Prior burn insult induces lethal acute lung injury in endotoxemic mice: effects of cytokine inhibition

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Sasaki, Junichi, Seitaro Fujishima, Hiroyuki Iwamura, Korekiyo Wakitani, Sadakazu Aiso, and Naoki Aikawa. Prior burn insult induces lethal acute lung injury in endotoxemic mice: effects of cytokine inhibition. Am J Physiol Lung Cell Mol Physiol 284:L270–L278, 2003. First published September 27, 2002; 10.1152/ajplung.00108.2002.— Many patients who experience surgical stress, including burn injury, become susceptible to severe sepsis and septic organ dysfunction, which remains the primary contributor to morbidity and mortality in burn patients. In the present study, we developed a murine model of burn-primed sublethal endotoxemia by producing a 15% BSA full-thickness burn on the dorsum of BALB/c mice under ether anesthesia and administering 10 mg/kg of LPS intravenously on day 11 to model endotoxemia. The prior burn injury in this model induced two-peaked production of cytokines, TNF-α, and macrophage inflammatory protein-2 at 2 and 12 h after LPS administration, and it was associated with increased mortality. We also assessed the effect of pharmacological modulation with cytokine synthesis inhibitors prednisolone and JTE-607 and found that pretreatment with them attenuated later cytokine production and decreased mortality after LPS administration. We speculate that the prior burn injury primed the mice for the secondary insult via cytokine production. These results also suggested that an anticytokine strategy might serve as a novel prophylactic therapy for septic organ dysfunction in burn-primed patients.

two-hit phenomenon; priming; systemic inflammatory response syndrome; chemokine; JTE-607

THE SEVERE COMPLICATIONS that occur after burn injury are divided into two phases according to the time after the burn insult. Early complications during the first week postburn are mainly characterized by hypovolemic shock, shock-induced renal failure, and burn wound infection (20). Great advances in fluid management based on hemodynamic monitoring (2, 33), widespread use of early collection, and the development of new wound care materials (30, 31) over the last three decades have reduced mortality, especially during the first week. The late complications are divided into two phases according to the time after the burn insult. Early complications during the first week postburn are mainly characterized by hypovolemic shock, shock-induced renal failure, and burn wound infection (20). Great advances in fluid management based on hemodynamic monitoring (2, 33), widespread use of early collection, and the development of new wound care materials (30, 31) over the last three decades have reduced mortality, especially during the first week. The late complications are divided into two phases according to the time after the burn insult.
glucocorticoids insofar as it does not inhibit the production of IL-2 and IFN-γ by T lymphocytes, phagocytosis, ROS production, or major histocompatibility complex class II antigen expression by human PBMCs (18).

In the present study, to elucidate the pathophysiological mechanisms of the two-hit phenomenon, we developed a murine model of burn-primed sublethal endotoxemia and analyzed plasma and lung cytokines in addition to mortality. We determined two proinflammatory cytokines, TNF-α and macrophage inflammatory protein-2 (MIP-2), in this study, because the former is the initial key mediator of sequential inflammatory events, and the latter is a murine homolog of human IL-8, a dominant neutrophil chemotactic mediator in various neutrophil-mediated acute inflammations (11, 29). We also assessed the effect of pharmacological modulation with the two cytokine synthesis inhibitors.

