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Efficacy of Erythromycin Lactobionate for Treating *Pseudomonas aeruginosa* Bacteremia in Mice

YOICHI HIRAKATA,[1]* MITSUO KAKU,[2] KAZUNORI TOMONO,[3] KAZUHIRO TATEDA,[1] NOBUHIKO FURUYA,[1] TETSUYA MATSUMOTO,[2] RYOUKO ARAKI,[2] AND KEIZO YAMAGUCHI[1]

Department of Microbiology, Toho University School of Medicine, Ohmorinishi, Ohta-Ku, Tokyo 143,[1] Department of Laboratory Medicine, Nagasaki University School of Medicine, Nagasaki 852,[2] and Department of Pulmonary Medicine, Jichi Medical School, Tochigi 329-04,[3] Japan

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We induced endogenous *Pseudomonas aeruginosa* bacteremia by administering cyclophosphamide and ampicillin to specific pathogen-free mice fed *P. aeruginosa*. Using this model, we evaluated the efficacy of erythromycin lactobionate (EML) in treating *P. aeruginosa* bacteremia. Treatment with EML at 50 and 100 mg/kg of body weight per day twice a day for 14 days significantly increased the survival rate. The most effective dose was 100 mg/kg/day, with a survival rate of 80% compared with a 20% survival rate in the control. However, the administration of EML at 500 mg/kg/day rather decreased the survival rate. In a model of intravenous infection, treatment with EML at 100 mg/kg/day twice a day for 7 days before the bacterial challenge also enhanced the survival rate. EML levels in serum, liver, and stool were apparently lower than the MIC (512 μg/ml). These observations suggest that EML is effective against *P. aeruginosa* bacteremia despite a lack of specific activity for this pathogen. Although the protective mechanism is still unclear, it is possible that a subinhibitory level of EML may affect the virulence of *P. aeruginosa* and enhance the host defense system.

Pseudomonas aeruginosa is a major opportunistic pathogen in patients with terminal burns, malignant diseases, and other forms of immunosuppression (20). In such hosts, *P. aeruginosa* frequently causes bacterial sepsis, carrying a higher fatality rate than any other gram-negative bacteria (5, 7). Additionally, we previously reported that *P. aeruginosa* is the major pathogen responsible for endogenous systemic bacteremia in neutropenic mice (14). *P. aeruginosa* is also the primary pulmonary pathogen in patients with cystic fibrosis (20, 26) and diffuse panbronchiolitis (15). Chemotherapy in such patients is often ineffective, since *P. aeruginosa* is typically refractory to most antibiotics (7, 20).

Recently, it has been reported that erythromycin (EM) improves the clinical symptoms and prognosis of patients with diffuse pulmonary panbronchiolitis, although it exhibits no direct antimicrobial activity against *P. aeruginosa* (18). However, the protective mechanism is still unclear. It is also unknown whether EM is effective against other *P. aeruginosa* infections such as bacteremia. In the present study, we evaluated the efficacy of EM in *P. aeruginosa* bacteremia by using murine experimental models.

MATERIALS AND METHODS

Antimicrobial agents. Erythromycin lactobionate (EML) for injection (Dainabot Co., Osaka, Japan) containing 18% (wt/wt) of benzyl alcohol as a preservative was used for animal studies, while EM base was used for in vitro studies. Both forms of EM were kindly provided by Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. EML was dissolved in distilled water for injection to a concentration of 100 mg/ml and then suspended in normal saline for injection at each final dose. EM base was dissolved with a small amount of ethanol (maximal final concentration, 0.5% [vol/vol]), and then suspended in sterile distilled water. Injectable ceftazidime (Modacin; Nippon Glaxo Ltd., Tokyo, Japan), gentamicin sulfate (Gentacin; Schering-Plough K.K., Osaka, Japan), and clindamycin phosphate (Dalacin P; Japan Upjohn Ltd., Tokyo, Japan) was dissolved, diluted with normal saline, and used in the animal studies. Basal forms of these antimicrobial agents were dissolved with distilled water and used for in vitro studies. The endotoxin content of EML preparation was less than 0.02 EU/ml (6.9 pg/ml) as determined by the chromogenic *Limulus* test (Toxicolor system; Seikagaku Co., Tokyo, Japan).

