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# Effect of Interleukin-10 on Gut-Derived Sepsis Caused by *Pseudomonas aeruginosa* in Mice

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**We evaluated the protective effect of interleukin-10 (IL-10) against murine gut-derived sepsis caused by *Pseudomonas aeruginosa*. Gut-derived sepsis was induced by administering cyclophosphamide and ampicillin while feeding *P. aeruginosa* to specific-pathogen-free mice. Treating mice with recombinant human IL-10 (rhIL-10) at 1.0 or 5.0 μg/mouse twice a day following the second cyclophosphamide administration significantly increased the survival rate compared to that of control mice treated with saline; however, treatment with rhIL-10 at 0.1 μg/mouse did not result in significant protection. Bacterial counts in the liver, spleen, and blood were all significantly lower in mice treated with rhIL-10 than in saline-treated control mice. Treatment with rhIL-10 significantly suppressed tumor necrosis factor alpha, interleukin-1β, interleukin-6, and gamma interferon levels in the serum of mice following induction of gut-derived sepsis. We also studied the effect of IL-10 on leukocyte recovery after cyclophosphamide treatment of mice. Administration of rhIL-10 intraperitoneally at 1.0 μg/mouse significantly accelerated the recovery of leukocytes in comparison with that of the group of saline-treated controls. These results indicate that IL-10 shows a protective effect against gut-derived *P. aeruginosa* sepsis. We suspect that the mechanism of this effect is that IL-10 regulates in vivo production of inflammatory cytokines. Furthermore, acceleration of leukocyte recovery by IL-10 after cyclophosphamide-induced depression may also play an important role in this protection.**

Septic shock is an often fatal condition, with death believed to result from excessive production of inflammatory cytokines (3, 38). Experimental data suggest that tumor necrosis factor (TNF) is a pivotal endogenous mediator of septic and endotoxic shock (3, 35, 38). In previous studies (25, 28), we evaluated the role of TNF-α and interleukin-1α (IL-1α) in murine gut-derived sepsis caused by *Pseudomonas aeruginosa* and concluded that these cytokines may facilitate bacterial translocation and cause deterioration due to gut-derived *P. aeruginosa* sepsis in mice.

IL-10, produced mainly by Th2 lymphocytes and monocytes/macrophages, is known to suppress lipopolysaccharide (LPS)-activated synthesis by human monocytes of several cytokines, including TNF-α, IL-1α, IL-1β, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF) (9). For example, a marked reduction in the amounts of LPS-induced TNF released into the circulation has been observed after IL-10 pretreatment; furthermore, IL-10 protects mice from lethal endotoxemia (16, 19). In vivo biologic and immunohistochemical analysis of murine experimental endotoxemia revealed that in this condition, hepatic sinusoidal macrophages (Kupffer cells) are a major source of cytokines such as TNF and IL-1 (5). Viral IL-10 gene therapy inhibits Kupffer cell production of TNF-α and IL-1β in response to LPS (11).

In addition to its potent anti-inflammatory properties, IL-10 causes depression of splenocyte functions in a murine model of gram-negative endotoxemia (12) and down-regulates macrophage function in a variety of experimental systems (4, 10, 13, 15, 31, 33). Its immunosuppressive effect may augment susceptibility to repeated or continuous invasion by microorganisms and may lead to exacerbation of disease, as is seen during clinical sepsis (12). Oswald et al. reported that IL-10 inhibits the ability of gamma interferon (IFN-γ) to activate macrophages for cytotoxicity against *Schistosoma mansoni* (33); they identified the mechanism of IL-10 action as inhibition of endogenous TNF-α production by macrophages.

We have previously reported that Kupffer cells play an important role in the occurrence of overwhelming systemic bacteremia in our animal model (18). Therefore, it could be postulated that while the anti-inflammatory properties of IL-10 provide benefits to the host, suppression of macrophage functions by IL-10 may induce exacerbation of infection. Therefore, although IL-10 probably plays a crucial role in the pathophysiology of sepsis, it has not been clearly determined whether IL-10 exacerbates or ameliorates the disease. These considerations led us to investigate the effect of IL-10 on gut-derived *P. aeruginosa* sepsis, and we further studied the mechanism of this effect of IL-10.

## MATERIALS AND METHODS

**Animals.** Specific-pathogen-free male ddY mice (Japan Shizuoka Laboratory Center Co., Ltd., Shizuoka, Japan) weighing 20 to 24 g were used in the experiments. The animals were housed in sterile cages and received sterile distilled water, except during the period when bacteria were being orally administered.

