Protective Effects of a Leukotriene Inhibitor and a Leukotriene Antagonist on Endotoxin-Induced Mortality in Carrageenan-Pretreated Mice

MASANORI OGATA,1* TAKAHIRO MATSUMOTO,1 MASAYUKI KAMOCHI,1 SHIN-ICHI YOSHIDA,2 YASUO Mizuguchi,2 and AKIO SHIGEMATSU1

Department of Anesthesiology1 and Department of Microbiology,2 School of Medicine, University of Occupational and Environmental Health, Kitakyushu 807, Japan

Received 18 November 1991/Accepted 17 March 1992

The leukotrienes and tumor necrosis factor (TNF) play an important role in the pathophysiology of septic shock, in which hypotension, leukopenia, thrombocytopenia, and hemoconcentration are observed. This study was performed to examine the effects of a 5-lipoxygenase inhibitor (AA-861), a selective leukotriene receptor antagonist (ONO-1078), and a cyclooxygenase inhibitor (indomethacin) on endotoxin-induced mortality and TNF production in mice. Mice were injected intraperitoneally with carrageenan (5 mg per mouse), which we previously reported as an effective priming agent for lipopolysaccharide (LPS)-induced TNF production and mortality (M. Ogata, S. Yoshida, M. Kamocho, A. Shigematsu, and Y. Mizuguchi, Infect. Immun. 59:679–683, 1991). The indicated doses of AA-861, ONO-1078, indomethacin, or controls were administrated subcutaneously 30 min before LPS (50 μg per mouse) provocation. The mortality of mice was significantly decreased by pretreatment with AA-861 (P < 0.001) or ONO-1078 (P < 0.01) but not by pretreatment with indomethacin. The 50% lethal dose of LPS in the mice treated with dimethyl sulfoxide or ethanol was 32 or 33 μg, respectively, and it increased to 83 μg with AA-861 or 59 μg with ONO-1078, respectively. Neither AA-861 nor ONO-1078 suppressed LPS-induced TNF production in sera. Treatment with AA-861 significantly decreased the leukopenia and thrombocytopenia, and ONO-1078 significantly decreased the hemoconcentration and thrombocytopenia. The role of endogenous TNF was also examined in the carrageenan-pretreated mice. Treatment with 2 × 108 U of rabbit anti TNF-α antibody intravenously 2 h before LPS challenge significantly suppressed the LPS-induced TNF activity and decreased the mortality. Therefore, both leukotrienes and TNF play important roles in endotoxin-induced shock and mortality.

The tumor necrosis factor (TNF) is an important cytokine that mediates endotoxin shock and causes multiple organ damage (5). It has been reported that patients with peritonitis or burns are apt to go into septic shock and to develop multiple organ failure (13). We previously reported that in mice pretreated intraperitoneally with carrageenan (CAR), a sulfated pagalactose, even a low dose of endotoxin (50 μg per mouse) caused enormous TNF production in serum and death (24). It was supposed that inflammation played an important role in the enhancement of lipopolysaccharide (LPS)-induced TNF production and mortality caused by CAR pretreatment, because CAR is used as an inflammatory agent as well as a reticuloendothelial system blocker. These findings suggest that the inflammation plays an important role in the development of low-dose endotoxin-induced mortality and multiple organ failure and that CAR-pretreated mice provide a useful model to analyze the pathophysiological mechanism.

There is increasing recent evidence that inflammatory mediators such as leukotrienes (LTs) (8–10, 33) and cyclooxygenase products (2, 20) are involved in the pathophysiology of endotoxemia. It was reported that LT inhibitors suppressed LPS-induced TNF production and mortality in galactosamine-treated mice (30).

