Time- and Dose-Dependent Effect of Fosfomycin on Suppression of Infection-Induced Endotoxin Shock in Mice

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Therapeutic effects of fosfomycin (FOF) and imipenem (IPM) were investigated in a novel model for endotoxin shock that was caused by intraperitoneal (i.p.) infection with 10⁹ colony forming units of attenuated Salmonella typhimurium. Acute lethal shock was observed in BALB/c and ddY but not in lipopolysaccharide (LPS)-nonresponder BALB/lpsd mice. Effects of FOF, but not its enantiomer, and IPM were dose- and time-dependent, since therapeutic efficacy was demonstrated in mice injected i.p. or orally at doses of more than 20 mg/kg 15 min before or 1 h after infection. Treatment with FOF 1 h postinfection (p.i.) resulted in significant decreases in bacterial numbers in spleen and liver, suggesting that the antimicrobial activity of FOF seems to closely correlate to suppression of infection-induced lethal shock. Regarding coagulation systems, FOF inhibited increase in the prothrombin time but upregulated fibrinogen concentration. Plasma levels of LPS released from bacilli were significantly higher in FOF- than IPM-treated mice and infection controls, but both antibiotics showed similar efficacy in protection.

Key words Salmonella-infection; endotoxin shock; fosfomycin; imipenem

Septic shock, systemic inflammatory response syndrome and multiple organ failure caused by infections correlate with high mortality. In infections with gram-negative bacteria, these systemic inflammatory responses are caused by lipopolysaccharide (LPS), the major component of the outer membrane. LPS triggers the production of various cytokines (TNF-α, IL-1β and IL-6 etc.), chemokines (IL-8 and monocyte chemotactant protein 1 etc.), and biologically active components implicated in inflammation (tissue factor, platelet activating factor, prostaglandins, thromboxanes, leukotrienes and nitric oxide etc.) via toll-like receptor 4 (TLR4)-dependent or -independent pathways.¹⁻³) These substances play a pivotal role for triggering septic shock.

Although several animal models for LPS-induced lethal shock have been developed,⁴⁻⁶) they are not always satisfactory in terms of rapid clearance of LPS from the circulation and necessity of large quantity of LPS for causing lethal shock. Since these aspects are different from clinical cases, infection-induced shock models are necessary for analyzing the protective effect of antibiotics in vivo. When LPS-sensitized mice were infected intraperitoneally (i.p.) with Escherichia coli or Pseudomonas aeruginosa and then treated with antibiotics, mice showed septic shock with differences in outcome, depending on different amounts of LPS released from bacilli by treatment with imipenem (IPM) and ceftazidime (CAZ).⁷⁻⁶

β-Lactam antibiotics such as IPM block the final step of peptidoglycan biosynthesis in the outer membrane and preferentially bind to penicillin-binding protein-2 (PBP-2).⁸) As a result, they cause rapid killing and sporeform formation of gram-negative bacteria with relatively little release of LPS.⁹) In contrast, fosfomycin (FOF) binds to UDP-N-acetyl-glucosamine enolpyruvate transferase and irreversibly inactivates this enzyme which catalyzes the first step of peptidoglycan biosynthesis in the bacterial cytoplasm.¹⁰) Recently, interesting studies showed that FOF suppressed TNF-α production by human monocytes and murine macrophages stimulated with LPS.¹¹,¹²)

We recently established a new endotoxin shock model based on infection with high numbers (10⁸ colony forming units (CFU)) of attenuated Salmonella typhimurium. In this model severe thrombosis and liver dysfunction following lethal shock is observed in LPS-responder but not in LPS-nonresponder mice, suggesting that this shock is potentially mediated by host responses to LPS released during infection. The result about suppression of TNF-α production by FOF and its enantiomer FOF+ encouraged us to investigate beneficial effects of FOF in infection-induced lethal models. We demonstrated in this study that the therapeutic effects of FOF and IPM were time- and dose-dependent using a novel endotoxin shock model.