**MATERIALS AND METHODS**

**Animal Preparations**

Male BALB/c mice (Charles River, Yokohama, Japan) weighing 23–28 g (7 wk old) were caged in an environment in which the temperature and relative humidity were controlled, and a 12-h light/dark cycle was maintained. They were given free access to standard mouse feed and water throughout the course of the experiments. Animals were randomized to a sham-burn group and a burn group. On the day before the experiments, the dorsum of all animals was shaved under pentobarbital anesthesia (50 mg/kg in 0.25 ml saline solution intraperitoneally) and the shaved area was exposed to steam at 100 °C. Immediately after burn injury (or sham), animals were similarly anesthetized and shaved but not exposed to steam. Immediately after burn injury (or sham), all animals received fluid resuscitation with 4 ml of normal saline solution intraperitoneally and were returned to the same conditions after the procedures. No analgesics were used during the experiment, because full-thickness burns do not cause pain, and analgesics affect immunological responses. On day 11 after the sham-burn or burn injury, a 10 mg/kg dose of LPS (Escherichia coli 0111:B4 endotoxin; Sigma Chemical, St. Louis, MO) in 10 ml/kg of normal saline solution was administered intravenously into all animals to model endotoxemia after burn injury. All animal studies were approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine.

**Preparation of Compounds to Modulate Cytokine Synthesis**

We used two independent anti-inflammatory drugs, PSL (Shionogi, Osaka, Japan) and JTE-607 (Japan Tobacco, Tokyo, Japan), to inhibit cytokine synthesis. PSL (3 mg/kg·day⁻¹) and JTE-607 (100 mg/kg·day⁻¹) were dissolved in 5% 10 ml/kg mannitol (Wako, Osaka, Japan).

**Experimental Protocols**

**Experiment 1.** The experimental protocol of the initial study is shown in Fig. 1. Mice were randomly divided into a sham-LPS group and a burn-LPS group, and LPS was administered intravenously on day 11 after the sham-burn or burn injury. The first series of experiments was performed to determine survival at 72 h after LPS administration in both groups (n = 4, each group). The second series of experiments was designed to determine the time course of the changes in cytokine levels up to 16 h after LPS administration to the sham-burned and burned mice. The animals were killed at 0, 2, 8, 12, and 16 h after LPS administration to obtain blood and lung samples (n = 4, each group).

**Experiment 2.** The protocol of experiment 2 was designed to determine whether cytokines are involved in the mechanism of the combined effect of burn insult and endotoxin in this murine model by investigating the effects of cytokine synthesis inhibitors PSL and JTE-607. The study design and experimental protocol of the second study are shown in Fig. 2. Mice were randomly divided into four groups: a sham-LPS (sham) group, burn-LPS group (positive control, PCTL), a PSL group, and a JTE-607 (JTE) group. In the PCTL, PSL, and JTE groups, 15% of TBSA full-thickness burn was created on the dorsum. After the burn injury, PSL in the PSL group and JTE-607 in the JTE group were injected subcutaneously daily for 10 days before LPS administration. A 10 ml/kg dose of 5% mannitol as the vehicle was injected by similar methods in the sham group and the PCTL group. On day 11, at 1 h after the final injection of PSL, JTE-607, or vehicle, LPS was administered intravenously to model endotoxemia. The first series of experiments was performed to determine survival up to 72 h after LPS administration in each group (n = 10, each group). The second series of experiments was designed to determine the cytokine levels (n = 8, each group), gene expression (n = 4, each group), myeloperoxidase (MPO) activity (n = 8, each group), and histopathological findings at 12 h after LPS administration to each group. Because the dose of LPS used in this experiment was sublethal, we did not examine the effect of two anti-inflammatory drugs, PSL and JTE-607, on endotoxemic mice without prior burn insult.
Sample Collection and Histopathological Examination

Mice were killed under effective ether anesthesia. Blood samples were collected by heart puncture. After being centrifuged at 400 g for 10 min, the plasma samples were removed and stored at −80°C for subsequent cytokine measurement. The right lobes of the lung were removed and used for cytokine measurement. The wet weight of the right lobe was determined before homogenization in five times the weight of 50 mM acetate buffer (pH = 3) containing 0.5% cetihl trimethylammonium bromide (Wako). After being centrifuged at 40,000 g for 10 min, the supernatants were removed and stored at −80°C for subsequent measurements. The left upper lobe of the lung was processed for histological examination. The tissue slices were fixed in 4% paraformaldehyde, and the histological sections were subsequently stained with hematoxylin and eosin. Sections were examined by light microscopy (×100 magnification). All other lobes of the lung were removed, flash-frozen in liquid nitrogen, and stored at −80°C for subsequent measurement.

