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ABSTRACT
Actions of glucocorticoids, cyclosporine A, and JTE-607 [(-)-ethyl-N-[3,5-dichloro-2-hydroxy-4-[2-(4-methylpiperazin-1-yl)ethoxy]benzoyl]-L-phenylalaninate dihydrochloride], a proinflammatory cytokine inhibitor that does not inhibit interleukin (IL)-2 or interferon-γ, were compared in a mouse septic shock model induced by cecal ligation and puncture (CLP). CLP caused elevation of macrophage inflammatory protein (MIP)-2 in lung, and MIP-2 and IL-6 in plasma and peritoneal fluid, reaching a peak 4 to 8 h after CLP. Acute treatment (subcutaneous injections 1 h before and 2 h after CLP) of mice with JTE-607 and methylprednisolone showed significant inhibition of elevated cytokine levels and MPO activity, plus increased survival rate. Similar treatment with cyclosporine A and prednisolone was ineffective. Chronic treatment (subcutaneous injection for seven consecutive days before CLP) of mice with JTE-607 also showed an inhibitory effect on cytokine production, MPO activity and mortality. In contrast, chronic treatment with cyclosporine A and prednisolone did not inhibit cytokine production or MPO activity, but rather exacerbated mortality. These results indicate that JTE-607 has protective effect on mouse mortality induced by CLP, correlating with inhibition of proinflammatory cytokines, whereas the immunosuppressants cyclosporine A and prednisolone do not. This suggests that JTE-607, a multiple cytokine inhibitor that does not cause adverse immunosuppression, is useful for treatment of septic shock.

Sepsis remains a serious clinical problems resulting in morbidity and high mortality. Human septic shock is reported to be associated with elevation of proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 (Casey et al., 1993; Lin and Lowry, 1999), representing an uncontrolled inflammatory response. Although infusion of these cytokines mimics many features of septic shock (Cannon et al., 1990), anti-TNF-α and anti-IL-1 therapies have not been successful in reducing mortality rate (Abraham, 1999), suggesting that mediators other than TNF-α and IL-1β may play important roles in the pathogenesis of septic shock.

Previous studies have suggested that lipopolysaccharide (LPS) exposure to sensitive animals results in systemic release of proinflammatory cytokines (e.g., IL-1, IL-6, and TNF-α), which are associated with death of the host (Mathison et al., 1988; Michie et al., 1988; Shalaby et al., 1989). Neutralization of TNF-α with antibody is effective in reducing LPS-induced lethality in mice (Remick et al., 1995). However, experimental results from using LPS do not always reflect human sepsis, because serum TNF-α levels are low and TNF-α antibodies in septic patients are inefficient (Abraham, 1999; Riedemann et al., 2003).

Cecal ligation and puncture (CLP) induces peritonitis due to mixed intestinal flora, closely resembling human septic shock (Wichterman et al., 1980). It has been reported that anti-TNF-α therapy is significantly less effective or even worse in reducing mortality of CLP compared with lethal LPS (Eskandari et al., 1992), and plasma levels of TNF-α after CLP are substantially lower than those observed in lethal LPS injection (Mathison et al., 1988; Michie et al., 1988; Eskandari et al., 1992; Remick et al., 1995; Remick et al., 2000; Riedemann et al., 2003). However, MIP-2, a member of C-X-C chemokine and IL-8 family in mice and rats,
possesses potent chemotactic activity for neutrophils (Watanabe et al., 1991) and has been suggested to be an important mediator in tissue damage (blockade of MIP-2 by anti-MIP-2 antibody significantly reduces lung injury and mortality after CLP) (Tsujimoto et al., 2002). IL-6 is also an important mediator of inflammation. IL-6 concentration is elevated in septic patients and correlated with severity and outcome of sepsis (Kantar et al., 2000), suggesting that IL-6 is a marker of severity. MIP-2 and IL-6 may play important roles in pathogenesis of CLP-induced septic shock, and CLP may represent a more clinically relevant model of sepsis.