MATERIALS AND METHODS

Chemicals, Glassware and Plastics Heparin (1000 units/ml, Novo Nordisk A/S, Denmark), Endospecy® (Seikagaku Corp., Tokyo), an endotoxin-free alkaline reagent consisting of 0.1 N KOH, 0.1% Triton X-100, 10 mM CaCl₂, 30 mM N,N'-bis(2-hydroxyethyl)glycine (Bicine), 0.07% ethyleneimine polymer and 0.1% polybrene (Seikagaku Corp.), the reference endotoxin standard of USP (Lot F) and E. coli 0111:B4 (Lot 735900, Difco Laboratories, Detroit) were used. A highly purified LPS preparation from Salmonella abortus equi was obtained from Dr. Chris Galanos (Max-Planck-Institute for Immunobiology, Freiburg in Breisgau, Germany). The stock solution (1 mg/ml) of S. abortus equi LPS dissolved in distilled water was clear. Working solutions were made with pyrogen-free saline (Otsuka Pharmaceutical Co., Tokushima, Japan). All glassware used in measurement of endotoxin was heated at 250 °C for 2 h to eliminate potentially contaminating environmental endotoxin. Endotoxin-free plastic tips and plastic pipettes were purchased from...
Seikagaku Corp. and Corning Inc. (Corning, NY, U.S.A.), respectively. FOF and FOF$^+$ (Meiji Seika Ltd., Tokyo, Japan) dissolved distilled water was mixed with saline (v/v = 1:3) before use. IPM (Merck-Banyu Pharmaceutical Co., Tokyo, Japan) was dissolved in saline for preparing a stock solution.

**Mice** Female BALB/c and ddY mice were purchased from Clea Japan Inc. (Tokyo, Japan) and Japan SLC Inc. (Hamamatsu, Japan), respectively. Breeding pairs of BALB/lps$^d$ mice were obtained from the Max-Planck-Institute for Immunobiology. All mice were kept at the animal facility of the Kitasato University School of Science under specific pathogen free conditions and fed autoclaved food and water.

For infections all mice were housed in plastic cages with filter setting in a clean-air streamed box of a P-2 level room and fed autoclaved food and water. The experiments described in this study were performed in adherence to National Institutes of Health guidelines on the use of experimental animals. Approval of the Animal Use Committee of the Kitasato University School of Science was obtained prior to initiating the experiments.

**Bacteria and Inoculation** An attenuated strain of *S. typhimurium* SL7207 araA, obtained from Dr. Bruce Stocker (Stanford University Medical School, Stanford, CA, U.S.A.) via Dr. Stephan Kaufmann (Max-Planck-Institute for Infection Biology, Berlin, Germany), was used. Bacteria were grown in tryptic soy broth (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) for 18 h at 37 °C. After washing with phosphate buffered saline (PBS), bacterial numbers were calculated from a standard curve made based on turbidity and counted colony numbers. Mice were injected i.p. with bacterial suspension (5×10$^8$ CFU/ml) in a volume of 0.2 ml. The inoculum size was checked by plating diluted aliquots on tryptic soy agar (Difco Laboratories, Detroit, MI, U.S.A.).

**Preparation of Blood Samples** Following deep anesthesia with diethyl ether, blood was taken from mice by cardiac puncture using a 1-ml syringe with 26G needle. For measuring endotoxin units and fibrinogen concentration, blood was immediately mixed with 20-μl heparin and then centrifuged at 10000×g for 1 min at room temperature. Plasma samples were collected carefully and used immediately or kept at −80 °C until use. For counting platelet numbers, blood samples (0.5-ml aliquots) were immediately mixed with 0.1 ml of 3.8% sodium citrate solution (Nipro Inc., Tokyo, Japan).

**Determination of Endotoxin in Plasma** Plasma components affecting the *Limulus* cascade reactions were eliminated before endotoxin determination as described previously.