Bacterial strain and media. *P. aeruginosa* D4 used in this study was isolated from cardiac blood of a mouse with systemic endogenous bacteremia as described previously (14). The isolate was grown on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 18 h. Bacteria were suspended in sterile normal or 0.45% saline and adjusted to a suitable concentration for each experiment by determining optical density with a spectrophotometer (UVIDEC-40; Jasco, Tokyo, Japan).

In vitro susceptibility. MICs were determined by a microbroth dilution method (23) with Mueller-Hinton broth supplemented with calcium and magnesium at concentrations of 50 and 25 mg/liter, respectively. Bacteria were inoculated at a final concentration of 5.0×10^5 CFU/ml.

Animals. Male ddY mice (Japan S.L.C. Co., Ltd., Shizuoka, Japan) weighing 20 to 24 g were used in the experiments. Fecal specimens were obtained before the study and examined to ensure the absence of *P. aeruginosa*. Mice were fed a sterile diet and received sterile distilled water except during the period of oral administration of the bacteria.

Experimental infections. (i) Endogenous bacteremia. Endogenous bacteremia was produced as previously described by Collins et al. (6), with modifications. Briefly, mice were administered 200 mg of sodium ampicillin per kg of body weight for injection (Viccillin; Meiji Seika Kaisha, Ltd., Tokyo, Japan) by intraperitoneal injection daily between days 1 and 5. Bacteria in sterile 0.45% saline at a concentra-

* Corresponding author.

tion of 10^7 CFU/ml were given in the drinking water between days 2 and 5. Mice were administered 200 mg of cyclophosphamide per kg for injection (Endoxan; Shionogi & Co., Ltd., Osaka, Japan) intraperitoneally on days 8 and 12. The experiments were performed by using this model to assess the therapeutic or prophylactic efficacy of EML.

In preliminary dose-response studies, 500, 200, 100, 50, or 10 mg of EML per kg per day was administered twice daily (b.i.d.), 12 h apart by intraperitoneal injection between days 7 and 20. Control mice were administered 0.2 ml of normal saline only.

In subsequent studies, 100 mg of EML per kg per day was administered b.i.d. as follows: (i) between days 7 and 13; (ii) between days 14 and 20; and (iii) between days 7 and 20. Control mice received saline containing a dose of sterile benzyl alcohol (Ishizu Pharmaceutical Co., Ltd., Osaka, Japan) equivalent to the EML solution between days 7 and 20.

In other studies, 500 or 50 mg of EML per kg per day, 400 or 40 mg of ceftazidime per kg per day, 16 or 1.6 mg of gentamicin sulfate per kg per day, 240 or 24 mg of clindamycin phosphate per kg per day, or normal saline was administered b.i.d. by intraperitoneal injection between days 7 and 20.

Moreover, we assessed the efficacy of combination therapy with EML and ceftazidime by intraperitoneal administration of 500 mg of EML per kg per day and 400 mg of ceftazidime per kg per day b.i.d. between days 7 and 20.

Mice were observed four times daily, and deaths were recorded up to day 21. Necropsies from cardiac blood were performed within 6 h on mice that died and on mice killed at the end of the experiments.

(ii) Intravenous infection. Both normal and granulocytopenic mice were used in the experiments. Granulocytopenic mice were produced by intraperitoneal administration of 200 mg of cyclophosphamide per kg on days 3, 6, 9, and 12. Normal mice were challenged intravenously with bacteria in saline at doses of 1.2×10^7, 6.0×10^6, or 3.0×10^6 CFU per mouse on day 8. Granulocytopenic mice received bacteria at doses of 4.4×10^3, 2.2×10^3, or 1.1×10^3 CFU per mouse. Treatment of mice with 100 mg of EML per kg per day b.i.d. or saline containing benzyl alcohol by intraperitoneal injection was performed as long as the mice lived (i) between days 1 and 7 and (ii) between days 8 and 14, respectively. Untreated mice were used as a control in the experiment with granulocytopenic mice. Mice were observed twice daily and deaths were recorded until day 20. To assess the nonspecific inflammatory response by EML, abdominal exudation cells were counted. For granulocytopenic mice, peripheral granulocyte counts and ratios of neutrophil between EML-treated and control mice were also compared.