JTE-607 suppresses IL-1β, TNF-α, IL-6, and IL-8 production from LPS-stimulated human peripheral blood mononuclear cells (PBMCs), plus protects mice and rats from LPS-induced lethality and lung injury if administered prophylactically (Kakutani et al., 1999; Iwamura et al., 2002). It would, therefore, be interesting to determine whether multiple inhibition of proinflammatory cytokines, rather than inhibiting single cytokine by JTE-607, would be beneficial in improving mortality induced by CLP.

Proinflammatory cytokines are a defense mechanism, and inhibition of cytokines can cause a risk for host defense (Sheppard et al., 1989; Kharazmi, 1991). Suppression of IL-2 and interferon (IFN)-γ production may cause serious immunosuppression (Wiskocil et al., 1985; Sazaki et al., 1992; Abe et al., 1996). Because JTE-607 has little effect on IL-2 and IFN-γ production in purified T lymphocytes, not like prednisolone (Kakutani et al., 1999), it would also be interesting to determine whether chronic administration of JTE-607 can cause immunosuppression, which is likely to be an undesirable effect in CLP-induced mortality.

In this study, we evaluated the effect of JTE-607 after acute (1 h before and 2 h after CLP) and chronic (once a day for a week before CLP) administration on CLP-induced septic shock in mice and compared with other immunosuppressants (prednisolone and cyclosporine A). Both acute and chronic administration of JTE-607 improved mortality of mice induced by CLP, correlating well to suppressed cytokine levels and MPO activity, whereas chronic administration of prednisolone and cyclosporine A exacerbated mortality.

Materials and Methods

Animals. Male BALB/c mice (8–9 weeks) were obtained from Charles River (Yokohama, Japan) and maintained under specific pathogen-free conditions at a room temperature of 23 ± 3°C and air humidity of 55 ± 15% in 12-h light/dark cycle environment.
CLP. Mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.) (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). The peritoneum was then opened and the cecum tied with 3-0 silk surgical thread 1 cm from the distal end. Care was taken not to obstruct the ileocecal valve. A single puncture was made with a 21-gauge needle on the mesenteric surface of the cecum at a point midway between the tip and ligated base. A small amount of feces was expressed through the puncture wound and intestines were returned to the abdominal cavity. The wound was closed with 3-0 silk thread. Mice were recovered from anesthesia within 30 min of CLP treatment. Sham-operated animals underwent identical laparotomy but did not undergo cecal ligation and puncture.

Preparation of Plasma, Peritoneal Lavage Fluid, and Lung Homogenate. Heparinized blood was collected by cardiac puncture. Lung was dissected, and the peritoneal cavity was washed with 2 ml of phosphate-buffered saline. Blood and peritoneal lavage fluid were centrifuged (1000g, 10 min, 4°C), and supernatants were frozen for later analysis. Dissected lung was minced with scissors and filled with 10-fold (w/v) 50 mM sodium acetate buffer (pH 6) containing 0.5% hexadecyltrimethylammonium bromide (Wako Pure Chemicals, Osaka, Japan), and then homogenated with a Polytron homogenizer (Kinematica, Littau, Switzerland). A portion of homogenate was centrifuged at 40,000g for 10 min, and then supernatant was used for measurement of MPO activity and cytokine levels.

Time Course Study after CLP Challenge. Mice were divided into two groups and treated with either CLP or a sham operation (n = 4). Mice were sacrificed 2, 4, 8, and 12 h after treatment, and then blood, lung, and peritoneal cavity was collected as described above.