2-(12-Hydroxydocosa-5,10-dinyl)-3,5,6-trimethyl-1,4-benzoquinone (AA-861) is a selective 5-lipoxygenase inhibitor that was reported to inhibit 5-lipoxygenase from guinea pig peritoneal polymorphonuclear leukocytes (36). This compound strongly inhibited allergic bronchoconstriction in guinea pigs and moderately reduced CAR-induced paw edema and pleurisy in rats (3). 4-Oxo-8-[p-(4-phenylbutyloxy)benzoylaminio]-2-tetrazol-(5-yl)-4H-1-benzopyran hemihydrate (ONO-1078) is an LTC4 receptor antagonist that has been shown to selectively antagonize LT-induced bronchoconstriction in guinea pig and human bronchial smooth muscles (23). The effect was 10 to 100 times stronger than that of the LT antagonist FPL-55712 (1). We therefore investigated the effect of AA-861, ONO-1078, and indomethacin on LPS-induced TNF production and mortality in CAR-pretreated mice. The present study shows that AA-861 and ONO-1078 cannot reduce the plasma TNF levels but can significantly reduce LPS-induced mortality of CAR-pretreated mice.

(Most of this study was presented at the Second International Congress on the Immune Consequences of Trauma, Shock and Sepsis Mechanisms and Therapeutic Approaches, Munich, Germany, 1991.)

MATERIALS AND METHODS

Animals. Male ddY mice were obtained from the Seiwa Experimental Animal Co., Oita, Japan. All mice used were 7 to 8 weeks of age. Mice were housed in groups of 10 and were allowed food and water ad libitum.

Reagents. Phenol-extracted Escherichia coli (O127:B8) LPS was purchased from Difco Laboratories, Detroit, Mich. Iota-carrageenan (lot 59C-0328) and indomethacin (lot 111F-

* Corresponding author.
LEUKOTRIENES IN LPS-INDUCED MORTALITY

TABLE 1. Protective effect of AA-861 or ONO-1078 on the LPS (50 μg)-induced mortality of CAR-pretreated mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mmol/kg)</th>
<th>Mortality rate (no. dead/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Exp 1</td>
</tr>
<tr>
<td>AA-861</td>
<td>20</td>
<td>0/10e</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3/10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4/10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8/10</td>
</tr>
<tr>
<td>AA-861 control (DMSO)</td>
<td>40</td>
<td>3/10</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4/10</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4/10</td>
</tr>
<tr>
<td>ONO-1078 control (ethanol)</td>
<td>8/10</td>
<td>8/10</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>40</td>
<td>9/10</td>
</tr>
<tr>
<td>Indomethacin control (saline)</td>
<td>8/10</td>
<td>ND</td>
</tr>
</tbody>
</table>

* CAR (5 mg per mouse) was injected i.p. 24 h before LPS (50 μg per mouse) was injected i.v. ND, not done.
* Each treatment was done 30 min before the LPS administration.
* P < 0.05 versus the control.
* P < 0.01 versus the control.

A significant difference was assumed at a probability value of <0.05.

RESULTS

Dose effects of 5-lipoxygenase inhibitor (AA-861) or LTC4D4 antagonist (ONO-1078) on mortality. CAR (5 mg per mouse) was injected i.p. 24 h before LPS (50 μg per mouse) was injected i.v. Various doses of ONO-1078 (40, 20, and 10 mmol/kg), AA-861 (20, 10, and 5 mmol/kg), indomethacin (40 mmol/kg), and the controls were injected i.c. into mice 30 min before they were challenged with 50 μg of LPS. The maximum soluble doses were 0.6 mmol/ml in 10% DMSO for AA-861 and 1.2 mmol/ml in 10% ethanol for ONO-1078. These solutions were used as the maximum doses for the treatments. The mortality of mice was significantly decreased in AA-861- ONO-1078-treated mice relative to that in the control mice (chi-square test; Table 1). However, we could not find any significant difference between vehicle- and indomethacin-treated mice in mortality. Although both AA-861 and ONO-1078 s.c. treatments 1 h before LPS (50 μg) injection significantly decreased the LPS-induced mortality, the treatments 1 h after the LPS injection did not do so (data not shown).

Effects of 5-lipoxygenase inhibitor (AA-861) or LTC4D4 antagonist (ONO-1078) on survival curves of mice in endotoxin shock. Pretreatment with CAR (5 mg i.p.) rendered the mice more sensitive to the effect of LPS, as previously reported (24). Although the survival rate of mice treated with each solvent was 20% at 72 h after LPS (50 μg per mouse) administration, s.c. treatment with AA-861 (20 mmol/kg) or ONO-1078 (40 mmol/kg) significantly increased the survival rate after the LPS administration (Fig. 1) (AA-861, P < 0.001; ONO-1078, P < 0.01); Kaplan Meier statistical method.

