contaminated with endotoxin as determined by endotoxin analysis (Toxicolor test; Seikagaku Kogyo, Co, Ltd, Tokyo, Japan).

Neutrophil counts in peritoneal lavage fluid. Peritoneal neutrophils were obtained by peritoneal lavage from rats 6 or 18 hours after CLP. After laparotomy and under direct vision, 10 mL phosphate-buffered saline (PBS) was injected into the peritoneal cavity with care to avoid puncturing any abdominal visera or lacerating the abdominal wall. Animals were gently agitated, after which lavage fluid was aspirated into a sterile 10-mL syringe, and the same procedure was then repeated. Cell separation was performed using a Ficoll-Paque density gradient that was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Cells were centrifuged at 450g for 25 minutes. Contaminating red blood cells were removed by hypotonic lysis and then centrifuged at 150g for 5 minutes. The resultant pellet that was rich in neutrophils was resuspended in cold PBS and centrifuged at 150g for 5 minutes. After the final wash, cells were resuspended in 1.0 mL PBS and counted using a hemocytometer.

Histologic examination. After rats were killed and bled, the heart was flushed with normal saline. The liver, lungs, cecum, spleen, and kidneys were fixed in buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. Histologic examinations were performed by a pathologist (N.M.) in a blind manner.

Statistical analysis. Each group was composed of six rats. Survival data were analyzed using the Kaplan-Meier method, and variations are expressed as the standard deviation of the mean. Two-tailed P values were calculated using Student's t-test for each analysis, and significance was determined or assumed when P < .05. Statistical analysis was performed on a Macintosh SE microcomputer using StatView SE+Graphics software (Abacus Concepts, Inc, Berkeley, CA).

RESULTS

Figure 1 shows the difference in survival rate after induction of CLP. Single subcutaneous administration of 20 μg rmGM-CSF 3 hours after induction of CLP showed no improvement in 48-hour survival rate compared with that for the control CLP group. The 12-hour survival rate in the rmGM-CSF group was significantly higher than that in the control group. The survival rate of rats treated with rmGM-CSF was 80%, while that of rats treated with saline was 0% (0 of 5; control, 16.7% (2 of 12)).

Fig 1. Survival rates after the induction of CLP in rats are shown. (C) rmGM-CSF (20 μg) or (S) saline (0.3 mL) was administered subcutaneously at 3 hours after surgery, which was performed at time 0.

Fig 2. The effect of rmGM-CSF on peripheral blood leukocyte counts (A) and neutrophil counts (B) after CLP is shown. (C) saline (0.3 mL) was administered subcutaneously at 3 hours after surgery, which was performed at time 0.

Fig 3. Histologic examination of the liver 12 hours after CLP is shown. (A) CLP treated with saline (0.3 mL); (B) CLP treated with rmGM-CSF (20 μg/body).
CSF group decreased to 66.7% (4 of 6) compared with that for the saline control group (100%, 12 of 12; \( P < .05 \)). The physical appearance of rats receiving GM-CSF was not different from those receiving physiologic saline, although some animals receiving GM-CSF showed symptoms of serious illness such as restlessness or crouching without motion.

A temporary decrease in circulating leukocyte counts was induced after CLP. There was no difference in circulating leukocyte and neutrophil counts between the two groups (Fig 2).

In the GM-CSF group, neutrophil counts in peritoneal lavage fluid were significantly lower than those in the control CLP group at 6 hours after CLP. (6 hours, 7.2 ± 0.4 \times 10^6/\text{mL} v 22.2 ± 4.9 \times 10^6/\text{mL}, \( P < .05 \); 18 hours, 18.6 ± 2.4 \times 10^6/\text{mL} v 22.5 ± 4.1 \times 10^6/\text{mL}, respectively).

Figure 3 shows the histologic liver findings in the control and GM-CSF groups. Dilated hepatic sinusoids around the central veins, neutrophil infiltration, and proliferation of Kupffer cells were found in the control group. This is the picture of mild hepatic injury observed in shock. In the GM-CSF group, the changes appeared a little more marked, and centrilobular degeneration and necrotic changes (nuclear defects and swelling of the cytoplasm) were also observed.

No apparent differences could be found in other organs. Numerous neutrophil and macrophage infiltrations were noted in the pulmonary septa showing the features of respiratory failure. Acute inflammatory change and abscess formation composed of marked neutrophil accumulation and necrotic tissue could be seen in the cecal serosa suggesting acute supplicative panperitonitis. Congestion and cellular infiltration in the red pulp were found in the spleen. Only mild tubular dilatation was found in the kidneys, without tubular necrosis or degeneration.

Figure 4 shows the effect of rmGM-CSF on changes in biochemical test values indicating organ dysfunction after the induction of CLP. Subcutaneous administration of rmGM-CSF in the CLP model significantly increased the plasma glutamic-pyruvic transaminase (GPT) level 8 hours after the induction of CLP (\( P < .05 \)). There were no significant change in the blood urea nitrogen (BUN) levels.

**DISCUSSION**

GM-CSF is now broadly used in immunocompromised patients suffering from various types of advanced cancer or acute immunodeficiency syndrome to increase neutrophil counts to the point where they can fight bacteria. However, it is not known whether GM-CSF administration is clinically safe and useful when it is administered as a host defense response modifier when infection is already present. Therefore, we induced severe peritonitis in a rat model and investigated the therapeutic effect of GM-CSF.