Cytokine Determination by ELISA

The TNF-α levels in the plasma samples and MIP-2 levels in the plasma and the supernatants of the lung tissue samples were quantitatively determined by specific sandwich ELISAs for mouse TNF-α (Genzyme, Cambridge, MA) and MIP-2 (R&D Systems, Minneapolis, MN) according to the manufacturers’ instructions. The minimum detectable levels of TNF-α and MIP-2 in these immunoassays were found to be typically <35 pg/ml and 7.8 pg/ml, respectively.

Quantitation of MIP-2 mRNA by Real-Time RT-PCR

mRNA expression in lung tissue was determined by a real-time RT-PCR. The MIP-2 mRNA level is expressed as a relative ratio to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level, as described previously (26). Briefly, total RNA was extracted from each lung tissue with RNAeasy kits (Qiagen, Germany), and single-stranded cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase (GIBCO BRL, Rockville, MD) with oligo(dT)20 primer. Gene expression was measured by a quantitative PCR method with a dual-labeled fluorogenic probe and 7900 Prism sequence detector (Perkin Elmer, Foster City, CA) as proposed by Gibson and coworkers (16). The probe was designed between the forward and reverse primer sites and labeled with a reporter fluorescent dye (6-carboxyfluorescein) at the 5’ end and a quencher fluorescent dye (6-carboxytetramethyl rhodamine) at the 3’ end. Accumulation of PCR products was detected directly by monitoring the increase in fluorescence of the reporter dye.

Measurement of MPO Activity

MPO activity in the supernatants of the lung tissue was assayed by a standard spectrophotometric technique (7). A 20-µl volume of test samples or standard MPO (Sigma Chemical) was mixed with 430 µl of acetate buffer (pH = 5.4) containing 1.56 mM tetramethylbenzidine (Dijin, Kumamoto, Japan), 50 µl of 3 mM hydrogen peroxide (Wako), and 20 µl of 300 U/ml catalase (Wako). Then, 2 ml of 0.2 N acetate was added to the samples, and the absorbance change at λ = 650 nm was measured with a spectrophotometer.

Statistics

The differences between cytokine levels at various times were analyzed by the unpaired t-test. Survival within 72 h was analyzed by the Cox-Mantel test. The cytokine levels, gene expression, and MPO activity of the samples were analyzed by one-way ANOVA followed by Fisher’s protected least significant differences test. A P value of <0.05 was considered significant. All data are expressed as means ± SE of the mean values unless otherwise specified.

RESULTS

Prior Burn Insult Decreases Survival and Induces a Two-Peaked Cytokine Increase After LPS administration

In the survival study, all four mice (100%) in the sham-LPS group had survived at 72 h after LPS administration, but none of the four mice (0%) in the burn-LPS group survived. LPS administration to burned mice resulted in poor survival. Figure 3, A and B, indicates the time course of the changes in plasma cytokine TNF-α and MIP-2 levels up to 16 h after LPS administration to the sham-burned and burned mice. Figure 3C shows the MIP-2 levels in the tissue homogenate of the right lung in both groups. All mice in both groups showed an increase in cytokine levels that peaked 2 h after LPS administration. There was no subsequent increase of plasma and lung cytokines in the sham-LPS group, but we observed at 12 h a second peak in cytokine levels in the burn-LPS group. In short, there was only one peak in cytokine levels 2 h
after LPS administration in the sham-LPS group, whereas two peaks occurred in the burn-LPS group at 2 and 12 h, respectively. The differences between the two groups in plasma TNF-α and lung MIP-2 levels at 12 h after LPS administration were significant (both \(P < 0.005\)). Although the difference in plasma MIP-2 levels between the two groups was not significant, the mean level in the burn-LPS group was higher than in the sham-LPS group.