Effects of 5-lipoxygenase inhibitor (AA-861) or LTC4D4 antagonist (ONO-1078) on cumulative percent mortality after LPS injection. The cumulative percent mortalities after various i.v. doses of LPS in mice treated s.c. with AA-861 (20 mmol/kg), ONO-1078 (40 mmol/kg), or one of the solvents (controls) were compared (Fig. 2). The 50% lethal doses (LD50s) of LPS in the AA-861 control (10% DMSO-treated mice) and the ONO-1078 control (10% ethanol-treated mice)
were 32 and 33 µg, respectively; the LD50s increased to 83 and 59 µg for mice treated s.c. with AA-861 (20 mmol/kg) and ONO-1078 (40 mmol/kg), respectively.

**Effects of AA-861 and ONO-1078 on kinetics of TNF production in sera.** CAR (5 mg per mouse) was injected i.p. 24 h before LPS was injected. The mice were treated s.c. with 20 mmol of AA-861 per kg, 40 mmol of ONO-1078 per kg, 10% DMSO, or 10% ethanol. After 30 min, the mice were injected i.v. with 50 µg of LPS and then bled at intervals. The kinetic studies revealed a monophasic TNF response with a peak at 2 h, as previously reported (24). The maximum activity of TNF in sera appeared 2 h after LPS injection. The level of TNF activity declined sharply thereafter and was hardly detectable after 5 h in all groups. Figure 3 shows the effect of AA-861 or ONO-1078 on the peak TNF activity in sera at 2 h after endotoxin injection. There was no significant difference between the TNF activities at 2 h after LPS challenge. We could not find any significant differences between the TNF levels in AA-861- and ONO-1078-treated mice and control mice at 1, 3, or 4 h (data not shown). There was also no significant difference between the TNF levels in mice treated with saline, DMSO, or ethanol (data not shown).

**Effects of anti-TNF-α antibody on LPS-induced mortality in CAR-pretreated mice.** For this experiment, each mouse received CAR (5 mg) and then LPS (50 µg) 24 h later. Control mice were injected i.v. with 0.2 ml of normal rabbit serum 2 h before the LPS challenge. The other mice were injected i.v. with 2 × 10^5 U of anti-TNF-α antibody 2 h before the LPS challenge. The treatment with 2 × 10^5 U of anti-TNF-α antibody significantly suppressed the LPS-induced TNF activity in sera and decreased the mortality (Table 2).

**Effects of AA-861 and ONO-1078 on the hematological response.** The effects of AA-861 or ONO-1078 on the hematological response are summarized in Table 3. The endotoxin-induced leukopenia and thrombocytopenia in the LPS-challenged group were significantly greater than those in the control group. The number of leukocytes in AA-861-treated mice was significantly higher than that in the control group at 30 min after LPS administration. The leukopenia remained until 4 h after LPS injection, but no significant difference was found between the AA-861- or ONO-1078-treated group and

---

**TABLE 2. Suppression of LPS-induced TNF activity and mortality by anti-TNF antibody treatment of CAR-pretreated mice**

<table>
<thead>
<tr>
<th>Mice</th>
<th>TNF activity (U/ml)</th>
<th>Mortality (no. dead; n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>112,400 ± 28,043</td>
<td>10</td>
</tr>
<tr>
<td>Anti-TNF antibody-treated</td>
<td>ND&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mice were pretreated i.p. with 5 mg of CAR 24 h prior to LPS (50 µg) challenge.

<sup>b</sup> TNF activities in sera were measured 2 h after LPS injection. Data are means ± standard errors.

<sup>c</sup> Deaths were recorded daily for 7 days.

<sup>d</sup> Normal rabbit serum in 0.2 ml was injected i.v. 2 h prior to LPS challenge.

<sup>e</sup> Each mouse was injected i.v. with 2 × 10^5 U of rabbit anti-TNF-α antibody in 0.2 ml 2 h prior to LPS injection.

<sup>f</sup> ND, not detected.