Acute and Chronic Administration of Test Compounds. JTE-607 (100 mg/kg; Japan Tobacco Inc., Osaka, Japan), cyclosporine A (30 mg/kg; Wako Pure Chemicals), prednisolone (3 mg/kg; Shionogi Pharmaceutical Co., Ltd., Osaka, Japan), and methylprednisolone (3 mg/kg; Toyama Chemical, Tokyo, Japan) were dissolved in 10% dimethyl sulfoxide/5% mannitol and used at indicated doses. Due to a very short half-life of JTE-607 after intravenous injection (t1/2 = 6 min), JTE-607 and other compounds were administered subcutaneously to the back of mice at a volume of 10 ml/kg. Mice were treated with test compounds either 1 h before and 2 h after CLP in the acute administration protocol, or once a day for a week before CLP in the chronic administration protocol (Fig. 1). Because methylprednisolone was used as a positive drug to validate experiments, only acute treatment was used for methylprednisolone both in acute and chronic administration protocols. Mortality of mice was studied with 12 animals per group, monitoring twice a day for 7 days after CLP. In separate experiments, eight animals per group were sacrificed 8 h after CLP in each protocol. Lung, blood, and peritoneal lavage fluid were collected and prepared for later analysis as described above.

Measurement of MPO Activity. For measurement of MPO activity in lung homogenate, supernatant (0.02 ml) was mixed with 0.43 ml of 0.16 M sodium phosphate buffer containing 0.56 mM tetramethylbenzidine (Wako Pure Chemicals) dissolved in N,N-dimethylformamide (Wako Pure Chemicals) [5.5% (v/v)]. After incubation at 37°C for 5 min, 3 mM H2O2 (0.05 ml) was added, and the mixture incubated again at 37°C for 3 min. The reaction was stopped by addition of 0.02 ml of catalase (300 mg/ml; Wako Pure Chemicals).

Fig. 3. Effect of acute administration of test compounds on survival rate after CLP challenge. Vehicle, JTE-607 (100 mg/kg), cyclosporine A (Cys.A; 30 mg/kg), prednisolone (Pred.; 3 mg/kg), or methylprednisolone (Methylpred.; 3 mg/kg) was subcutaneously administered 1 h before and 2 h after CLP challenge. Survival rate was observed for 1 week after CLP (n = 12). Data are expressed as percentage of live animals. *, p < 0.05 versus vehicle (Wilcoxon test).

Fig. 4. Effect of acute administration of test compounds on MIP-2 level and MPO activity in lung 8 h after CLP challenge. Vehicle, JTE-607 (100 mg/kg), cyclosporine A (Cys.A; 30 mg/kg), prednisolone (Pred.; 3 mg/kg), or methylprednisolone (Methylpred.; 3 mg/kg) was subcutaneously administered 1 h before and 2 h after CLP challenge. MIP-2 level (A) in lung was measured by ELISA. MPO activity (B) in lung was measured as described under Materials and Methods. Data are expressed as mean ± S.E.M. (n = 8). ###, p < 0.001 versus sham; **, p < 0.01; and *, p < 0.05 versus vehicle (Student’s t test).
The mixture was diluted with 2 ml of 0.2 M acetic acid, and optical density was measured using a plate reader (THERMOmax microplate reader; Molecular Devices, Menlo Park, CA) at 650 nm with Softmax software (Molecular Devices).

**Measurement of Cytokines.** MIP-2 in lung homogenate, plasma, and peritoneal lavage fluid, and IL-6 and TNF-α in plasma and peritoneal lavage fluid were measured by ELISA (R&D Systems, Minneapolis, MN).

**Measurement of Platelet and White Blood Cell (WBC) Count in Blood.** After collecting heparinized blood by cardiac puncture, an aliquot of blood was used for measurement of platelet and WBC count using an automatic blood cell counter (F-820; Toa Medical Electronics Co., Tokyo, Japan).

**Statistics.** Mortality data were analyzed using the Wilcoxon test. Data were represented as mean ± S.E.M. Data between groups were analyzed using Student’s unpaired t test. Probability of \( p < 0.05 \) was considered to be significant.