EML concentrations in serum, liver, and stool. Mice were administered a single dose of 50 mg of EML per kg intraperitoneally. Blood and liver were obtained aseptically before antibiotic administration and at 3, 10, and 30 min and 1, 2, 4, 6, 8, and 12 h after dosing. Stool was collected before drug administration and for the following intervals: 0 to 6, 6 to 12, 12 to 24, 24 to 36, and 36 to 48 h. Liver and stool were homogenized with an adequate volume of phosphate-buffered saline (10 Mm, pH 8.0). Supernatants of homogenates were passed through a 0.22-μm-pore-size filter (Millex-GV; Japan Millipore Ltd., Tokyo, Japan), and used for assay. EML concentrations in specimens were determined by bioassay with an agar diffusion method (8) with metal cylinders (diameter, 7 mm). Mueller-Hinton agar was inoculated with *Micrococcus luteus* ATCC 9341 at a concentration of 10^5 CFU/ml as the indicator organism. Standards and samples were run in duplicate. The limited sensitivity of the test system was 0.031 μg/ml. To determine whether the kinetics of repeated doses of EML differ from those of EML given as a single dose, we also studied mice given 100 mg of EML per kg per day b.i.d. for 14 days. After the last administration of EML, specimens were collected from these mice, and EML levels were determined as described above.

Statistics. The chi-square test and Student's *t* test were used to compare survival rates and means, respectively. A level of 5% was accepted as statistically significant.

RESULTS

In vitro susceptibility. The MICs of EM base and benzyl alcohol against *P. aeruginosa* D4 were 512 and >2,048 μg/ml, respectively. The MICs of ceftazidime, gentamicin sulfate, and clindamycin hydrochloride were 1.0, 2.0, and >2,048 μg/ml, respectively.

Efficacy of EML in experimental infections. (i) Endogenous bacteremia. The survival kinetics of mice in the dose-response studies are shown in Fig. 1. The survival rate of control mice given only saline was 20%, and deaths were observed by day 18. Treatment with EML at doses of 100 and 50 mg/kg/day b.i.d. for 14 days significantly increased the survival rate ($P < 0.05$). The most effective dose was 100 mg/kg/day, with a survival rate of 80%. In contrast, treatment of mice with 500 mg of EML per kg per day lowered the survival rate to 0%, and these mice died by day 16. This dosage of EML was not lethal for healthy mice; however, healthy mice did show apparent weight loss (mean weight was 78.9% of that of control mice) and appetite loss (70.7% compared with that of control). Figure 2 shows that treatment of mice with 100 mg of EML per kg per day b.i.d. between days 7 and 13 (50% survival) and between days 7 and 20 (45% survival) significantly enhanced their survival rates compared with that (0%) of control mice ($P < 0.01$).

As shown in Table 1, treatment of mice with 50 mg of EML per kg per day and 400 or 40 mg of ceftazidime per kg given b.i.d. for 14 days significantly enhanced their survival rates ($P < 0.01$). No increase in survival rate was observed in mice treated with 500 mg of EML per kg per day, 16 or 1.6 mg of gentamicin sulfate per kg per day or 240 or 24 mg of clindamycin phosphate per kg per day.

Table 2 shows that combination treatment of 500 mg of EML per kg/day and 400 mg of ceftazidime per kg per day decreased the survival rate of mice compared with that of those given ceftazidime only ($P < 0.05$).

All cultures of cardiac blood obtained from dead mice were positive for the challenge strain. There were no differences in the positive ratio of cultures (10 to 30%) from survivors in each groups at the end of the experiments.