<sup>g</sup> P < 0.01 versus control mice.
the respective control group. The numbers of platelets in the ONO-1078-treated group were significantly higher than those in the control group at 30 min and 4 h, but that in the AA-861-treated group was significantly higher only at 4 h after LPS administration. The platelet counts of AA-861- or ONO-1078-treated mice at 4 h after LPS injection returned to the control value. ONO-1078 significantly decreased the hemoconcentration after 30 min of LPS injection, but AA-861 did not. At 4 h after the LPS challenge, the percent value of hematocrit in the various experimental groups returned to that of the control group.

**DISCUSSION**

The present data show that both AA-861 and ONO-1078 reduce the mortality rate without suppressing endotoxin-induced TNF production. The drugs attenuated the thrombocytopenia, leukopenia, and the early vascular permeability that were caused by endotoxin in CAR-pretreated mice. These findings suggest that LTs exerted an important role in determining the endotoxin-induced mortality in CAR-pretreated mice.

It has been reported that lipoxigenase inhibitors suppress TNF production in galactosamine-treated mice (30). Our present data, however, showed that the lipoxigenase inhibitor AA-861 or the LTC₄D₄ antagonist ONO-1078 suppressed endotoxin-induced mortality despite the elevated TNF activity in sera, indicating that LTs influenced the LPS-induced mortality rate but not LPS-induced TNF production in CAR-pretreated mice. It has been reported that interleukin-6 in serum is dramatically elevated by LPS stimulation in CAR-pretreated mice (12). These findings suggest that inflammatory mediators play a key role in cyclooxygenase-induced mortality in CAR-pretreated mice. Our study, however, demonstrated that a cyclooxygenase inhibitor (indomethacin) could not decrease the rate of LPS-induced mortality in CAR-pretreated mice. We conclude that the role of LTs is more important than that of cyclooxygenase products in the mortality of CAR-pretreated mice.

Recently, Franks et al. reported that anti-TNF-α antibody could not prevent LPS-induced mortality in CAR-pretreated mice. In their experiment, 4 × 10⁸ U of rabbit anti-TNF-α antibody was injected i.p. 5 h before LPS challenge. Our data, however, demonstrated that treatment with 2 × 10⁸ U of rabbit anti-TNF-α antibody i.v. 2 h before LPS challenge significantly reduced LPS-induced mortality and the TNF activity in sera. The discrepancy between the data of Franks et al. and our data might be due to differences in the time intervals and routes of the anti-TNF-α antibody injection and the experimental model with CAR. Franks et al. injected lambda-CAR (1 mg) i.p. into BALB/c mice and challenged the mice with LPS (2 µg) 1 h later. In our experiment, anti-TNF-α antibody significantly suppressed the TNF activity in sera and prevented death. Thus, TNF plays an important role in low-dose endotoxin-induced shock and death even in CAR-pretreated mice. We wanted to know how AA-861 or ONO-1078 affected the reduction of the endotoxin-induced mortality in CAR-pretreated mice. Hagemann et al. reported that production of LTs was enhanced by endotoxin (18) and that LTs were eliminated into the bile (17). Endotoxin is known to cause hemoconcentration, leukopenia, and thrombocytopenia, indicating increased vascular permeability, margination of leukocytes, and platelets in blood vessels, respectively. Our present data showed that the LT antagonist ONO-1078 somewhat decreased the LPS-induced hemoconcentration and thrombocytopenia and that the LT inhibitor AA-861 also decreased LPS-induced leukopenia and thrombocytopenia.