**PSL and JTE-607 Improved Survival Within 72 h After LPS Administration**

The Kaplan-Meier survival curves up to 72 h after LPS administration are shown in Fig. 4. All 10 mice (100%) in the sham group survived at 72 h after LPS administration, whereas none of the 10 (0%) survived in the PCTL group, the same as in the previous results. Five of the 10 mice (50%) in the PSL group and 7 of the 10 mice (70%) in the JTE group survived. Pretreatment with PSL and JTE-607 significantly improved survival within 72 h after LPS administration.

**PSL and JTE-607 Attenuated Acute Lung Injury and Decreased Lung MPO Activity**

The histopathological findings in the lung in the animals with and without prior burn injury are shown in Fig. 5, A and B. LPS administration resulted in pulmonary edema associated with neutrophil emigration into the interstitium and alveolar space. The severity of pulmonary edema and the increase in neutrophil emigration into the lungs were more prominent in the PCTL group (Fig. 5B) than in the sham group (Fig. 5A) but were obviously attenuated in the PSL group (Fig. 5C) and the JTE group (Fig. 5D). Figure 7C shows the MPO activity in the lung tissue in each group. MPO activity in the PCTL group (0.574 ± 0.051 U/mg) was significantly higher than in the sham group (0.405 ± 0.022 U/mg, \(P < 0.005\)), also indicating neutrophil accumulation in the lungs. Lung MPO activity in the PSL group (0.244 ± 0.016 U/mg) and JTE group (0.257 ± 0.032 U/mg) was significantly lower (\(P < 0.005\), respectively), corroborating the mitigation of neutrophil accumulation.

**Plasma TNF-α and MIP-2 Levels Were Attenuated by PSL and JTE-607 at 12 h After LPS Administration**

At 12 h after LPS administration, the TNF-α levels in the PCTL group (3,024.0 ± 600.6 pg/ml) were higher than in the sham group (1,190.1 ± 405.7 pg/ml, \(P < \))
0.005), but the levels in the PSL group (748.6 ± 137.3 pg/ml) and JTE group (979.5 ± 153.0 pg/ml) were lower than in the PCTL group (P < 0.0005, respectively; Fig. 6A). The TNF-α values of three mice in the sham group were higher (3,726, 1,929, and 1,205 pg/ml) than in the other animals in the sham group (724, 576, 567, 421, and 373 pg/ml), but since there was no valid reason to exclude them, we calculated the average value of all eight mice. The MIP-2 levels in the PCTL group (13,765.6 ± 3,120.3 pg/ml) were higher than in the sham group (6,395.5 ± 1,179.7 pg/ml, P < 0.005), but the levels in the PSL group (790.5 ± 45.5 pg/ml) and JTE group (1,712.2 ± 385.7 pg/ml) were lower than in the PCTL group (P < 0.005, respectively; Fig. 6B).

**Lung MIP-2 Levels and Gene Expression Were Attenuated by PSL and JTE-607 at 12 h After LPS Administration**

Figure 7A shows the MIP-2 levels in the right lung tissue homogenate of each group. The MIP-2 levels in the PCTL group (142.5 ± 12.4 pg/mg) were higher than in the sham group (70.0 ± 7.5 pg/mg, P < 0.005), but the levels in the PSL group (11.6 ± 1.5 pg/mg) and JTE group (38.9 ± 7.3 pg/mg) were lower than in the PCTL group (P < 0.005, respectively). Figure 7B shows the expression of MIP-2 mRNA in the right lung in each group as a ratio to that of GAPDH (MIP-2/GAPDH mRNA ratio). The MIP-2/GAPDH mRNA ratio in the PCTL group (0.975 ± 0.048) was higher than in the sham group (0.345 ± 0.135, P < 0.05), but the ratios in the PSL group (0.052 ± 0.009) and JTE group (0.075 ± 0.033) were lower than in the PCTL group (P < 0.005, respectively).

