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Immunomodulating Effect of Fosfomycin on Gut-Derived Sepsis Caused by *Pseudomonas aeruginosa* in Mice

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We evaluated the protective effect of fosfomycin (FOM) and an enantiomer of fosfomycin [FOM(+); an isomer of FOM with no bactericidal activity] on murine gut-derived sepsis caused by *Pseudomonas aeruginosa*. Endogenous bacteremia was induced by administering cyclophosphamide (CY) and ampicillin to specific-pathogen-free mice fed *P. aeruginosa*. Treatment of mice with FOM at 250 mg/kg of body weight per day twice a day after the second CY administration significantly increased the survival rate compared to that for control mice treated with saline. Treatment with FOM(+) at 20 and 100 mg/kg also significantly increased the survival rate (from 30% for control mice to 80% for treated mice). The bacterial counts in the liver and blood were both significantly lower in FOM(+)-treated mice in comparison with those in the liver and blood of saline-treated control mice. FOM(+) administration affected neither the bacterial colonization in the intestinal tract nor the leukocyte counts in the peripheral blood of the mice. After intravascular inoculation of *P. aeruginosa*, treatment of mice with FOM(+) did not enhance bacterial clearance from the blood of mice pretreated or not pretreated with CY. FOM(+) significantly suppressed tumor necrosis factor alpha, interleukin-1β, and interleukin-6 levels in the serum of mice after gut-derived sepsis. These results indicate that both FOM and FOM(+) have protective effects against *P. aeruginosa* bacteremia, despite a lack of specific activity of FOM(+), and suggest that FOM may possess immunomodulating activity and that it induces a protective effect. The protective mechanism is speculated to be that FOM modulates the in vivo production of inflammatory cytokines.

Septic shock usually results from gram-negative bacteria that are rarely pathogenic in healthy hosts. *Pseudomonas aeruginosa* is frequently isolated as a causative agent of septicemia in immunocompromised hosts (6, 8), and septicemia caused by *P. aeruginosa* has a higher fatality rate than that caused by any other gram-negative bacteria (6, 8, 44). We induced endogenous *P. aeruginosa* bacteremia by administering cyclophosphamide (CY) and ampicillin to specific-pathogen-free mice fed *P. aeruginosa*. This model incorporated oral inoculation of bacteria, subsequent bacterial colonization, overgrowth in the intestinal tract, and invasion into the bloodstream. Consequently, this animal model closely mimics the pathophysiology of septicemia in humans (10).

Recently, several antimicrobial agents have been shown to affect the host immunologic responses, such as lymphocyte proliferation and cytokine production (31–34). Fosfomycin (FOM), *l-cis*-1,2-epoxypropylphosphoric acid, is a broad-spectrum bactericidal antibiotic that is not structurally related to other classes of antimicrobial agents. It acts against gram-positive and gram-negative bacteria by inhibiting the first step in bacterial cell wall synthesis (13). Some investigations have demonstrated that FOM affects host immunologic responses. For example, FOM inhibits immunoglobulin E-mediated histamine release from peripheral blood basophils (15) and pulmonary histamine release from lungs isolated from guinea pigs (28). FOM, in combination with steroids, improves the clinical symptoms of patients with severe bronchial asthma (24). Moreover, Morikawa et al. (21, 22) have demonstrated that FOM inhibits the proliferative response of resting B cells and immunoglobulin secretion (21) and suppresses the proliferation of human lymphocytes and the mixed lymphocyte reaction and interleukin-2 (IL-2) production by T cells (22).

Septic shock is an often fatal condition, and the excessive production of inflammatory cytokines is thought to be responsible for the lethality (2, 5, 16, 40, 42). We hypothesized that if host immunologic responses including cytokines are modulated by an isomer of FOM with no bactericidal activity [FOM(+)], we could demonstrate the protective effect of FOM in our murine gut-derived sepsis model. Interestingly, the protective effect may not be produced by the antibacterial activity but rather may be produced by the immunomodulatory effect of FOM, because the enantiomer of fosfomycin [FOM(+)] also showed a protective effect, despite a lack of specific activity for this pathogen.