Exogenous administration of LTC₄ and LTD₄ has been reported to increase vascular permeability (22, 32). Filep et al. demonstrated that LTC₄ or LTD₄ increased the hematocrit in conscious rats (11). Wendel and Tieg reported that LTD₄ plays an important role in galactosamine-endotoxin-induced hepatitis (35). Recently, an LT antagonist, LY 203647, prevented extravascular water accumulation in pig lungs in a septic shock model (8). In addition, endotoxin-induced hemoconcentration was reduced by the LT antagonist LY 171883 (9) or SK&F 104353 (33). LTD₄ also causes an increase in peripheral vascular permeability in rabbits, rats, guinea pigs, and hamsters (6). LTB₄ injected alone has little permeability-increasing effect in the skin; however, the LTB₄ response is markedly enhanced by the presence of a vasodilator such as prostaglandin E₂ (6). In our results, the reduction of the endotoxin-induced hemocrit by the LTC₄D₄ antagonist ONO-1078 was stronger than that of the 5-lipoxygenase inhibitor AA-861. Therefore, these findings suggest that LTC₄ and LTD₄ play a predominant role in the enhancement of vascular permeability in an early phase after endotoxin administration. Schulz et al. et al. reported that leukocytes are margined in the pulmonary microcirculation and release protease, oxidants, and arachidonic acid metabolites during endotoxin shock (31). LTB₄ is one of the most potent chemoattractants of leukocytes (6). We demonstrated that the 5-lipoxygenase inhibitor AA-861 attenuated endotoxin-induced leukopenia at 30 min after LPS injection, but the LTC₄D₄ antagonist
ONO-1078 could not do so. These findings are in agreement with those of Puranapanda et al. (25) and Smith et al. (33), who reported that LTC4D4 antagonists had no effect on the leukopenia in septic shock. We suggest that leukocyte sticking and emigration are not direct consequences of LTC4 or LTB4 release but are caused by LTB4 release.

Although endotoxin induced a significant thrombocytopenia, both ONO-1078 and AA-861 significantly prevented thrombocytopenia at 30 min and 4 h after LPS administration, which coincides with the results of Smith et al. (33). Thus LTB4 and LTC4D4 are the most potent mediators of endotoxin shock in CAR-pretreated mice.

Both AA-861 and ONO-1078 are barely soluble even in the solvents used. Although the pharmacokinetics of these drugs after s.c. injection have not been investigated, we assume that neither can diffuse easily into the bloodstream. Therefore, the development of more soluble agents will be required for clinical application.

Recently, it was proven that recombinant human TNF-α directly stimulated arachidonic acid release in human neutrophils (4) and LT production in vitro (19). It is supposed that AA-861 or ONO-1078 reduces the LPS-induced mortality of CAR-pretreated mice mainly by preventing the effect of LTB4 and LTC4D4, despite the high TNF level in sera. Fuerstein et al. demonstrated that there can be a substantial discrepancy between the plasma TNF level and mortality rate (14). Our results suggest that inflammation is closely related to endotoxin-induced mortality in CAR-pretreated mice. Recently, it was reported that recombinant human TNF-α alone was not lethal, but that synergy between TNF and bacterial products caused lethal shock in mice (26). We believe that TNF probably involves synergism with LPS and other inflammatory mediators such as interleukin-1, interleukin-6, platelet-activating factor, and LTs and that such synergisms lead to the associated platelet activation, bronchoconstriction, hypotension, increased vascular permeability, and, eventually, death.

Although we usually inject a large amount of LPS into experimental animals to elicit endotoxin shock, clinical observation suggested that a high level of serum endotoxin is not essential to develop shock and multiple organ failure (15, 16, 34). It is supposed that inflammation (7, 13, 15, 16) and depression of the reticuloendothelial system (28, 29) are important factors in developing endotoxin-induced mortality and multiple organ damage in human patients. CAR-pretreated mice are available for the study of the pathophysiologic mechanism of the low-dose endotoxin-induced shock and multiple organ failure, because even a small dose of LPS can enhance both the plasma TNF levels and the mortality rate in CAR-pretreated mice, as we previously reported (24).

In summary, both an LT inhibitor (AA-861) and an LT antagonist (ONO-1078) significantly decreased endotoxin-induced mortality in CAR-pretreated mice without suppressing the serum TNF activity. Treatment with anti-TNF-α antibody i.v. also decreased the LPS-induced mortality and suppressed TNF activity. We conclude that LTs and TNF are important mediators of endotoxin-induced mortality in CAR-pretreated mice.

ACKNOWLEDGMENTS

This study was supported by a grant-in-aid for Scientific Research 03077013 from the Ministry of Education, Science, and Culture, Japan.

We thank Eri Takahashi and Rieko Maeda for their technical assistance.

REFERENCES