**Bacterial strain.** *P. aeruginosa* D4 isolated from the blood of a neutropenic mouse with bacteremia (17) was used. The strain was maintained frozen at −80°C in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) containing 15% glycerol.

**Reagents.** Recombinant human IL-10 (rhIL-10) was a kind gift from Schering-Plough K.K., Osaka, Japan. The reagent was dissolved with pyrogen-free saline, at various final concentrations, prior to injection.

**Murine gut-derived *P. aeruginosa* D4 sepsis: induction and survival rates.** Murine gut-derived sepsis was produced as described previously (24, 26, 27). Briefly, bacteria were grown on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 18 h, suspended in sterile 0.45% saline, and adjusted to a concentration of $10^7$ CFU/ml. This bacterial suspension was given in the drinking water between days 1 and 3. To aid in the colonization of *P.*

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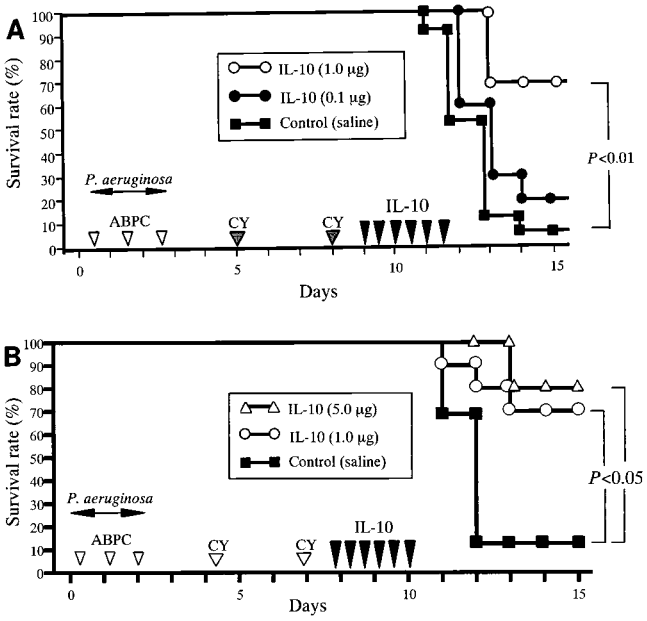


FIG. 1. Effect of IL-10 on survival of mice with gut-derived sepsis caused by *P. aeruginosa*. Beginning after the second cyclophosphamide administration, mice in groups of 10 were intraperitoneally given IL-10 at 1.0 or 0.1 μg/mouse twice a day (A). Furthermore, the effect of a larger dose of IL-10 was evaluated by administration of IL-10 at 5.0 ($n = 5$) or 1.0 ($n = 10$) μg/mouse at the same intervals (B). Control mice ($n = 15$) were given pyrogen-free saline intraperitoneally at the same intervals. ABPC, ampicillin; CY, cyclophosphamide treatment.

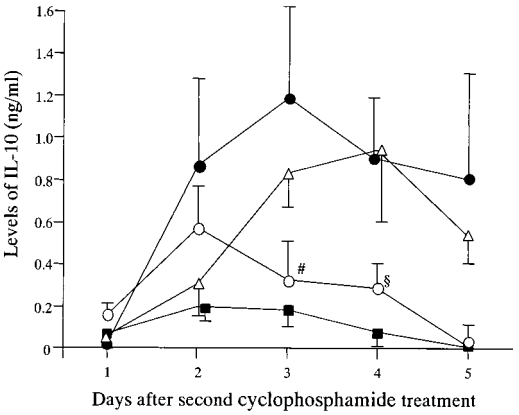


FIG. 2. IL-10 levels in serum after cyclophosphamide treatment with or without *P. aeruginosa* infection. Endogenous IL-10 levels in serum were evaluated for 5 days beginning on the day following the second administration of cyclophosphamide in the following four groups of mice: mice without *P. aeruginosa* (■), mice with *P. aeruginosa* plus saline treatment (●), mice with *P. aeruginosa* plus IL-10 treatment (0.1 μg; △), and mice with *P. aeruginosa* plus IL-10 treatment (1.0 μg; ○). Values are means ± the standard errors of the means (six different mice per time point). The IL-10 levels in the sera of mice 3 and 4 days after cyclophosphamide administration were significantly lower than the levels of saline-treated controls (Symbols: #, $P < 0.05$; §, $P < 0.01$).