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 of oral administration of the bacteria.

Bacterial strain. *P. aeruginosa* D4 isolated from the blood of a neutropenic mouse with bacteremia (11) was used. The strain was maintained frozen at −80°C in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) containing 15% glycerol.

Reagents. FOM sodium, FOM(+), and ampicillin (ABPC) were supplied by Meiji Seika, Ltd., Tokyo, Japan. These drugs were dissolved in pyrogen-free water and were then suspended in saline for injection.

In vitro susceptibility. The MICs of FOM and FOM(+) for *P. aeruginosa* D4 were determined by an agar dilution method (9) by using nutrient agar (Difco Laboratories). Bacterial suspensions cultured overnight in nutrient broth were inoculated at a final concentration of 5.0×10^5 CFU/ml. Each inoculated agar plate was incubated at 37°C for 18 h, and the lowest drug concentration at which the growth was completely suppressed was expressed as the MIC.

Survival study of mice with gut-derived sepsis due to *P. aeruginosa* D4. Murine gut-derived sepsis was produced as described previously (10). 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

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of 10^7 CFU/ml. The bacterial suspension was given in the drinking water between days 1 and 3. To aid in the colonization of *P. aeruginosa*, the normal intestinal flora of the mice was disturbed by administering 200 mg of ABPC per kg of body weight by intraperitoneal injection daily between days 1 and 3. Mice were then given 150 to 200 mg of CY per kg 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 CY administration.

To determine the effect of FOM or FOM(+), each group of mice was given either of these drugs twice a day after the second CY treatment by intraperitoneally injection. 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 confirm whether the administration of FOM(+) ameliorates the infection, we measured viable bacterial counts in liver and blood. Mice from each treatment group were killed by inhalation of ether at the indicated time points, and cardiac blood and liver samples were obtained aseptically. The liver was homogenized in sterile saline. A portion 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 were then centrifuged at 2,000 × *g* for 15 min. Serum samples were preserved at −80°C until cytokine level measurement.

Bacterial clearance study. *P. aeruginosa* D4, which is relatively resistant to clearance from the blood of healthy mice (11), was incubated with Mueller-Hinton agar at 37°C for 24 h. The cells were suspended in pyrogen-free saline at a concentration of 7.5×10^7 CFU/ml for CY-treated mice and 1.6×10^9 CFU/ml for CY-untreated mice. Clearance from blood was evaluated as described previously (11). Briefly, 0.2 ml of bacterial suspension was injected into the tail vein of the mice. At 15 and 30 min after injection, 20-μl samples of blood were obtained from the retro-orbital plexus, and the blood was diluted in saline and plated onto Trypticase soy agar. The values at time zero were calculated by estimating the blood volume as 8% (vol/wt) of the body weight of each mouse used. FOM(+), at concentrations of 20 and 100 mg/kg, was intraperitoneally administered every 12 h six times before the clearance assay. The last administration of FOM(+) was done 1 h before bacterial challenge. CY at 200 mg/kg was administered by intraperitoneal injection 3 days before the clearance assay.

Influence of FOM(+) on phagocytic activity of murine peritoneal macrophages. The effect of FOM(+) on phagocytosis of *P. aeruginosa* D4 by murine peritoneal macrophages was assessed as follows. Mice were intraperitoneally administered either 20 or 100 mg of FOM(+) per kg or saline every 12 h for six administrations. Peritoneal macrophages were freshly drawn from each group of mice and were incubated with RPMI 1640 medium containing 5% normal mouse serum and logarithmic-phase bacteria. The mixtures were incubated in a 24-well tissue culture plate (Falcon 3047; Becton Dickinson & Co.) with rocking for 2 h at 37°C in 5% CO_2. The number of unphagocytosed viable bacteria in the medium was determined by plating the culture supernatant on Mueller-Hinton agar and by incubation at 37°C for 24 h. The phagocytic activities of the macrophages were estimated by the reduction rates, calculated as follows: reduction rate (percent) = 100 − [(number of CFU in the medium at 2 h)/(number of CFU in the medium at time 0)] × 100.

