Limitation of Polymyxin B on Suppression of Endotoxin Shock Induced by *Salmonella* Infection in Mice

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Received June 21, 2004; accepted August 16, 2004

The protective effects of an antibiotic polymyxin B (PLB), having lipopolysaccharide (LPS)-binding activity, on infection-induced endotoxin shock in mice were investigated. Infection with 10⁸ colony forming units of an attenuated *Salmonella typhimurium* aroA strain caused lethal endotoxin shock to ddY mice. Treatment with PLB 1 h post infection (p.i.) resulted in significant reduction of mortality and bacterial numbers in livers. In addition, treatment with PLB 1 h p.i. resulted in a transient increase at the early stage and gradual decline in plasma LPS levels. Although plasma levels of sCD14 and high mobility group box chromosomal protein-1 (HMGB-1) increased according with progression of infection, increases in plasma levels of sCD14 and HMGB-1 were downregulated by treatment with PLB 1 h p.i. However, the lethal shock was not blocked by treatment with anti-CD14 monoclonal antibody at 3 h and 6 h p.i. Interestingly, administration of PLB 6 h p.i. did not show any protective activities, indicating that a time window for effective PLB action is present.

Key words *Salmonella; endotoxin; shock; polymyxin B; CD14; high mobility group box chromosomal protein-1 (HMGB-1)

Endotoxic lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is a pivotal molecule for triggering production of proinflammatory cytokines such as TNF-α and IL-1β. ⁵ TNF-α is a representative cytokine capable of initiating systemic inflammation response syndrome, multiple organ failure and septic shock, conditions which correlate with a high mortality. ², ³—⁴

In previous studies, ⁵, ⁶ we reported that the clearance of smooth form LPS from the circulation occurred rapidly since 99% of intravenously (i.v.)-injected *Salmonella abortus equi* LPS disappeared within 6 h. Therefore, we tried to establish a model in which endotoxin shock is triggered by bacterial infection rather than LPS administration, thus mimicking the situation found in clinical cases more closely. In intraperitoneal (i.p.) infection with *Escherichia coli* O111: B4, ¹ the mortality closely depended on a challenge dose, since all mice infected with 10⁸ colony forming units (CFU) succumbed but no mice died when infected with 10⁷ CFU. Treatment with an antibiotic polymyxin B (PLB) 1 h post infection (p.i.) with 10⁴ CFU resulted in significant protection. Recently we established a mouse model for endotoxin shock caused by a systemic infection using high numbers of an attenuated *Salmonella typhimurium* aroA. ⁸—¹⁰ We reasoned that a higher infection dose might enhance LPS-triggered effects and allow the dissection of events contributing to endotoxin shock, thus serving as suitable model for the purpose outlined above. Thus we tested whether this infection model is suitable for assessing the potential of anti-shock agents such as PLB, which in clinical cases is used for removing LPS from the circulation utilizing the LPS-binding property. Here we describe that the action of PLB shows a time-window in terms of therapeutic effect, since treatment with PLB 1 h but not 6 h p.i. blocked induction of lethal shock and suppressed release of soluble CD14 (sCD14) and high mobility group box protein-1 (HMGB-1) to plasma.

**MATERIALS AND METHODS**

**Mice** Female ddY and BALB/c mice were purchased from Japan SLC Inc. (Hamamatsu) and used at 8 to 10 weeks of age. All mice were housed under specific pathogen free conditions in the animal facility of School of Science and Graduate School for Fundamental Life Science of Kitasato University, and fed autoclaved food and water. For infections all mice were housed in plastic cages with a filter set 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 with the 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** The *S. typhimurium* aroA (SL7207) strain ¹¹ was obtained from Professor Bruce Stocker (Stanford University School of Medicine, CA, U.S.A.) and used for all experiments and grown for 18 h at 37°C in tryptic soy broth (Nissui Pharmaceutical Co., Tokyo). Aliquots were stored in −80°C until use. For infection, aliquots in a vial were rapidly thawed and bacteria were cultured for 18 h at 37°C in tryptic soy broth. After centrifugation, washing with phosphate buffered saline (PBS), and resuspended into PBS, turbidity was measured. Bacterial cell suspensions were adjusted with PBS to the desired bacterial number based on a standard curve of optical density versus bacterial number. Mice were infected i.p. with 10⁶ CFU of *S. typhimurium* aroA. The injected CFU number was confirmed by plating on tryptic soy agar (Difco Laboratories, Detroit, MI, U.S.A.).

**Chemicals** A highly purified LPS preparation from *S. abortus equi* was a kind gift from Dr. C. Galanos (Max-Planck Institute for Immunobiology, Freiburg in Breisgau, Germany). The endotoxin unit (EU) value of this preparation equivalent to 1 ng was 10.2. A stock solution of polymyxin B sulfate (PLB, Pfizer Pharmaceuticals Inc., Tokyo) was made...
at a concentration of 100000 units/ml in saline.