(ii) Intravenous infection. Table 3 shows the survival rates of normal mice infected intravenously. In mice infected with 6.0×10^6 CFU per mouse, treatment with 100 mg of EML per kg per day b.i.d. between days 1 and 7 significantly enhanced the survival rate ($P < 0.01$). In the granulocytopenic mice infected with 2.2×10^3 CFU per mouse, treatment with EML between days 1 and 7 significantly increased the survival rate ($P < 0.05$) (Table 4). In these studies, deaths were observed between day 9 and 15. In both normal and granulocytopenic mice, the late treatment did not work. EML did not change the number of peripheral granulocytes or the ratio of neutrophil in mice treated with cyclophosphamide through the experiments. For example,

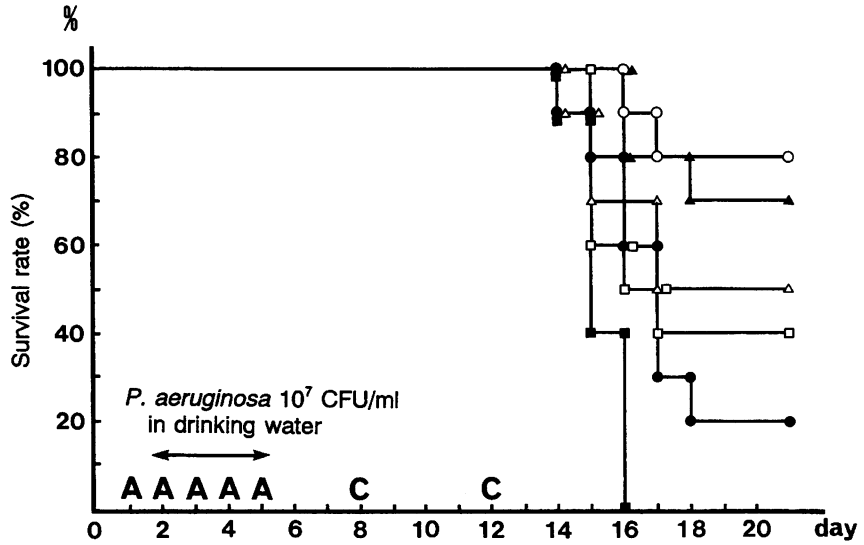

FIG. 1. Influence of various doses of EML on survival rates of mice with endogenous *P. aeruginosa* bacteremia (10 mice per group). Mice were given 500, 200, 100, 50, or 10 mg of EML per kg per day or saline (control) b.i.d. between days 7 and 20. Survival rates of mice treated with 100 and 50 mg of EML per kg per day significantly exceeded that of control mice ($P < 0.05$, respectively). Symbols: ■, 500 mg of EML per kg per day; □, 200 mg of EML per kg per day; ○, 100 mg of EML per kg per day; ▲, 50 mg of EML per kg per day; △, 10 mg of EML per kg per day; ●, control (saline); A, ampicillin at 200 mg/kg i.p.; C, cyclophosphamide at 200 mg/kg i.p.

the granulocyte count (mean ± standard deviation; percentage of neutrophil) of control and EML-treated mice was 840 ± 428/μl (7.3% ± 1.1%) and 800 ± 322/μl (6.7% ± 2.1%), respectively, on day 9. A slight increase of the number of abdominal exudation cells in EML-treated mice was observed on day 2 ($8.8 \times 10^6 \pm 1.0 \times 10^6$ versus $8.0 \times 10^6 \pm 1.2 \times 10^6$ cells per mouse); however, there were no differences on other days.

EML concentrations in serum, liver, and stool. Peak concentrations of EML in serum and liver in mice given a single dose of EML were 6.42 ± 3.52 μg/ml and 11.29 ± 3.84 μg/g, respectively, 10 min after injection. In mice inoculated with repeated doses of EML, peak concentration in serum was 6.95 ± 2.45 μg/ml 10 min after injection. In liver, EML reached a maximal concentration of 13.64 ± 4.33 μg/g 3 min after injection. No differences in peak antibiotic concentrations in both serum and liver between mice inoculated with a single dose and repeated doses of EML were observed. However, EML was detected for somewhat longer intervals in mice administered repeated doses of EML (Fig. 3). In