**Results**

**Time Course Study of Cytokine Level, MPO Activity, and Blood Cell Count in CLP-Treated Mice.** Changes over time of MIP-2 level and MPO activity in lung, MIP-2 and IL-6 levels in plasma and peritoneal fluid, and platelet and WBC count in blood were studied after CLP in mice. MIP-2 level in lung gradually increased after CLP and reached a peak 8 h after CLP (Fig. 2A). MPO activity in lung also increased time-dependently and almost reached its peak 8 h after CLP (Fig. 2B). MIP-2 and IL-6 levels in plasma reached their peaks 4 h, and levels were sustained until 8 h, after which they began to decline (Fig. 2, C and D). MIP-2 and IL-6 levels in peritoneal lavage fluid reached their peaks at 4 h, and levels were sustained until 12 h (Fig. 2, E and F). TNF-α was not detected in either plasma or peritoneal lavage fluid at any time point. CLP induced a drop in platelet count in peripheral blood and was significantly lower 8 and 12 h after CLP challenge than sham-operated mice (Fig. 2G). A decrease in WBC count was observed from 4 h; however, there was no significant change between CLP and sham-operated mice (Fig. 2H). Because most parameter changes reached a peak 8 h after CLP challenge, the effect of test compounds on these parameters was evaluated 8 h after CLP.

**Effect of Acute Administration of Test Compounds on CLP-Induced Mortality.** The effect of JTE-607 by administering 1 h before and 2 h after CLP on mortality was evaluated and compared with cyclosporine A, prednisolone, and methylprednisolone. CLP-treated animals died in a time-dependent manner, and survival rate 7 days after CLP

![Fig. 5. Effect of acute administration of test compounds on MIP-2 and IL-6 levels in plasma and peritoneal fluid 8 h after CLP challenge. Vehicle, JTE-607 (100 mg/kg), cyclosporine A (Cys.A; 30 mg/kg), prednisolone (Pred.; 3 mg/kg), or methylprednisolone (Methylpred; 3 mg/kg) was subcutaneously administered 1 h before and 2 h after CLP challenge. MIP-2 level (A and B) and IL-6 level (C and D) in plasma and peritoneal fluid were measured by ELISA. Data are expressed as mean ± S.E.M. (n = 8). ###, \( p < 0.001 \) versus sham; *, \( p < 0.05 \); **, \( p < 0.01 \); and ***, \( p < 0.001 \) versus vehicle (Student’s t test).]
was 17%. JTE-607 and methylprednisolone significantly improved survival rate after CLP compared with vehicle treatment, whereas cyclosporine A and prednisolone did not (Fig. 3).

Effect of Acute Administration of Test Compounds on Cytokine Level, MPO Activity, and Blood Cell Count in CLP-Treated Mice. The increase in MIP-2 level and MPO activity in lung 8 h after CLP was significantly inhibited by acute administration of JTE-607 and methylprednisolone (Fig. 4). MIP-2 and IL-6 levels in plasma and peritoneal lavage fluid were also significantly suppressed by JTE-607 and methylprednisolone (Fig. 5). Prednisolone showed significant inhibition of plasma IL-6 and peritoneal MIP-2 and IL-6 but not plasma MIP-2. Cyclosporine A did not suppress these cytokines, except for plasma IL-6 (Fig. 5). None of the compounds affected decreased platelet and WBC count in peripheral blood; however, methylprednisolone showed a significant effect on platelet count (Fig. 6).

Effect of Chronic Administration of Test Compounds on CLP-Induced Mortality. CLP-induced mortality was observed after 7 days of consecutive administration of test compounds. Vehicle-treated animals died after CLP challenge, and survival rate 7 days after CLP was 8.3%. Chronic administration of JTE-607 significantly improved survival rate as did acutely administered methylprednisolone (positive control). In contrast, pretreatment with cyclosporine A and prednisolone significantly exacerbated mortality (all animals died within 48 h) (Fig. 7).