*aeruginosa*, the normal intestinal flora of the mice was disturbed by administering 200 mg of ampicillin per kg of body weight by intraperitoneal injection daily between days 1 and 3. Mice were then given 150 to 200 mg of cyclophosphamide per kg of body weight by intraperitoneal injection on days 5 and 8. Each experiment was repeated at least twice. The animals were scored for mortality every 24 h for up to 7 days after the second cyclophosphamide administration. To determine the effect of IL-10, each group of mice was given rhIL-10 by intraperitoneal injection twice a day after the second cyclophosphamide treatment. Control mice were given pyrogen-free saline by intraperitoneal injection.

The experimental protocols were approved by the Institutional Animal Care and Use Committee at the Toho University School of Medicine.

**Determination of viable bacteria in blood and liver and preparation of serum samples.** To determine whether administration of IL-10 ameliorates the infection, we measured viable bacterial counts in liver and blood. Mice from each treatment group were killed by ether inhalation at the indicated time points, and cardiac blood and liver samples were obtained aseptically. The liver was homogenized in sterile saline. Portions of the blood samples and liver homogenates were plated onto Trypticase soy agar, and the samples were cultured at 37°C for 24 h for detection of the challenge *P. aeruginosa* strain. The rest of the blood samples were allowed to clot at 4°C in sterile glass tubes and then centrifuged at 2,000 × *g* for 15 min. Serum samples were preserved at −80°C until cytokine levels were measured.

**Cytokine assay.** IL-10, TNF-α, IL-6, and IFN-γ levels in mouse serum were determined with enzyme-linked immunosorbent assay (ELISA) kits (Endogen Inc., Boston, Mass.). IL-1β and GM-CSF concentrations were assessed with a commercially available ELISA kit (Genzyme Corp., Boston, Mass.). The assays were performed exactly as described by the manufacturers, and the levels in each sample were determined in duplicate.

**Statistical analysis.** The differences between the survival rates of groups of mice were evaluated by the chi-square test. Cytokine levels in serum and viable bacterial counts in liver, spleen, and blood were compared by the Mann-Whitney U test. A probability level of 5% was considered to be significant.

## RESULTS

**Effect of rhIL-10 on mouse survival.** Figure 1 presents the survival kinetics of mice with gut-derived sepsis given rhIL-10 or saline. We found that treatment with rhIL-10 at 1.0 μg/mouse twice a day after the second cyclophosphamide administration significantly protected mice against mortality (70% survival compared to 6.7% survival of saline-treated control mice). However, there was no significant protection following treatment with rhIL-10 at 0.1 μg/mouse (Fig. 1A). Furthermore, the effect of a larger dose of IL-10 was evaluated by administration of IL-10 at 5.0 or 1.0 μg/mouse at the same intervals, and the result revealed that the larger dose of rhIL-10 also showed a protective effect against murine sepsis (Fig. 1B).

**Changes in endogenous IL-10 levels in serum.** We determined the endogenous production of IL-10 after cyclophosphamide treatment. The results depicted in Fig. 2 show that cyclophosphamide treatment induced a slight increase in the levels of IL-10 in serum without infection with *P. aeruginosa*. The results also revealed that administration of 1.0 μg of rhIL-10 significantly suppressed the levels of endogenous IL-10 in serum 3 and 4 days after cyclophosphamide treatment. On the other hand, the 0.1-μg rhIL-10 treatment showed no significant effect on the IL-10 levels in serum.

We also studied the influence of rhIL-10 treatment on endogenous IL-10 levels in serum after cyclophosphamide treatment of mice without *P. aeruginosa* infection and found no significant difference in the IL-10 levels in serum between the groups with and without rhIL-10 treatment (data not shown).

**Effect of rhIL-10 on the numbers of viable bacteria in liver, spleen, and blood.** Figure 3 presents the numbers of viable bacteria in the livers, spleens, and heart blood of mice after the second cyclophosphamide treatment. On the second and third days following this treatment, the average numbers of viable bacteria in organs of mice treated with rhIL-10 were significantly lower than those in organs of saline-treated mice.