In the present model, early death, ie, before 12 hours after the onset of CLP, is rare. Progressive peritonitis leads to endotoxin shock and to mortality in the late septic phase. However, after GM-CSF administration, centrilobular necrosis in the liver was seen in the early stage, and the plasma GPT concentration increased earlier than in the control group. This suggests that the liver dysfunction may contribute to the earlier death seen in the GM-CSF–treated animals. Spolarics et al\(^{22}\) showed that neutrophils accumulated in the liver and lungs in normal rats 15 minutes after GM-CSF administration and that metabolic changes in the liver were also observed. For neutrophil-related liver injury, Hewett et al\(^{11}\) showed that administration of antibody to leukocytes reduced liver toxicity after injection of lipopolysaccharide (LPS) in a rat model. We previously showed that neutrophils activated by TNF could damage hepatocytes in vitro.\(^{23}\) Pathologic changes such as hepatic necrosis and cellular infiltration observed after the administration of LPS and TNF were almost the same as those in our present model.\(^{24}\) When GM-CSF was administered alone, these changes were not observed. Thus, endotoxin or TNF might be produced after CLP, and they might act along with GM-CSF. Because GM-CSF could induce production of TNF by fibroblasts in vitro,\(^{25}\) these pathologic changes in the liver might be mediated secondarily by the production of TNF. Görgen et al\(^{26}\) reported that circulating TNF increased after the administration of GM-CSF to a galactosamine-induced liver dysfunction model, whereas it decreased after the administration of G-CSF. This result supports the finding that TNF along with GM-CSF may take part in liver toxicity.\(^{27}\)

In the present study, neutrophil counts in peritoneal lavage...
fluid were lower in the GM-CSF group. This agrees with the in vitro finding that GM-CSF inhibits the migration of neutrophils. Yong et al. showed, using an in vitro model of the vascular endothelial barrier, that GM-CSF enhances neutrophil migration across unstimulated endothelium but inhibits neutrophil migration across IL-1-activated endothelium. In our model, after the induction of sepsis, there may be a high concentration of IL-1 in the vascular compartment, and GM-CSF may act as a neutrophil migration inhibitor. Therefore, when GM-CSF is administered systemically in an infection, neutrophils may not be able to sequester into the inflammatory site. Instead, neutrophils may be activated by GM-CSF along with TNF and IL-1 produced in macrophages to destroy tissue cells in various vital organs in a septic state. We previously showed the therapeutic efficacy of G-CSF in the same CLP model in which G-CSF stimulated the chemotactic activity of neutrophils that easily sequester to the inflammatory site and increases the bactericidal effect. Both G-CSF and GM-CSF have a priming effect on mature neutrophils, but GM-CSF is more potent, and adverse effects occur along with its direct action. The administration of GM-CSF in a clinical study during chemotherapy induced neutropenia, but the occurrence of febrile events or infection was neither reduced nor prevented, although G-CSF could prevent and reduce the frequency of these events in various clinical studies.

In terms of the side effects of GM-CSF, systemic responses such as rash, hypoxia, liver dysfunction, and ARDS were reported in a large number of clinical studies. It is thought that eosinophils may also be important in these adverse effects of GM-CSF, and a slight increase in peripheral eosinophils was observed in the present model. We could not clarify if activated eosinophils are related to early death.

There are some other reports showing that G-CSF pretreatment did not enhance but rather attenuated endotoxin-induced acute lung injury in an animal model. Therefore, the administration of G-CSF is clinically safer and more useful for the treatment of infection than the administration of GM-CSF.

In the present study, the dose of GM-CSF was thought to be enough to induce neutrophilia in normal rats. Because the CLP model was a good sepsis model for induction of neutrophil sequestration into the peritoneal cavity, peripheral neutrophil counts did not increase even when the full dose of GM-CSF (20 μg) was administered after the onset of peritonitis. However, when half the dose of GM-CSF (10 μg) was administered after the onset of peritonitis, no adverse effects were observed in the present model (data not shown). This suggests that tissue injury may not be caused by activated neutrophils alone. When GM-CSF is administered to neutropenic animals before infectious episodes, neutrophil counts can return to the normal range, and endogenous cytokines induced by GM-CSF will increase the host defense against infection. TNF, which is induced by GM-CSF, induces tolerance to infection, and we can see the prophylactic effect of GM-CSF. However, GM-CSF administration after the onset of infection could stimulate a vicious cascade of cytokine production via macrophage stimulation, and then a detrimental effect might arise. This strongly indicates that the timing of administration is quite important in cytokine modulation for the treatment of infection.

Granovitz and Tretter et al. showed that when endotoxin was injected into human volunteers, serum GM-CSF could not be detected. Recently, we showed that serum IL-6 increased after surgery and during infection. We could also detect a simultaneous increase in serum G-CSF after surgery (unpublished data). Until now, we have speculated that IL-6 and G-CSF might act as host defense mediators similar to hormones. However, we could not detect any circulating TNF and IL-1 in these patients. This indicates that TNF and IL-1 along with GM-CSF may act only locally at the site of inflammation and mediate only via locally activated immune cells. Therefore, the systemic administration of such locally acting cytokines might be dangerous when infection is already present.

In the present study, we showed that administration of rmGM-CSF after the onset of peritonitis-induced sepsis failed to increase the survival rate in the rat CLP model. This indicates that the administration of GM-CSF for the treatment of severe peritonitis and its application to patients suffering from sepsis is not clinically recommended.

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