Effect of Chronic Administration of Test Compounds on Cytokine Level, MPO Activity, and Blood Cell Count in CLP-Treated Mice. Chronic pretreatment with JTE-607 and acute treatment of methylprednisolone strongly suppressed increased MIP-2 level and MPO activity in lung after CLP, whereas cyclosporine A and prednisolone increased these parameters (Fig. 8). MIP-2 and IL-6 levels in plasma and peritoneal lavage fluid were also suppressed by pretreatment with JTE-607 and methylprednisolone. However, the cytokine levels were not suppressed by pretreatment with cyclosporine A and prednisolone, although IL-6 level in plasma was slightly inhibited (Fig. 9). Decreased platelet and WBC count in peripheral blood was significantly recovered by JTE-607, and significant platelet recovery was also observed in methylprednisolone-treated animals. Cyclosporine A and prednisolone did not show any significant effect on platelet and WBC count (Fig. 10).

Discussion

As indicated by previous reports, there are many differences between lethal LPS injection and CLP, although mortality in both models is equivalent and similar (Remick et al., 2000). In fact, we demonstrated that CLP caused a slower induction of cytokines such as MIP-2 and IL-6, which reached their peaks at 4 to 8 h, and TNF-α was not detectable. Although there are some reports that showed increased TNF-α levels in CLP models, levels vary depending on experi-
Fig. 8. Effect of chronic and acute administration of test compounds on MIP-2 level and MPO activity in lung 8 h after CLP challenge. Vehicle, JTE-607 (100 mg/kg), cyclosporine A (Cys.A; 30 mg/kg), or prednisolone (Pred.; 3 mg/kg) was administered subcutaneously once daily for 1 week before CLP. Methylprednisolone (Methylpred.; 3 mg/kg) was administered subcutaneously 1 h before and 2 h after CLP. MIP-2 level (A) in lung was measured by ELISA. MPO activity (B) in lung was measured as described under Materials and Methods. Data are expressed as mean ± S.E.M. (n = 8). ###, p < 0.001 versus sham; *, p < 0.05; **, p < 0.01; and ***, p < 0.001 versus vehicle (Student’s t test).

Fig. 9. Effect of chronic and acute administration of test compounds on MIP-2 and IL-6 levels in plasma and peritoneal fluid 8 h after CLP challenge. Vehicle, JTE-607 (100 mg/kg), cyclosporine A (Cys.A; 30 mg/kg), or prednisolone (Pred.; 3 mg/kg) was administered subcutaneously once daily for 1 week before CLP. Methylprednisolone (Methylpred.; 3 mg/kg) was administered subcutaneously 1 h before and 2 h after CLP. MIP-2 level (A and B) and IL-6 level (C and D) in plasma and peritoneal fluid were measured by ELISA. Data are expressed as mean ± S.E.M. (n = 8). ###, p < 0.001 versus sham; *, p < 0.05; **, p < 0.01; and ***, p < 0.001 versus vehicle (Student’s t test).
Methylprednisolone (Methylpred.; 3 mg/kg) was administered subcutaneously 1 h before and 2 h after CLP. Numbers of platelets (A) and WBCs (B) were counted using an automatic blood cell counter. Data are expressed as mean ± S.E.M. (n = 8). ##, p < 0.01 versus sham; *, p < 0.05; and ***, p < 0.001 versus vehicle (Student’s t test).

Inflammatory conditions, such as animal strain and severity of CLP, and are very low in general compared with LPS models, causing rapid induction of cytokines and peaking at 1.5 h (Villa et al., 1995; Remick et al., 2000; Vianna et al., 2004; Yan et al., 2004).

JTE-607 was discovered as a multiple cytokine inhibitor showing high selectivity to PBMCs; i.e., it suppressed human inflammatory cytokines, including IL-1β, TNF-α, IL-6, and IL-8, produced by LPS-stimulated human PMBCs (Kakutani et al., 1999). We demonstrated that acute administration of JTE-607 and methylprednisolone improved survival rate after CLP, with significant suppression of MIP-2 (a member of the IL-8 family in mice) and IL-6 levels in lung, plasma, and peritoneal lavage fluid, plus MPO activity in lung. Inhibition of cytokine production, including MIP-2, by JTE-607 and methylprednisolone in lung lead to decreases in neutrophil migration, represented by reduced MPO activity. Neutrophil-induced damage to lung was reduced, and animals showed an improved survival rate. These results suggest that MIP-2 and IL-6, but not TNF-α, play an important role in the CLP model, as reported previously (Eskandari et al., 1992; Remick et al., 1995), and that JTE-607 and methylprednisolone have protective effects on septic shock, correlated to the inhibition of inflammatory cytokines.