**Effect of IL-10 on cytokine levels in serum during gut-derived sepsis.** Since the inflammatory cytokines TNF-α, IL-1β, IL-6, and IFN-γ are thought to be important mediators of septic shock, we examined the effect of rhIL-10 on their production. As depicted in Fig. 4, the results demonstrated significant suppression of cytokine production by rhIL-10. Our preliminary study revealed that cytokine levels in the sera of untreated healthy mice were below the limits of detection by the methods used.

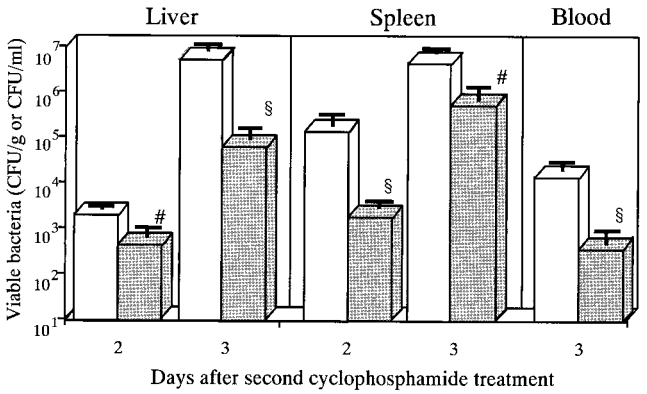


FIG. 3. Effects of IL-10 on viable bacterial counts in liver, spleen, and blood during gut-derived *P. aeruginosa* sepsis. Viable bacterial counts in the livers, spleens, and blood of mice treated with rhIL-10 at 1.0 μg/mouse (closed columns) and saline-treated controls (open columns) 2 or 3 days after the second cyclophosphamide treatment are shown. The values, expressed as numbers of CFU per gram or per milliliter, are means ± the standard errors of the means (six mice in each group). Symbols: #, $P < 0.05$; §, $P < 0.01$.

**Effect of rhIL-10 on leukocyte recovery after cyclophosphamide treatment of mice.** Recovery of leukocytes after cyclophosphamide-induced depression may also influence the prognosis of this infection. Therefore, we determined the effect of rhIL-10 on the recovery of leukocytes after cyclophosphamide treatment of mice. As shown in Fig. 5, administration of rhIL-10 intraperitoneally at 1.0 μg/mouse significantly accelerated the recovery of leukocytes in comparison with the group of saline-treated controls. We found that the leukocytes recovered after cyclophosphamide treatment were composed mainly, more than 70%, of neutrophils (data not shown).

**Effect of IL-10 on GM-CSF levels in serum during gut-derived sepsis.** Since there is a possibility that acceleration of leukocyte recovery after rhIL-10 treatment was induced by other cytokines, it would be important to determine the levels of other cytokines, especially GM-CSF, G-CSF, or IL-3, in serum after IL-10 treatment. We studied of GM-CSF levels in serum by using a commercial ELISA kit. Contrary to our expectation, the results showed that administration of rhIL-10 significantly reduced the levels of GM-CSF in serum (Fig. 6).

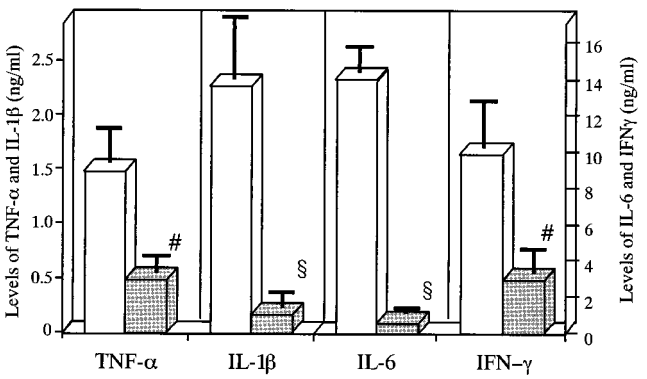


FIG. 4. Effects of IL-10 on cytokine levels in the serum of mice with gut-derived sepsis due to *P. aeruginosa*. IL-10 was administered intraperitoneally at 1.0 μg/mouse (closed columns) twice a day; saline was administered to control animals (open columns) at the same intervals. Serum samples were collected 3 days after the second cyclophosphamide administration. Values are means ± the standard errors of the means (six mice in each group). Symbols: #, $P < 0.05$; §, $P < 0.01$.