Cytokine assay. Serum samples were obtained as described above. Tumor necrosis factor alpha (TNF-α) and IL-6 levels in mouse serum were determined with enzyme-linked immunosorbent assay (ELISA) kits (Endogen Inc., Boston, Mass.). IL-1β 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. Serum cytokine levels, leukocyte counts, reduction rates, and viable bacterial counts in blood, liver, and feces were compared by the Mann-Whitney U test. A probability level of 5% was considered significant.

RESULTS

In vitro susceptibility of *P. aeruginosa*. The MICs of FOM and FOM(+) for *P. aeruginosa* D4 were 256 and >8,000 μg/ml, respectively. These data indicate that the strain used in this study was apparently resistant to FOM and that FOM(+) had no antimicrobial effect on this organism. We evaluated the effect of glucose-6-phosphate, which enhances uptake of this drug (9), on the MICs. The results revealed that no effect on the MICs was observed from addition of 50 μg of glucose-6-phosphate per ml (data not shown).

We furthermore studied the in vitro activities of FOM and FOM(+) in combination with CY against *P. aeruginosa* D4. Then we found that the addition of 100 μg of CY per ml did not enhance the activity of FOM or FOM(+) (data not shown).

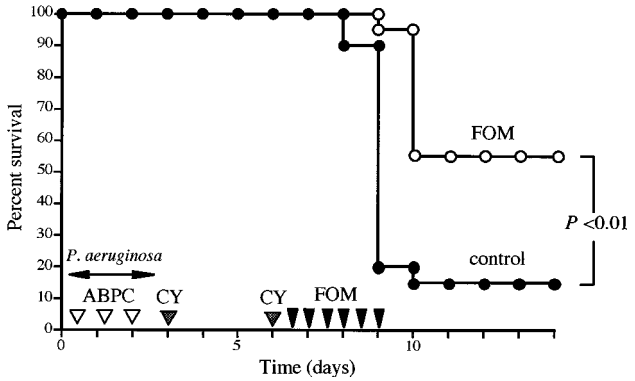

FIG. 1. Effect of FOM on survival of mice after gut-derived sepsis caused by *P. aeruginosa*. Mice in groups of 16 mice each were administered FOM at 250 mg/kg intraperitoneally (open circles) or saline (controls; solid circles) twice a day on the indicated days (arrowheads).

Effect of FOM or FOM(+) on the survival of mice after gut-derived sepsis caused by *P. aeruginosa*. We evaluated the effect of FOM on the survival of mice with gut-derived sepsis. Figure 1 presents the survival kinetics of mice given FOM or saline. We found that treatment with 250 mg of FOM per kg protected mice against mortality in comparison with saline treatment.

Treatment with FOM(+) at 20 and 100 mg/kg twice a day significantly increased the survival rate (80%, compared to 30% for the control mice; Fig. 2).

There is a possibility that the sodium load included in FOM and FOM(+) influence the survival of mice. Therefore, we evaluated the effect of sodium load on the survival of mice in this model. However, we could not demonstrate a significant increase in the survival rate (60%, compared to 50% for the control) of mice by treatment with NaCl at a concentration of 160 mg/kg, which is equal to the sodium volume included in 250 mg of FOM per kg.

Kinetics of viable bacterial counts in liver and blood of mice after gut-derived sepsis with *P. aeruginosa*. Figure 3A presents the kinetics of viable bacterial counts in the liver after the second CY treatment. On the second and third days after the second CY treatment, the average number of viable bacteria in