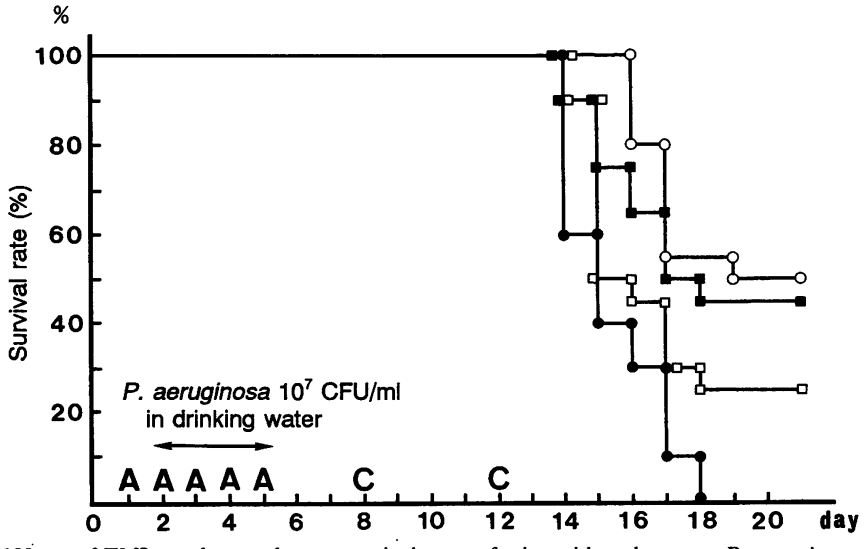

FIG. 2. Influence of 100 mg of EML per kg per day on survival rates of mice with endogenous *P. aeruginosa* bacteremia (20 mice per group). Survival rates of mice treated between days 7 and 20 and between days 7 and 13 significantly exceeded that of control mice ($P < 0.01$). Survival rate of mice treated between day 13 and day 20 also significantly exceeded that of control mice ($P < 0.05$). Symbols: ■, 100 mg of EML per kg per day b.i.d. between day 7 and 20; ○, treated between days 7 and 13; □, treated between days 13 and 20; ●, control (dose of sterile benzyl alcohol equivalent to dose of EML between days 7 and 20); A, ampicillin at 200 mg/kg i.p.; C, cyclophosphamide at 200 mg/kg i.p.

TABLE 1. Influence of EML and other antibiotics on survival rate of mice with endogenous *P. aeruginosa* bacteremia

Agent	MIC (μg/ml)	Dose[a] (mg/kg/day)	No. of mice per group	Survival rate (%)
EML	512	500	10	20
		50	10	100[b]
Ceftazidime	1.0	400	10	100[b]
		40	10	100[b]
Gentamicin sulfate	2.0	16	5	40
		1.6	5	40
Clindamycin phosphate	>2,048	240	5	40
		24	5	40
Control (saline)			10	20

[a] Given b.i.d., 12 h apart by intraperitoneal injection between days 7 and 20.
[b] Significantly higher than the survival rate of control mice ($P < 0.01$).

stool, EML reached a maximal concentration of 95.12 ± 25.30 μg/g between 6 and 12 h after a single-dose inoculation and then declined. No accumulation of EML in stool was observed (Fig. 4). No differences between normal and granulocytopenic mice were noted (data not shown).

DISCUSSION

The present study confirms that EM has a protective activity against *P. aeruginosa* bacteremia. In animal studies, EML levels in serum and liver were apparently lower than the MIC against the infecting bacterial strain at all points in time. Although the stool contained a high level of EML, the level never reached the MIC. Moreover, in both the endogenous and the intravenous infection models, early treatment, especially before infection in the latter model, revealed apparent efficacy. These observations may exclude a direct antimicrobial effect of EM against the pathogen. While its protective mechanisms are unclear, they may include (i) an influence of EM on the composition and number of intestinal flora, (ii) an altered virulence of *P. aeruginosa* by EM, and (iii) an effect of EM on the immune defense system of the hosts.

The most significant difference between endogenous and intravenous infection model is that the former involves the steps of bacterial colonization, overgrowth, and invasion and consequently closely mimic the pathophysiology of septicemia in human.

In this endogenous bacteremia model, administration of ampicillin alters the normal intestinal flora and promotes superinfection with *P. aeruginosa* (13, 24). It is therefore possible that EM maintains or restores the composition and number of protective flora.