**Measurement of Platelet Numbers, Prothrombin Time and Fibrinogen Concentration** Platelet numbers were differentially counted by a SS-3000 autoanalyzer (Sysmex Co., Tokyo, Japan). The prothrombin time and fibrinogen concentration in plasma was measured by a Coagulex 100 autoanalyzer (Sysmex Co., Tokyo, Japan). The prothrombin time and fibrinogen concentration in plasma were measured by a Coagulex 100 autoanalyzer (Sysmex Co., Tokyo, Japan). The prothrombin time and fibrinogen concentration in plasma were measured by a Coagulex 100 autoanalyzer (Sysmex Co., Tokyo, Japan).

**Statistical Analysis** Statistical significance of the data was determined by the Scheffé of post-hoc and log-rank tests. A P value of less than 0.05 was taken as significant.

RESULTS AND DISCUSSION

Reduction of LPS-induced TNF-α production by macrophages after *in vitro* incubation with the antibiotic FOF and its non-bactericidal enantiomer FOF$^+$ has previously been reported. In a murine model of gut-derived sepsis with *Pseudomonas aeruginosa* Matsumoto et al. found that treatment with FOF and FOF$^+$ resulted in long-term survival due to reduction of TNF-α levels. Since it is generally accepted that TNF-α plays a central role for triggering of endotoxin shock, we investigated the suitability of FOF for shock treatment after intraperitoneal (i.p.) infection with 10$^9$ CFU of attenuated *S. typhimurium* araA. BALB/c mice died from shock within 24 h, whereas LPS-nonresponder BALB/lps$^d$ mice were resistant in this period, proving that mortality closely related to the LPS effects (Fig. 1A). BALB/lps$^d$ mice finally succumbed to bacteremia one week later. Administration of 200 mg/kg FOF$^+$ i.p. to BALB/c mice 15 min before infection was ineffective. In contrast, oral pretreatment with 20 mg/kg FOF 15 min before infection conferred resistance comparable to that of BALB/lps$^d$ mice, while i.p. administration with 20 mg/kg FOF 15 min before infection protected half of the animals from infection-based mortality (Fig. 1A). For further experiments the oral route, although less effective, was chosen as the one later more suitable for patients. To base our results not only on inbred mice but also on more robust outbred strains and ensure compatibility to other studies, ddY mice were used, which were found to die from LPS shock within 48 h (Fig. 1B). The time dependency of the FOF effect was determined by administering 200 mg/kg FOF 15 min before or 1, 3 or 6 h after infection and counting surviving mice in 6 h-intervals until 48 h post infection (p.i.) and then daily. As shown in Table 1, treatment 6 h p.i. was ineffective for prevention of endotoxin shock, whereas adminis-

![Fig. 1. (A) Survival of BALB/lps$^d$ and BALB/c Mice after *S. typhimurium* araA Infection with or without FOF Treatment](image-url)
tration at all other time points extended survival over the 48 h-limit typical for control mice (vs. the infection controls, by the log-rank test). Treatment 15 min before or 1 h after infection also conferred full or partial protection against infection, respectively, as all or some mice survived until day 14 p.i. To measure dose effects, various amounts of FOF were administered 1 h p.i. and mice were evaluated as before. Resistance to lethal shock was achieved by a dose of 2 mg/kg or more (Table 1). In order to address the determinants contributing to protection, the effects of FOF and IPM were compared. IPM has been identified as releasing comparatively low amounts of LPS during bacterial killing.16–18) Determined by a chromogenic Limulus test,14) a comparison of plasma LPS in mice treated orally with 20 mg/kg FOF or i.p. with 20 mg/kg IPM 1 h p.i. indeed confirmed lower values for IPM (Fig. 2A). Oral administration of 20 mg/kg FOF 1 h p.i. significantly increased plasma LPS levels until 12 h p.i. compared to infection control and IPM-treated mice. Nevertheless, both antibiotics protected mice to a similar degree (Fig. 1B). Treatment with FOF or IPM 1 h p.i. significantly reduced bacterial numbers in spleens between 1.5 h and 36 h p.i. (Fig. 2B). Bacterial kinetics was very similar in livers (data not shown). To link survival to the suppression of shock-related mortality factors, we then monitored coagulation, which ultimately leads to organ failure due to disseminated intravascular coagulation, a hallmark of septic shock.19) Prothrombine time and fibrinogen levels as indicators of thrombosis20) were also measured. Analysing plasma taken 14 h p.i. from mice treated with 20 mg/kg FOF or IPM 1 h p.i., reduction of the infection-induced increase of prothrombin time as well as the decrease of fibrinogen was evident compared to infection control mice (Fig. 3).