**Preparation of Plasma Samples** Following deep anesthesia with diethyl ether, blood was taken from mice by cardiac puncture using a 1-ml syringe with 26G needle. After bleeding blood samples were immediately mixed with 20-µl heparin (Novo Nordisk A/S, Roskilde, Denmark) and then centrifuged at 7200g for 1 min at room temperature. Plasma samples were collected carefully and used immediately or kept at −80°C until use.

**Endotoxin Determination** The concentration of endotoxin in plasma samples was measured by a kinetic-chromogenic assay after treatment with an alkaline reagent, as described previously.5) Aliquots (20 µl) of plasma samples were transferred to endotoxin-free test tubes, mixed with 80-µl alkaline reagent (Endospecy® ES TEST MK, Seikagaku Corp., Tokyo) and incubated for 10 min at 37°C. After treatment plasma samples were diluted with pyrogen-free water (Otsuka Pharmaceutical Co., Tokushima). Aliquots (25 µl) of test samples and standard endotoxin solution (Seikagaku Corp., Tokyo) and incubated for 10 min at 37°C. After treatment values of absorbance at 405 nm were measured automatically every 15 s and EU values were calculated by a computerized well-reader. All glassware used in this assay was heated to 250°C for 2 h to eliminate potentially contaminating environmental endotoxin. Endotoxin-free plastic tips and 96-well plates were obtained from Seikagaku Corp.

**Electrophoresis and Western Blotting** Plasma samples were mixed with a sample buffer, heated in boiling water for 2 min and analyzed using a 12.5% gel by SDS-PAGE. After blotting, scCD14 and HMGB-1 were identified using antimouse CD14 monoclonal antibody (mAb) (rmC5-3) and affinity-purified rabbit anti-HMG-1 polyclonal antibody (BD Pharmingen, San Diego CA, U.S.A.), respectively, and visualized using a VECTASTAIN ABC-PO kit (Vector Laboratories Inc., Burlingam, CA, U.S.A.).

**Anti-CD14 mAb** For in vivo study, anti-CD14 mAb (4C1) was prepared as described previously.13) As an isotype control, rat IgG (Chemicon International, Temecula, CA, U.S.A.) was used.

**Statistical Analysis** Statistical significance of the data was determined by Scheffé of Post-hoc-, the log-rank (Kaplan–Meier)- and Student’s t-tests. A p value of less than 0.05 was taken as significant.

**RESULTS**

**Time-Dependent Effect of PLB on Protection from Infection-Induced Lethal Shock** When ddY mice were infected intraperitoneally (i.p.) with 10⁸ CFU of *S. typhimurium* aroA, similar endotoxin shock phenomena were observed as BALB/c and C57BL/10ScSn mice (data not shown). Therefore mice were similarly infected with 10⁸ CFU *S. typhimurium* aroA and treated i.p. with 10000 units/kg PLB or saline 1, 3 and 6 h p.i. Infection control (saline, closed circle, n=8), treatment with PLB 1 h p.i. (open circle, n=10), PLB 3 h p.i. (open triangle, n=10) and PLB 6 h p.i. (open square, n=10). PLB 1 h p.i. or 3 h p.i. vs. infection control (p<0.001 by the log-rank test of Kaplan–Meier).

**Bacterial Numbers and Endotoxin Levels** When infected ddY mice were treated with PLB 1 h after infection, bacterial numbers in the livers markedly decreased at 3 to 12 h after infection (Fig. 2). On the other hand, LPS levels released into plasma by treatment with PLB peaked 3 h after infection and were significantly higher than infection controls (p<0.001) (Fig. 3). At 6 h p.i. the LPS levels in PLB-treated group were still significantly higher (p<0.05), although infection-induced lethal shock was completely blocked by PLB treatment.

**Plasma scCD14 Levels in Infected Mice** LPS bound to scCD14 molecule is pattern-recognized by TLR4. In a previous study using Western blot analysis,6) we demonstrated that scCD14 levels in plasma were not detectable in naive mice but increased 6 to 9 h after LPS administration. To compare scCD14 levels between LPS administration and *Salmonella* infection, BALB/c mice were administered i.v. 10 µg *S. abortus* equi LPS or infected i.p. with 10⁸ CFU *S. typhimurium* aroA. As shown in Fig. 4A, scCD14 levels peaked at 9 h and persisted higher levels by 12 h p.i. Much
higher levels of sCD14 were detected in infected mice than in LPS-administered mice all time points. Maximum expression of sCD14 in infected mice was seen at 9 to 12 h p.i.