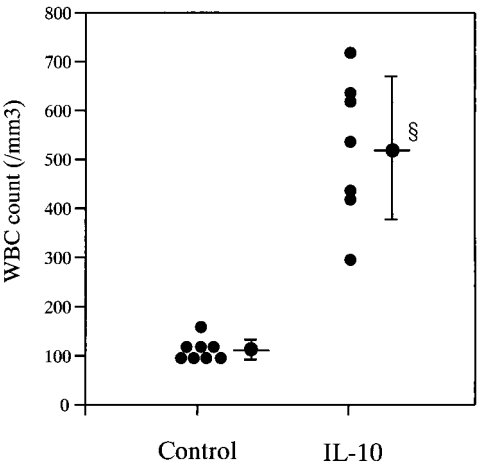


FIG. 5. Effect of IL-10 on leukocyte recovery after cyclophosphamide treatment of mice. IL-10 was administered intraperitoneally at 1.0 μg/mouse twice a day; saline was administered to control animals at the same intervals. Heart blood were collected 3 days after the second cyclophosphamide administration. The numbers of leukocytes (WBC) in the blood of IL-10-treated mice ($n = 7$) and saline-treated mice ($n = 8$) are depicted. Bars are means ± the standard deviations of the means. Symbol: §, $P < 0.01$.

These results demonstrated that administration of IL-10 had a protective effect against gut-derived sepsis with *P. aeruginosa*. The mechanism by which IL-10 protects is probably suppression of in vivo production of inflammatory cytokines. Furthermore, acceleration of leukocyte recovery by IL-10 after cyclophosphamide-induced depression may also play an important role in this protection.

## DISCUSSION

Clinical studies that use surveillance cultures of fecal samples from immunocompromised patients suggest that the gastrointestinal tract is a primary reservoir for opportunistic bacteria (38). Berg and Garlington (2) and Deitch et al. (6) have demonstrated that bacteria contained within the gut can cross the gastrointestinal mucosal barrier and spread systemically by

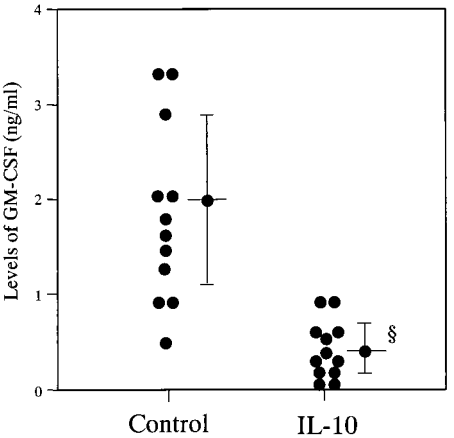


FIG. 6. Effects of IL-10 on GM-CSF levels in the serum of mice with gut-derived sepsis due to *P. aeruginosa*. IL-10 was administered intraperitoneally at 1.0 μg/mouse twice a day ($n = 12$); saline was administered to control animals ($n = 12$) at the same intervals. Serum samples were collected 3 days after the second cyclophosphamide administration. Bars are means ± the standard deviations of the means. Symbol: §, $P < 0.01$.

a process termed bacterial translocation. Bacterial translocation may occur with alterations of the host defense, disruption of the normal indigenous bacterial flora, or loss of the mucosal barrier (6, 22, 23). We induced gut-derived sepsis with *P. aeruginosa* by administering cyclophosphamide and ampicillin to specific-pathogen-free mice fed *P. aeruginosa* (17, 18, 24, 26, 27). This model incorporated oral inoculation of bacteria, subsequent bacterial colonization, overgrowth in the intestinal tract, and invasion of the bloodstream. Consequently, this animal model closely mimics the pathophysiology of septicemia in humans (17).

Several recent studies have suggested that IL-10 inhibits functions related to the microbicidal activity of macrophages and to cellular immunity (30, 33). Therefore, a negative effect of IL-10 on protection was predicted—a hypothesis supported by some studies. For example, in murine models of infection with *Mycobacterium avium* (7) and *Candida albicans* (36), anti-IL-10 antibodies prevented lethality. Transgenic mice that secrete IL-10 from the T-cell compartment were unable to clear infection with the Calmette-Guerin bacillus (*Mycobacterium bovis*) and developed large bacterial burdens (31). However, the role of IL-10 in infection is considerably more complex. Kato et al. studied the therapeutic efficacy of IL-10 by testing its effect on the survival rate in a murine cecal ligation-and-puncture model, and the results revealed that treatment with IL-10 increased the survival of mice after cecal ligation and puncture (20).

