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Enhancement of Lipopolysaccharide-Induced Tumor Necrosis Factor Production in Mice by Carrageenan Pretreatment

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Tumor necrosis factor (TNF) is a cytokine which mediates endotoxin shock and causes multiple organ damage. It is thought that macrophage (MP) activation is necessary to increase lipopolysaccharide (LPS)-induced TNF production and lethality. Carrageenan (CAR) is sulfated polygalactose which destroys MP; it is used as a MP blocker. We found that CAR pretreatment can increase both endotoxin-induced TNF production and the mortality rate in mice. The ddY mice (7 to 8 weeks old) were injected intraperitoneally with CAR (5-mg dose) and challenged intravenously with LPS 24 h later. Without CAR pretreatment, LPS doses of less than 10 μg did not induce TNF in sera. After pretreatment, however, about 3×10^3 to 4×10^4 U of TNF per ml was produced after LPS injection at doses of 0.1 to 10 μg, respectively. TNF production was significantly increased by CAR pretreatment at LPS doses of more than 10 μg. CAR pretreatment rendered the mice more sensitive to the lethal effect of LPS; 50% lethal doses of LPS in CAR-pretreated mice and nonpretreated mice were 26.9 and 227 μg, respectively. The mortality of the two groups was significantly different at doses of 50, 100, and 200 μg of LPS. CAR increased LPS-induced TNF production and mortality within 2 h, much earlier than MP activators, which needed at least 4 days. Our results made clear that TNF production is enhanced not only by a MP activator but also by a MP blocker.

Bacterial lipopolysaccharide (LPS) can induce shock, which is characterized by hypotension, disseminated intravascular coagulation, diarrhea, and multiple organ damage (12). Tumor necrosis factor (TNF) has been shown to be the principal cytokine in causing endotoxin-induced shock (1, 35, 36), because TNF induces mortality with symptoms typical for endotoxin shock and anti-TNF antibodies can prevent lethal toxicity produced by LPS (5). Macrophage (MP) activators such as *Mycobacterium bovis* BCG (18) and *Propionibacterium acnes* (*Corynebacterium parvum*) (13) were used as priming agents for LPS-induced TNF production. TNF has been considered to be a product of activated MP (18, 25), and numerous reports suggest that MP activation is the common underlying requirement for increased sensitivity to endotoxin (4, 15, 33, 34).

In contrast, it has been shown that sensitivity to endotoxin is enhanced by a MP blocker such as silica (37) and carrageenan (CAR) (2). There is, however, no published work reporting the effect of CAR or silica on LPS-induced TNF production. We therefore examined the effect of CAR pretreatment on LPS-induced TNF production in sera and on mouse mortality. We found that CAR pretreatment increased both LPS-induced TNF production and mouse mortality by endotoxin shock.

MATERIALS AND METHODS

Animals. Male ddY mice were obtained from Seiwa Experimental Animal Co., Ooita, Japan. C3H/HeJ and C3H/HeN mice were obtained from Nihon Crea, Tokyo, 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* lipopolysaccharide (O127:B8) was purchased from Difco Laboratories, Detroit, Mich. Iota-carrageenan (lot 59C-0328) was purchased from Sigma, St. Louis, Mo. Both LPS and CAR were dissolved in pyrogen-free physiological saline (Otsuka Pharmaceutical Co., Naruto, Japan). CAR solution was autoclaved at 121°C for 15 min before use.

Antiserum. Polyclonal rabbit anti-murine TNF (lot B8269) was obtained from Genzyme Corporation, Boston, Mass. As little as 0.1 μl of this antibody can neutralize approximately 1,000 U of TNF.

Priming for and induction of endotoxin shock. CAR (0.01 to 5 mg in 0.5 ml of physiological saline) was injected intraperitoneally (i.p.) as a priming agent. LPS (0.001 to 1,000 μg in 0.5 ml of physiological saline) was injected intravenously (i.v.) into the tail vein as an inducing agent. The blood was collected by cardiac puncture, and it was kept at room temperature for clotting. The sera were stored at −80°C until they were used for TNF assay.

Mortality. The cumulative percentages of mortality were determined by counting the numbers of dead mice at 6, 18, 24, 36, and 72 h after the administration of endotoxin, with 10 mice in each group.

TNF assay. TNF activity was assayed by the colorimetric determination of cytotoxicity to L929 cells as previously reported (24). The titer of TNF was expressed in units per milliliter, which is the reciprocal of the dilution necessary to cause lysis of 50% cells. The blood samples for TNF assay were usually collected 2 h after LPS administration.