Some investigators have reported that sub-MICs of cephem, aminoglycosides, and quinolone suppress the expression of several exoenzymes (12, 25). Moreover, Kita et al. (18) have recently shown that EM stearate suppresses the production of proteases and leukocidin by *P. aeruginosa* without affecting cell growth. On the other hand, other investigators have demonstrated that sub-MICs of antibiotics enhance phagocytosis, killing by macrophages, and serum sensitivity of bacteria (1, 3). Because EM interferes with bacterial protein synthesis, it is possible that a subinhibitory concentration of this antibiotic may alter superficial components, have an effect on extracellular products, and consequently, reduce the virulence of *P. aeruginosa*.

Antibiotic treatment can have a direct effect not only on bacterial virulence, but also on the host's defense system (22). Macrolide antibiotics penetrate and concentrate in polymorphonuclear neutrophils (PMN) and macrophages (16, 22). Some investigators have reported that EM enhances such PMN functions as chemotaxis, ingestion, and bactericidal activity (10, 11). Concerning the interaction of EM with PMN functions, controversial data have been reported (9, 19). Although the adjustment of the inoculum size is so delicate that the model may easily lead to misinterpretation

TABLE 2. Influence of EML and ceftazidime on survival rate of mice with endogenous *P. aeruginosa* bacteremia

Agent	Dose[a] (mg/kg/day)	Survival rate (%)
EML	500	10
Ceftazidime	400	90[b]
EML + ceftazidime	500 + 400	50[c]
Control (saline)		15

[a] Given b.i.d. 12 h apart by intraperitoneal injection between days 7 and 20 (20 mice per group).
[b] Significantly higher than the survival rate of control mice ($P < 0.01$).
[c] Significantly higher than the survival rate of control mice ($P < 0.05$); however, significantly lower than the survival rate of mice treated with ceftazidime alone ($P < 0.05$).

TABLE 3. Influence of EML on survival rate of normal mice infected intravenously with *P. aeruginosa*

No. of bacteria[a] (CFU/mouse)	Survival rate (%) of mice		
	Treated with EML[b] between:		Control[c]
	Day 1 and 7	Day 8 and 14	
1.2×10^7	50.0	0.0	16.7
6.0×10^6	100.0[d]	50.0	50.0
3.0×10^6	100.0	100.0	97.2

[a] Mice were challenged intravenously with the specified number of bacteria on day 8 (18 mice per group).
[b] Mice were inoculated intraperitoneally with 100 mg of EML per kg/day b.i.d., 12 h apart.
[c] Mice were inoculated intraperitoneally with a dose of sterile benzyl alcohol equivalent to the dose of EML solution.
[d] Significantly higher than the survival rate of control mice ($P < 0.01$).

TABLE 4. Influence of EML on survival rate of granulocytopenic mice infected intravenously with *P. aeruginosa*

No. of bacteria[a] (10^3 CFU/mouse)	Survival rate (%) of mice		
	Treated with EML[b] between:		Control[c]
	Day 1 and 7	Day 8 and 14	
4.4	13.3	6.7	13.3
2.2	40.0[d]	6.7	6.7
1.1	73.3	66.7	77.3

[a] Mice were challenged intravenously with the specified number of bacteria on day 8 (15 mice per group).
[b] Mice were inoculated intraperitoneally with 100 mg of EML per kg/day b.i.d., 12 h apart.
[c] Untreated mice were used as a control.
[d] Significantly higher than the survival rate of control mice ($P < 0.05$).