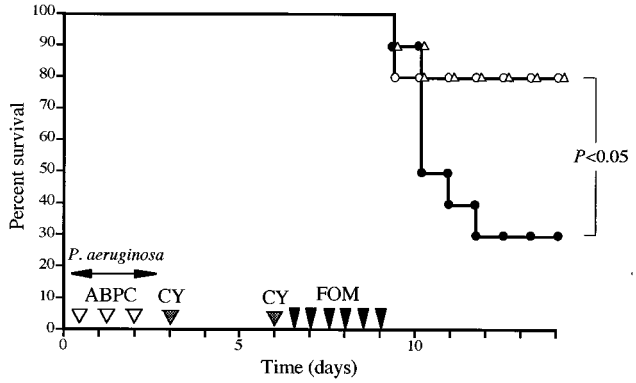

FIG. 2. Effect of FOM(+) (FOM) on survival of mice after gut-derived sepsis caused by *P. aeruginosa*. Mice in groups of 10 mice each were administered FOM(+) intraperitoneally at 20 mg/kg (open triangle) or 100 mg/kg (open circles) or saline (solid circles) twice a day on the indicated days (arrowheads).

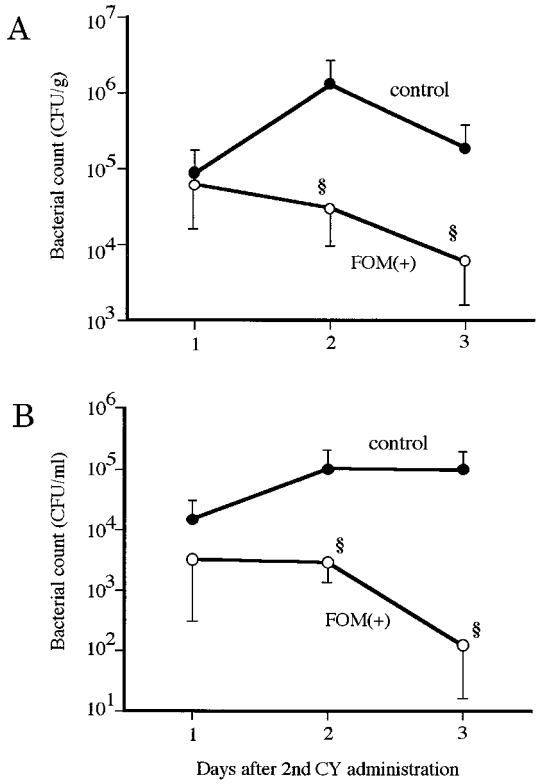

FIG. 3. Effect of FOM(+) at 100 mg/kg (open circles) or saline (control; solid circles) on viable bacterial counts in liver tissue (A) and blood (B) after gut-derived sepsis in mice. Viable bacterial counts in liver tissue are expressed as the numbers of CFU per gram of liver (wet weight). Values are means ± standard errors of the means (six mice in each group). §, $P < 0.05$.

the livers of FOM(+)-treated mice was significantly lower than that in the livers of saline-treated mice. Furthermore, FOM(+) significantly decreased the number of viable bacteria in blood in comparison with that in the blood of saline-treated mice (Fig. 3B).

Influence of FOM(+) on bacterial colonization in the intestinal tract. We examined the effect of FOM(+) on *P. aeruginosa* colonization in the intestinal tract, because the intestinal tract is the first reservoir of the bacteria in this model. However, FOM did not affect the bacterial colonization in the intestinal tracts of the mice (Fig. 4).

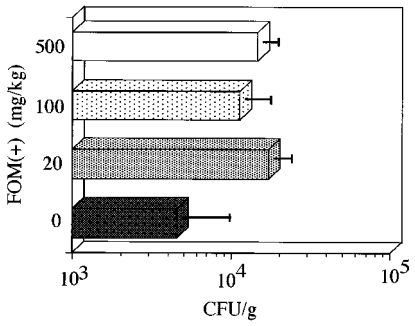

FIG. 4. Effects of various doses of FOM(+) on *P. aeruginosa* colonization of the intestinal tracts of mice. Values are means ± standard errors of the means (eight mice in each group). There was no significant difference between the groups.