Possible reasons for these contradictory results include (i) differences in the pathogen causing infection, (ii) the pathophysiologic differences between endotoxin shock and septic shock, and (iii) the question of whether IL-10's main effect in a particular infection is its anti-inflammatory or its antimacrophage activity. In connection with the first point, we suspect that infection with intracellular organisms such as *M. avium* (7) and *Listeria monocytogenes* (39) may lead to a negative effect for IL-10 because such pathogens are managed primarily by macrophages. Mosmann also commented that IL-10 is associated with a poor or absent response against infections whose elimination requires a cell-mediated response; excess production of IL-10 may be harmful to animals infected with a number of intracellular pathogens (30).

On the second point, Bagby et al. revealed that passive immunization with neutralizing goat anti-TNF-α immunoglobulin G significantly improved the survival of rats administered LPS intravenously but was completely ineffective in protecting rats from lethal *Escherichia coli* peritonitis (1). It is therefore reasonable that the effect of IL-10 in suppressing inflammatory cytokine production may lead to different results in endotoxin shock and in septic infection.

Finally, the relative importance of IL-10's anti-inflammatory and antimacrophage activities under different circumstances merits consideration. Comparison of the periods of infection in our gut-derived sepsis model, the cecal ligation and puncture model, and various other models of infection suggests to us that IL-10 plays a beneficial role in acute infections (that is, models in which most mice die within 3 or 4 days of the onset) but may play a detrimental role in chronic infections in which the time course covers more than 1 week.

Concerning the IL-10 dosing range, Kato et al. reported that administration of 1.0 μg or more of recombinant IL-10 significantly decreased lethality in septic mice (20). We therefore first adopted the dose of 1.0 μg of IL-10. However, larger doses of IL-10 may have immunosuppressing effects. Therefore, we further studied the influence of a larger dose, 5.0 μg/mouse, of rhIL-10 on the survival of mice. The results revealed that this dose of rhIL-10 also showed a protective effect against murine sepsis.

Concerning the administration time of IL-10, we think neutrophils play important roles in the host defense in this model. In our preliminary experiment, the leukocyte count decreased to less than 1,000/mm$^3$ for 3 to 4 days after cyclophosphamide treatment. Therefore, we concluded that this period is the most important and decided to administer rhIL-10 for 3 days after cyclophosphamide treatment.

Since there was no significant difference between the IL-10 levels in the serum of the groups of mice with or without rhIL-10 treatment and without *P. aeruginosa* infection, we suspect that rhIL-10 administration had no significant influence on endogenous IL-10 production in mice without infection. We also suspect that elevation of IL-10 levels in serum reflects a severe inflammation induced by *P. aeruginosa* sepsis.

In regard to the effect of IL-10 on leukocyte recovery after cyclophosphamide treatment, our present study revealed that administration of IL-10 significantly increased the number of leukocytes in mice. We therefore suspect that this effect also plays an important role in this protection in our model. Although a double-blind, placebo-controlled study with healthy humans revealed that an intravenous bolus injection of rhIL-10 induced transient leukocytosis (14), as far as we know, this is the first report to mention the acceleration of leukocyte recovery by IL-10 after cyclophosphamide-induced depression.

It would be interesting to determine the level of GM-CSF, G-CSF, or IL-3 in serum after IL-10 treatment. We studied GM-CSF levels in serum by using a commercial ELISA kit. Contrary to our expectation, administration of rhIL-10 significantly reduced the levels of GM-CSF in serum. This result is supported by the reports of Oehler et al. and Lenhoff et al. (21, 32). We therefore suspect that acceleration of leukocyte recovery by rhIL-10 is independent of the effect of GM-CSF.

In summary, this study provides evidence that treatment with rhIL-10 induces protective effects in mice with gut-derived sepsis. The beneficial function appears to be associated with IL-10's ability to suppress inflammatory cytokine production and accelerate leukocyte recovery after leukopenia. It has previously been shown that IL-10 has great potential therapeutic utility for treating diseases (8) such as autoimmune diseases (29, 30), transplant rejection (34), bacterial peritonitis (20), and inflammatory bowel disease (37). However, we must recognize that since endogenous IL-10 has both beneficial and detrimental effects on the host response to bacterial infection in mice (39), administration of the compound may induce unexpected effects. Thus, further investigation is needed to determine the conditions under which IL-10 treatment will produce the maximum protective effect in humans with sepsis.

## ACKNOWLEDGMENTS

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