Decreases in sCD14 Levels by Treatment with PLB
Since infection with $10^8$ CFU of *S. typhimurium* aroA also increased expression of plasma sCD14 levels in BALB/c mice, the effect of PLB on sCD14 production was determined at 6, 12 and 24 h p.i. As shown in Fig. 4B, plasma sCD14 levels in infection controls peaked at 12 h after infection. Treatment with PLB 1 h p.i. suppressed plasma sCD14 levels at 12 and 24 h p.i. Treatment with PLB 6 h p.i. did not reduce sCD14 levels at any time points tested. These results suggest that downregulation of sCD14 might be involved in the rescue from endotoxin shock by PLB. As new therapeutic targets the utility of anti-CD14 mAb in septic shock is strongly suggested. In order to estimate effects of neutralizing anti-CD14 mAb (4C1) on our shock model, six BALB/c mice per group were treated i.v. twice with 100 mg 4C1 3 h and 6 h p.i. Pooled samples at the indicated time points were prepared by mixing an equal volume of each plasma sample from 3 mice per group.

Decreases in HMGB-1 Levels by Treatment with PLB
Wang *et al.* reported that HMGB-1 is a late mediator of endotoxin shock. In a previous study, however, we showed that plasma levels of HMGB-1 increased the late stage of infection with *S. typhimurium* aroA. Therefore, we estimated effects of PLB on plasma HMGB-1 expression in this model. BALB/c mice were infected i.p. with $10^8$ CFU of *S. typhimurium* aroA and treated i.p. with 10000 units/kg PLB or saline 1 h p.i. Pooled samples at the indicated time points were prepared by mixing an equal volume of each plasma sample from 3 mice per group.
DISCUSSION

The present study clearly showed that i.p. infection with 10^7 CFU of S. typhimurium aroA caused acute lethal shock in ddY mice. We demonstrated that the outcome between LPS-responder BALB/c and LPS-nonresponder BALB/lpsd in ddY mice. We demonstrated that the outcome between both LPS-administration and infection is important for the induction of acute lethal shock also in our Salmonella-based shock model.

Animal shock models that have been reported previously are mostly based on administration of LPS. As reported in previous studies, less than 1% of intravenously injected LPS can be detected after 6 h. In S. typhimurium-infected mice, plasma LPS levels reached a plateau (approximately 1000 EU/ml) 3 h p.i., and the high levels persisted until 12 h p.i. In addition, plasma sCD14 levels peaked at 9 to 12 h and were higher in infected mice than LPS-administered mice (Fig. 4A). It is therefore reasonable to assume that continuous production of sCD14 molecules allows the formation of lasting complexes with LPS from 6 to 12 h after infection. By contrast, complex formation between LPS and sCD14 is limited in LPS-administered mice, because more than 99% of the LPS injected have already disappeared from the circulation at 6 h after administration. Consequently, a big difference exists between both LPS-administration and infection models. Extensive LPS/sCD14 complex formation has been shown to trigger signaling via TLR4, a receptor implicated in the induction of innate immunity. Treatment with neutralizing anti-CD14 mAb 3 and 6 h p.i. did not block lethal shock caused by Salmonella infection, but plasma LPS levels significantly increased in the mice treated with anti-CD14 mAb (4C1) (Fig. 5). Le Roy et al. reported that treatment with 4C1 mAb resulted in blocking of LPS-induced lethal shock in the galactosamine model, but in Klebsiella pneumoniae infection, this mAb did not work effectively. This is coincided with our result that 4C1 dysfunctions in infection model.

Treatment with antibiotics during infections enhances release of endotoxin and production of cytokines. Also in our model treatment with PLB 1 h p.i. caused a significant increase in plasma LPS levels compared to infection controls, evident as early as 2 h later. At the same time bacterial numbers decreased (Fig. 2), and suppression of sCD14 expression was initiated at 12 h p.i. Taken together, our results suggest that in addition to the well-known LPS-binding activity of PLB, a reduction of bacterial numbers in combination with downregulation of sCD14 as molecule participating in LPS-signaling is another mechanism by which PLB counters endotoxin shock. Our model also showed a time dependence of the therapeutic effect of PLB. Administration of PLB 1 h p.i. strongly blocked the lethal shock, but treatment with PLB 6 h p.i. did not show inhibitory effects on infection-induced lethal shock anymore, even though expression of plasma HMGB-1 levels was suppressed at 24 and 36 h p.i. by administration of PLB 6 h p.i. This result suggests that HMGB-1 is not a late mediator of endotoxin shock. Treatment with PLB 3 h after infection caused significant suppression but the efficacy was insufficient. These results suggest that a sensitive period for PLB treatment exists, and if the period exceeds a good therapeutic outcome would not be expected. While PLB is not used for patient treatment because of its toxicity, its inclusion as test substance in this study showed that our infection-induced shock model could be valuable for establishing the therapeutic efficacy and optimizing administration regimens of other antibiotics or drugs.

In summary, we present a new infection-based model for the evaluation of endotoxin shock, which could become a valuable basic research tool for identifying factors contributing to this medical condition. This new system is suitable for evaluating the activity of anti-septic shock agents under more realistic and therefore more stringent conditions than the widely used LPS-based models.

Acknowledgements This study was supported in part by a Grant-in-Aid for Scientific Research (C) from JSPS (15590397 to R.H.), and a Grant-in-Aid for Scientific Research Collaboration of Kitasato University (15-3 to Y.K.).

REFERENCES