**DISCUSSION**

We developed a murine model of burn-primed sublethal endotoxemia in this study and found that prior burn injury induced two-peaked production of cytokines after LPS administration 11 days after the burn injury and that it was associated with increased mortality. We also showed that pretreatment with two independent anti-inflammatory drugs, PSL and JTE-607, attenuated the later cytokine production and decreased mortality after LPS administration. We therefore speculate that prior burn injury primed the mice for secondary insult through production of inflammatory cytokines, such as TNF-α and MIP-2. The results also suggest that an anticytokine strategy might serve as a novel prophylactic therapy for septic organ dysfunction in patients primed by burns or other insults.
Burn-Primed Endotoxemia and the Two-Hit Phenomenon

This study demonstrates that secondary administration of a sublethal dose of LPS 11 days after the injury induced sustained production of TNF-α and MIP-2, acute lung injury (ALI), and lethality after burn injury. The augmented response to secondary insults within a few hours to a few weeks after the initial insults has been referred to as the two-hit phenomenon (9). The initial insults described as the first hit in this phenomenon have been various injuries, such as hemorrhagic shock, burn insult, major trauma, and surgery, and they primed patients to a second hit in the form of a subsequent local and systemic bacterial infection or sepsis. Although it has been speculated that inflammatory mediators, particularly cytokines, are involved in the pathogenesis of this phenomenon, the precise mechanisms are still undetermined. In the present study, we showed that prior burn insult induced a two-peaked and augmented production of TNF-α and MIP-2 after administration of a sublethal dose of LPS, and the lethality was prevented by pretreatment with anti-inflammatory drugs. Thus it is reasonable to hypothesize that the sustained cytokine production is the key mechanism responsible for increased mortality. Although other mechanisms, such as coagulation and complement cascades, are involved in septic organ dysfunction and survival was only partially improved by cytokine inhibition, this hypothesis is also supported.
by the recent clinical observation that modulation of sustained cytokine production by glucocorticoids is associated with improved outcome in patients with ALI (23).

With regard to initial burn insult, it is reasonable to hypothesize that the injured skin or subcutaneous tissue plays a major role in inducing the augmented inflammatory response, including dysregulated cytokine production, which resulted in organ dysfunction, particularly ALI. This hypothesis is supported by the finding that early excision and grafting reduce TNF-α production in animal models (10, 28) and improve the survival in clinical studies (30, 31). The mechanisms of augmented cytokine production may also be related to the ischemia-reperfusion injury of the related area, and it is quite unlikely that cytokine synthesis inhibitors were directly associated with wound healing during the observation period in this study, since we did not find any differences in the burned area among treated and untreated groups.

In the present study, we administered LPS intravenously as the second hit, and the increased mortality was associated with sustained production of TNF-α and MIP-2 in our model. Clinically, systemic infection as a complication or detection of plasma LPS frequently occur in burn patients, and they are thought to be attributable to the burn wound or bacterial translocation (34). There is a study showing that higher levels of plasma endotoxin are associated with poorer outcome (36). Although it did not show a direct association between the augmented cytokine production and poor outcome of burn patients, it is possible to hypothesize that higher levels of LPS induced higher production of proinflammatory cytokines.

**ALI as the Outcome of Accelerated Inflammatory Response**

An augmented inflammatory response has also been observed in animal models of ALI. Hemorrhagic shock and resuscitation 1 h before LPS administration has been reported to induce augmented production of lung cytokine-induced neutrophil chemoattractant (CINC), but not MIP-2, and to subsequently induce ALI (12). Acid aspiration 24 h before LPS administration has been shown to exaggerate ALI and induce a greater increase in plasma TNF-α and bronchoalveolar lavage (BAL) fluid CINC (35). In the present study, increased mortality and ALI were observed when LPS was administered 11 days after the burn insult. Although the intervals between the initial and secondary insults differed, we hypothesize that there is a common mechanism that augmented the response to the secondary attack.