Glucocorticoids are widely used for experimental shock models and are effective against mortality after lethal LPS challenge (Hall-Angeras et al., 1986; Kakutani et al., 1999). We used two glucocorticoids, methylprednisolone and prednisolone, in our experimental protocol; however, the results from acute administration protocol of prednisolone and methylprednisolone were in contrast. Methylprednisolone clearly inhibited mortality and cytokine production, whereas prednisolone did not. Transportation of methylprednisolone to lung after intravenous administration is greater than prednisolone (Greos et al., 1991). Although we did not compare them by intravenous administration in our experiments, the difference in effective concentration to inhibit cytokine production in lung between the two corticosteroids may have affected contrasting results. Although we used methylprednisolone as a positive drug to validate our experiments, it has been reported that high-dose methylprednisolone did not improve survival rate after septic shock in human patients (Sprung et al., 1984; Bone et al., 1987). It is not clear why there is a discrepancy between animal models and human patients, but we speculate that dose, timing, and duration of the corticosteroid treatment may affect the results as we demonstrated in this report. In this respect, experiments with high dose or chronic treatment of methylprednisolone would be of interest.

Inhibiting inflammatory cytokines may impair host defense mechanisms. JTE-607 was shown to have little effect on IL-8 production from fibroblasts, endothelial cells and mesangial cells, and IL-2 and IFN-γ production from purified human T cells (Kakutani et al., 1999). We therefore expect that long-term treatment with JTE-607 might not cause serious immunosuppression, unlike glucocorticoids and cyclosporine A (Van Wauwe et al., 1995; Fessler et al., 1996). This study was designed to assess the effect of 1-week pretreatment with JTE-607 before CLP on mortality to compare with prednisolone and the immunosuppressant cyclosporine A. JTE-607 reduced mortality rate, whereas prednisolone and cyclosporine A significantly exacerbated mortality. These results suggest that long-term treatment with prednisolone and cyclosporine A weakened host defense by “immunosuppression”. Thus, the differential effects on IFN-γ and IL-2 production between JTE-607 and immunosuppressants prednisolone and cyclosporine A may reflect these contrasting results on mortality after CLP.

The beneficial effect of chronic treatment with JTE-607 on survival rate was accompanied by reduced MIP-2 and IL-6 levels in lung, plasma, and peritoneal fluid, and MPO activity in lung, as was the case for acute treatment. These results suggest that the effect of chronic treatment with JTE-607 on mortality by CLP was due to inhibition of inflammatory cytokines.

The change in platelet and WBC count in peripheral blood is another important parameter in septic shock. We observed a significant decrease in platelet and WBC count, although...
the decrease in WBC count was not significant in a time course study. The lack of reproducibility in decrease of WBC count may be because the change is marginal. The effect of JTE-607 on platelet and WBC count was not impressive in acute treatment, although significant recovery was seen from chronic treatment. Decreased platelet and WBC counts may be a part of the disease process of CLP and may not be strongly related to animal death.

In conclusion, we showed that inhibition of inflammatory cytokines without causing immunosuppression is useful for CLP-induced mortality. JTE-607 had beneficial effects, but immunosuppressants prednisolone and cyclosporine A did not. Further investigation of JTE-607 by post-treatment will be necessary. Moreover, care has to be taken in discussing efficacies in human sepsis from the CLP model. However, our results indicate that JTE-607 infusion therapy could be a novel strategy for treatment of sepsis without causing adverse immunosuppression. The effective dose of JTE-607 in human should not be so high, because there is approximately 150 times higher activity of JTE-607 on human than mouse cytokines (Kakutani et al., 1999).

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References


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Improved Mortality of Septic Mice by JTE-607