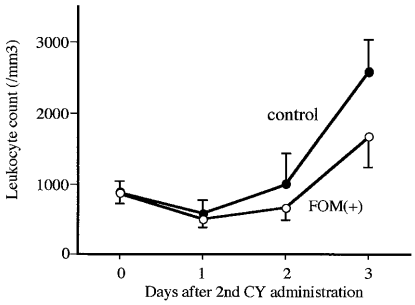

FIG. 5. Effect of FOM(+) at 100 mg/kg (open circles) or saline (solid circles) on leukocyte counts after the second CY treatment. Values are means ± standard errors of the means (eight mice in each group). There was no significant difference between the groups.

Influence of FOM(+) on leukocyte counts. We also examined the effect of FOM(+) on leukocyte recovery in the peripheral blood after the second CY administration, because the number of leukocytes influences the mortality of mice. As shown in Fig. 5, FOM(+) did not accelerate the leukocyte recovery.

Influence of FOM(+) on bacterial clearance from blood. As shown in Fig. 6A, treatment of FOM(+) did not significantly enhance the bacterial clearance from the blood of healthy mice at all time points. There was no significant difference between the groups of CY-treated mice at any time point (Fig. 6B).

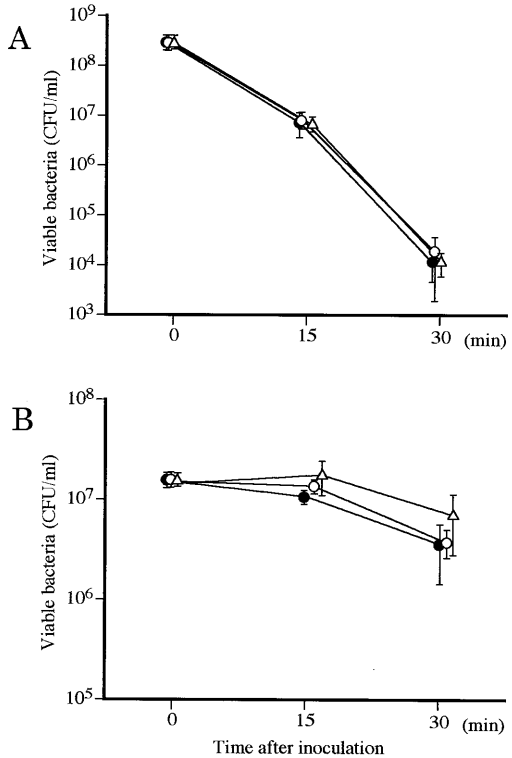

FIG. 6. Effect of FOM(+) at 20 mg/kg (open circles) or 100 mg/kg (open triangles) or saline (solid circles) on bacterial clearance in CY-treated and untreated mice. Each mouse in the CY-treated group (A) and the untreated group (B) was inoculated with 3.2×10^8 and 1.5×10^7 CFU of *P. aeruginosa* D4, respectively. Data are means ± standard deviations (five mice in each group). There was no significant difference between the groups at any time point.

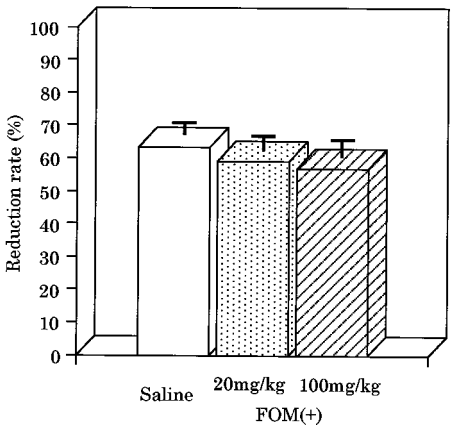

FIG. 7. Influence of FOM(+) on phagocytosis of bacteria by murine peritoneal macrophages. After treatment of mice with either 20 or 100 mg of FOM(+) per kg or saline, 5×10^5 cells of peritoneal macrophages from each group of mice per ml were incubated with 4×10^4 CFU of *P. aeruginosa* D4 per ml. Rates of reduction were calculated as described in Materials and Methods. Values are means ± standard errors of the means (eight mice in each group). There was no significant difference in the rates of reduction among the groups.