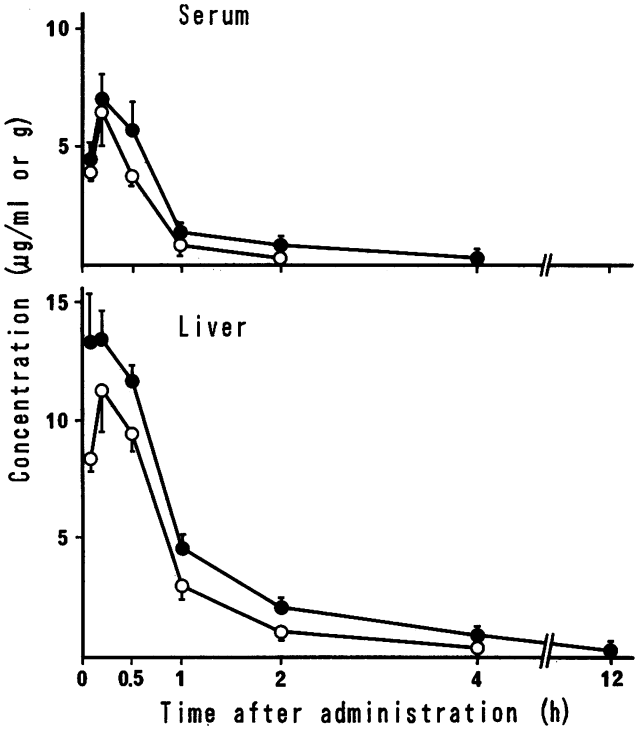

FIG. 3. Concentrations of EML in sera and livers of mice after single and repeated doses (mean ± standard error of results for five mice). Symbols: ○, a single dose of 50 mg of EML per kg given intraperitoneally; ●, 100 mg of EML per kg per day given b.i.d. for 14 days.

in our intravenous infection model, treatment with EML showed protective effect against *P. aeruginosa* infection in both normal and granulocytopenic mice. Because the efficacy was higher in normal mice, it is possible that EM may affect the PMN function. Anderson et al. (2) reported that pretreatment with EM increased the mean survival times of mice lethally infected with *Candida albicans*. Our findings are similar to theirs; however, EM was also effective in granulocytopenic mice with endogenous or intravenous infections. Our results suggest that EM may possess an immunostimulating activity in addition to its effect on PMN. Kita et al. (17) have shown that the long-term administration of EM to mice enhanced the production of interleukin-1 (IL-1) by macrophages and that of IL-2 and IL-4 by splenocytes. With regard to IL-1, it is known that this cytokine increases the survival rate of mice systemically infected with *P. aeruginosa* (21). Recently, Bailly et al. have reported that EM increased IL-6 production by human monocytes in vitro (4). Therefore, it is possible that EM possesses a lot of immunopotentiating effects, including the enhancement of cytokines production, and subsequently enhances the host defense system.

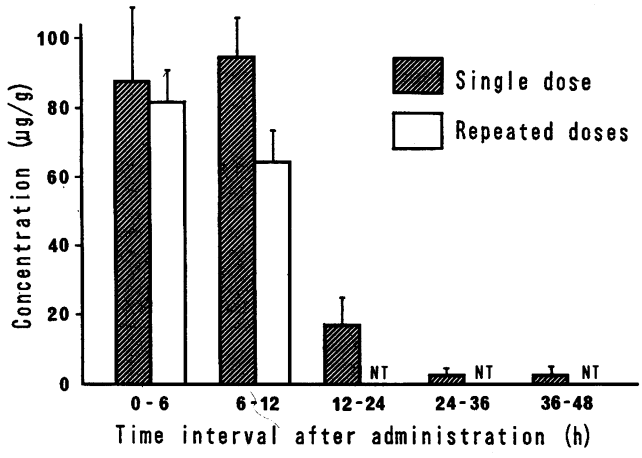

FIG. 4. Concentrations of EML in stools of mice following single and repeated doses (mean ± standard error of results for five mice). Mice were given a single dose of 50 mg of EML per kg once or 100 mg of EML per kg per day b.i.d. for 14 days. NT, not tested.

Probably, *P. aeruginosa* produce various extracellular virulent factors in the intestine with their growth. Therefore, we speculate that sub-MIC of EM may influence the organism mainly in the intestine as well as the host defense system before bacterial invasion into blood in our endogenous bacteremia model.

Clindamycin interferes with the synthesis of bacterial protein by binding to the 50S subunit of ribosomes, as does EM. However, clindamycin was ineffective against *P. aeruginosa* bacteremia. It is possible that the protective effect against *P. aeruginosa* infection by antibiotics lacking specific activity against this pathogen is specific to EM or macrolides.

Although 500 mg of EML per kg per day was not lethal for healthy mice, it caused apparent weight and appetite loss. This dosage of EML might have been toxic to infected animals because the addition of the high dosage of EML to ceftazidime sharply reduced the beneficial effect of ceftazidime.

In conclusion, we have demonstrated a protective activity of EM against *P. aeruginosa* bacteremia in mice. However, further studies will be required to clarify the protective mechanism and clinical usefulness of EM in this respect.

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