In previous studies we showed that 99% of LPS disappeared from the circulation within 6 h when mice were injected intravenously with 10 μg S. abortus equi LPS14) and that plasma LPS levels decreased faster in mice injected with heat-killed S. typhimurium arsA (equivalent to 3 × 10^8 CFU) than in mice infected with the same numbers of viable cells (data not shown). High levels of plasma LPS (2000–3000 EU/ml) persisted in infected mice observed even 36 h p.i. (Fig. 2A).

In summary, we identified FOF as potentially suitable agent to counter endotoxin shock using our model. Although the central role of LPS for this clinical syndrome is undisputed, our results indicate that high LPS levels in the initial phase of infection are not necessarily the only determinants for shock: both FOF and IPM, releasing high and low levels of LPS, respectively, but initially equally suppressing bacterial growth were able to counteract shock, while FOF as FOF-enantiomer without bactericidal activity was without effect. In a study of Matsumoto et al. FOF improved survival

<table>
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<tr>
<th>Timing</th>
<th>Dose (mg/kg)</th>
<th>Dead/tested</th>
<th>Mean survival time (d)</th>
<th>p value</th>
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<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>12/12</td>
<td>1.06±0.07</td>
<td>n.s.</td>
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<tr>
<td>+6 h</td>
<td>200</td>
<td>11/11</td>
<td>1.23±0.10</td>
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<td>9.50±1.02</td>
<td>&lt;0.0001</td>
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<tr>
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<td>4/13</td>
<td>12.85±0.50</td>
<td>&lt;0.0001</td>
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<tr>
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<td>7.40±0.70</td>
<td>&lt;0.0001</td>
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<tr>
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<tr>
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<td>10/10</td>
<td>1.57±0.30</td>
<td>0.0391</td>
</tr>
<tr>
<td>–15 min</td>
<td>200</td>
<td>0/8</td>
<td>&gt;14.00</td>
<td>&lt;0.0001</td>
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n.s.: not significant.
of mice after gut-derived sepsis, an effect explained by suppression of cytokines in a presumably less severe infection situation (10^7 orally administered *P. aeruginosa* compared to i.p. infection with 10^8 *S. typhimurium*). Whereas the system of Matsumoto *et al.* mimicked a natural infection including the colonisation step, our system is designed to cause endotoxin shock while avoiding the artificial system of LPS administration and allows the discrimination between endotoxin lethality (fast: within 24—48 h depending on the mouse strain) and sepsis (slow: several days). As argued in Matsumoto *et al.*, the presence of whole bacteria providing a multitude of additional stimuli rather than LPS alone might affect the pathophysiology of shock. This might explain the relative importance of bacterial loads in our complex model system, despite suppressive effects of FOF or even FOF^1^ on key cytokines for shock development in *in vitro* and *in vivo* studies solely relying on LPS. Our conclusion is also in agreement with a very recent study of Giacometti *et al.* where several cecropins and IPM provided significant protection against i.p. infection-mediated shock by equally reducing bacterial loads, even though IPM caused significantly higher release of endotoxin. Future research is certainly required to address this point. In the present study we could clearly establish therapeutic efficacy of FOF by determining doses which extended life span over the period of endotoxin lethality. Higher doses additionally conferred protection against later bacteremia. Taken together, our results warrant further research about FOF as anti-shock agent.

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