Influence of FOM(+) on phagocytic activity of murine peritoneal macrophages. Decreased bacterial counts in liver and blood after FOM(+) treatments may indicate an enhancement of phagocytic activity of murine macrophages against bacteria. Therefore, we studied the influence of FOM(+) on the antibacterial phagocytic activity of murine peritoneal macrophages. The results are depicted in Fig. 7 and indicate that in vivo treatment with FOM(+) did not significantly enhance the antibacterial phagocytic activity of peritoneal macrophages.

Kinetics of cytokines in the serum of mice after gut-derived sepsis with *P. aeruginosa*. In our preliminary study, we confirmed that neither FOM nor FOM(+) administration to healthy mice induce elevated cytokine (TNF-α, IL-1β, and IL-6) levels in serum (data not shown). As indicated in Table 1, the mean serum TNF-α level in FOM(+)-treated mice was significantly lower than that in control mice ($P < 0.05$). Furthermore, the mean levels of IL-1β and IL-6 in serum were both significantly suppressed in comparison with those in control mice ($P < 0.05$).

TABLE 1. Effect of FOM(+) on serum cytokine levels after gut-derived *P. aeruginosa* sepsis in mice[a]

Day	Group	Level (ng/ml)		
		TNF-α	IL-1	IL-6
1	Control	1.10 ± 0.92	0.32 ± 0.15	3.58 ± 1.41
	FOM(+)	1.08 ± 0.41	0.07 ± 0.07	0.33 ± 0.32
2	Control	1.09 ± 0.32	1.83 ± 0.54	17.67 ± 5.51
	FOM(+)	1.05 ± 0.56	0.10 ± 0.05[b]	1.27 ± 0.59[b]
3	Control	2.36 ± 0.93	2.42 ± 0.26	8.91 ± 3.90
	FOM(+)	0.76 ± 0.45[b]	0.34 ± 0.21[b]	0.37 ± 0.28[b]

[a] Values are means ± standard errors of the means (five mice in each group). FOM(+) was administered intraperitoneally at 100 mg/kg twice a day. The sensitivities of the ELISA methods for determination of the TNF-α, IL-1, and IL-6 levels were 50, 15, and 25 pg/ml, respectively. Our preliminary study revealed that serum cytokine levels for untreated healthy mice were below the detection levels by the methods that we used.

[b] $P < 0.05$.

DISCUSSION

Clinical studies that use surveillance cultures of fecal samples from immunocompromised patients suggest that the gastrointestinal tract may be a primary reservoir for opportunistic bacteria (39). Berg and Garlington (4) and Deitch et al. (7) have demonstrated that bacteria contained within the gut can cross the gastrointestinal mucosal barrier and spread systemically, a process termed bacterial translocation.

We first studied the effect of FOM on the survival of mice with gut-derived sepsis and found a protective effect associated with this drug. However, treatment of bacteria with subinhibitory concentrations of antimicrobial agents frequently increases bacterial phagocytosis, intracellular killing, and susceptibility to serum (1, 3). For example, pretreatment of *Staphylococcus aureus* with FOM clearly sensitized the bacteria to leukocytic killing in the presence of normal human serum (27). Thus, we have to confirm that the survival results for mice treated with FOM were not reflective of the sub-MIC effect of the drug against the bacterial strain used in this study. Therefore, after the initial series of experiments, we decided to use an enantiomer of fosfomycin [FOM(+)], which is structurally an isomer of FOM and which lacks antibacterial activity against *P. aeruginosa*.

Bacterial translocation may occur with alterations of host defense, disruption of the normal indigenous bacterial flora, or loss of the mucosal barrier (7, 17, 18). Knothe et al. (14) studied the influence of FOM on the intestinal flora in healthy volunteers. Although the counts of *Escherichia coli* and enterococci were reduced, no other bacteria, including anaerobic bacteria, were selected while the volunteers were on the medication (14). From our results, there was no significant change in the colonization of *P. aeruginosa* in the intestinal tract after treatment with FOM(+). Therefore, we speculate that the mechanism of the protective effect of FOM and FOM(+) is not due to an influence on the microbial intestinal flora.