Sepsis and sepsis-related MODS, particularly ALI, have recently become major complications after burn injury, and they are considered to be the primary contributor to morbidity and mortality (3). Because the onset of ALI usually occurs more than a week later (8), we think our model is relevant to actual burn patients clinically.

**Chemokines in ALI**

The proinflammatory cytokine MIP-2, chosen as a major parameter in our investigation, is a member of the C-X-C chemokine family and a murine homolog of human IL-8 (11, 29). IL-8 is well recognized as a key mediator in various inflammatory lung diseases, including acute respiratory distress syndrome (ARDS) (24) and inhalation injury (14). High levels of proinflammatory cytokines, TNF-α, IL-1β, IL-6, and IL-8, have been demonstrated in the plasma and BAL fluid of experimental animals and humans with ARDS (15, 22). Augmented production of IL-8 has also been reported in rabbit lungs collapsed for 3 days and then re-expanded for 2 h, and its neutralization attenuated the development of re-expansion pulmonary edema (26). In their rabbit model of re-expansion lung injury, collapsing may correspond to the first hit and re-expansion to the second hit.

The IL-8 family, including MIP-2, exhibits potent neutrophil chemotactic activity, and its members are key mediators in recruiting neutrophils, which play a major role in tissue injury and host defense against various infections (13, 17). The accumulation of neutrophils in the lung tissue of PCTL mice was indicated from the increase in lung MPO activity, and ALI was confirmed histopathologically after LPS administration. On the basis of these findings that lung MIP-2 production was increased and its inhibition attenuated ALI, we speculate that MIP-2 induced neutrophil-mediated ALI in our model. By contrast, these findings were minimal in the sham group. We conclude that the prior burn insult triggered an exaggerated response to sublethal LPS through an as yet unidentified mechanism.

**Pharmacological Modulation With Cytokine Synthesis Inhibitors**

In the two-hit phenomenon, initially less severely injured patients eventually develop MODS as a result of an exaggeration of their inflammatory response caused by a second or subsequent insult, such as infection (9, 25). If our hypothesis explaining the two-hit phenomenon is valid, that is, if the burn-primed hosts produce greater amounts of cytokines than in ordinary sepsis, the modulation of the exaggerated response with anti-inflammatory drugs, particularly cytokine synthesis inhibitors, should improve organ dysfunction.

Glucocorticoids, a class of multifunctional anti-inflammatory drugs, are well known for their ability to potently inhibit the synthesis of various proinflammatory cytokines (19). Administration of dexamethasone inhibited TNF and IL-1 synthesis and improved survival in a murine model of septic shock (5). In corticosteroid-treated patients with ARDS, survivors were demonstrated to show greater reductions in plasma and BAL TNF-α, IL-1β, IL-6, and IL-8 levels, especially in the later phase of ARDS, compared with the nonsurvivors, indicating a dominant role of inflammatory...
cytokines in worsening the outcome of ARDS (21). JTE-607 is a newly developed small molecule that targets proinflammatory cytokine production by monocytes (18). Pretreatment with two independent cytokine synthesis inhibitors, PSL and JTE-607, decreased the plasma and lung cytokine levels as well as lung cytokine production, and these changes were associated with attenuation of ALI and decreased mortality.

Although the mechanism by which prior burn insult induces sustained cytokine production is unknown, we hypothesize that augmented production of these cytokines is the major mechanism responsible for the ALI and high mortality in this model. Furthermore, we predict that pharmacological modulation of cytokine synthesis immediately after the burn insult will serve as an effective means of preventing ALI and other types of organ dysfunction in primed patients. However, it is unknown whether pretreatment with cytokine synthesis inhibitors, particularly PSL, will increase the risk of infection in the long term.

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