Singer et al. (37) studied patients with bacteremia and fungemia as complications of neoplastic disease and found that mortality was higher among patients with leukopenia than among those with normal leukocyte counts. Therefore, we think that the leukocyte counts are also important in the prognosis of this animal model. However, in this study, FOM(+) administration did not affect the leukocyte counts. Milatovic (20) studied the effect of FOM on phagocytosis of *P. aeruginosa* by human polymorphonuclear leukocytes and revealed that the rates of uptake of untreated and FOM-treated bacteria were not significantly different. Therefore, we thought that neither the effect of FOM(+) on the number of leukocytes nor the direct effect of FOM(+) against *P. aeruginosa* in supporting bacterial phagocytosis may be the mechanism of the phenomenon that we observed.

The clearance of bacteria from blood by Kupffer cells plays an important role in protecting against systemic bacteremia in this animal model (11). From our results, treatment of mice with FOM(+) did not enhance the clearance of bacteria from blood. Therefore, we thought that a direct effect of FOM(+) on the Kupffer cells was unlikely.

There is also a possibility that FOM(+) enhances the antibacterial phagocytic activities of murine macrophages. Therefore, we evaluated the influence of FOM(+) on phagocytosis of *P. aeruginosa* D4 by murine peritoneal macrophages. The results revealed that treatment with FOM(+) did not significantly enhance the antibacterial phagocytic activities of murine peritoneal macrophages. Therefore, we thought that an enhancement of the phagocytic activities of murine macrophages by FOM(+) was also unlikely.

Septic shock is often a fatal condition, and the excessive production of host inflammatory mediators is thought to be responsible for the lethality. Several studies have indicated that septic shock is mediated mainly by inflammatory cytokines. In particular, TNF-α has been shown to be an important mediator of endotoxic shock (5, 29, 40). Various reports have demonstrated that other cytokines such as IL-1β and IL-6 also play important roles in the pathologic manifestations of endotoxic or septic shock (2, 16, 38, 42, 43). Therefore, we further studied the influence of FOM(+) on the levels of TNF-α, IL-1β, and IL-6 in the serum of mice, and the results revealed that FOM(+) significantly suppresses the endogenous production of these cytokines. Although we could not directly demonstrate that the survival of mice correlated with cytokine levels, we think that alteration of cytokine production by FOM and FOM(+) may potentially affect the pathophysiology of septic shock.

Some investigators have revealed the possibility that supplemental treatment with FOM reduces cisplatin- or aminoglycoside-induced nephrotoxicity and ototoxicity (12, 25, 26, 35, 36, 41). Administration of antineoplastic drugs, including CY, may also induce mucosal damage to the intestinal tract and facilitate bacterial translocation. Therefore, we speculated that one of the mechanisms of the protective effect of FOM against gut-derived sepsis is the lessening of mucosal damage, and we are now investigating this possibility.

Morikawa et al. (23) demonstrated that FOM increases the synthesis of IL-6 and IL-10 and decreases the synthesis of IL-1, TNF, and granulocyte-macrophage colony-stimulating factor by lipopolysaccharide (LPS)-stimulated human monocytes in vitro. The results concerning IL-1 and TNF were coincident with our results, although we demonstrated suppression of IL-6 production in vivo. We speculate that this conflict may resulted from differences in experimental design. We confirmed that FOM (+) increased the levels of IL-6 in serum after LPS stimulation in vivo (data not shown). Therefore, differences in the pathophysiology between LPS-inoculated endotoxic shock and gut-derived sepsis may reflect the conflicting results.

We have previously reported that erythromycin and amphotericin B may enhance the host defense system and induce resistance to *P. aeruginosa* infection in mice (10, 19). Some investigators have reported that quinolones and β-lactams also affect host immunologic responses (30, 31). These immunomodulating effects of antibiotics may be useful in treating patients with septic syndrome, septic shock, and acute respiratory distress syndrome, because these patients have significant alterations in inflammatory and immune responses. Because, we cannot directly translate our results to humans, we also have to determine conditions of drug administration, such as the timing or doses of FOM, to induce the maximum protective effect of the drug in humans.

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