RESULTS

Effect of CAR on lethality in endotoxin shock. The mortality rate of ddY mice injected i.v. with LPS was dose dependent. Cumulative percent lethality of ddY mice by LPS injection is shown in Fig. 1. The fifty percent lethal dose was 227 μg. When the mice were pretreated with 5 mg of CAR 24 h prior to LPS challenge, the treatment rendered the mice more

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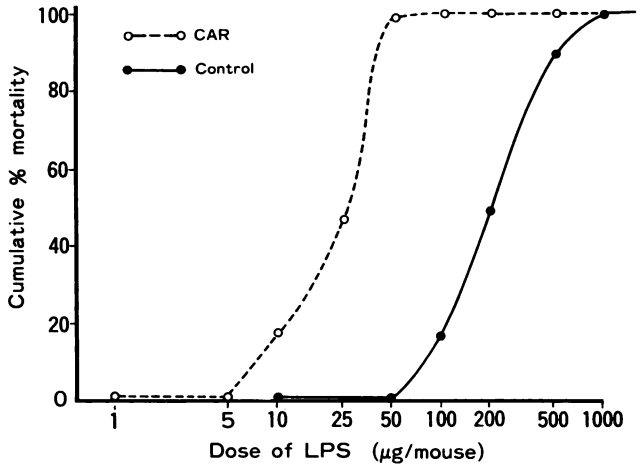

FIG. 1. Cumulative percent mortality after LPS injection in CAR-treated and nontreated mice. CAR (5 mg) was injected i.p. 24 h prior to LPS i.v. injection. Each group receiving LPS doses consisted of 10 mice. Deaths were recorded daily for 3 days. Because mice that were alive at day 3 remained healthy through day 10, observation was terminated after day 3.

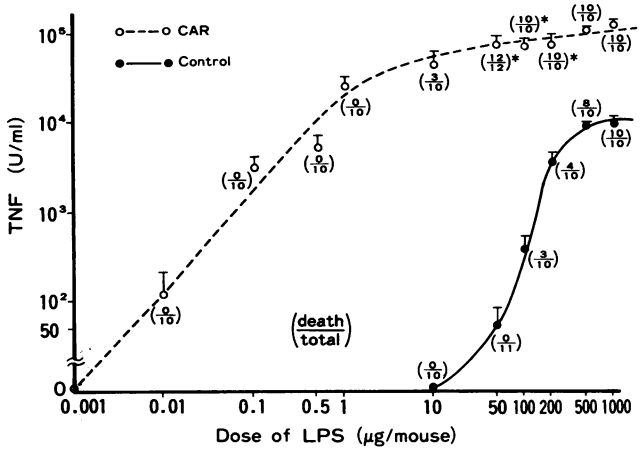

FIG. 2. Augmentation of LPS-induced TNF production in sera in CAR-treated mice. Mice were treated i.p. with 5 mg of CAR or phosphate-buffered saline, and various doses of LPS were injected i.v. 24 h after pretreatment. Two hours after LPS administration, mice were bled and sera were used for TNF assay. Data are expressed as the means ± standard deviations for 8 to 10 mice. Numbers in parentheses represent numbers of dead mice/total mice used at each dose of LPS. The numbers were obtained from the experiment shown in Fig. 1. TNF titers were significantly higher ($P < 0.01$ by χ^2 test) in CAR-treated mice at LPS doses of 50, 100, and 200 μg.

sensitive to the effect of LPS (Fig. 1). LPS doses of more than 50 μg killed 100% of the mice. The 50% lethal dose of LPS in CAR-treated mice was only 26.9 μg. The mortality rate of CAR-pretreated mice and nonpretreated mice was significantly different at LPS doses of 50, 100, and 200 μg by the chi-square test ($P < 0.01$, Fig. 2). Survival time was shortened in CAR-treated mice; that is, mice challenged with 100, 200, or 500 μg of LPS died within 24 h (data not shown).

Effect of CAR on LPS-induced TNF production in sera. Various doses (0.001 to 1,000 μg) of LPS were injected i.v. and TNF activities in sera of CAR (5-mg dose)-treated and nontreated mice were compared. TNF activity was measured by using sera obtained 2 h after LPS administration. Although LPS could induce TNF in a dose-dependent fashion in both groups, TNF activity was significantly increased by CAR pretreatment at all doses of LPS (Fig. 2). Although TNF activity in nonpretreated mice was at most 10^4 U/ml, TNF activity in CAR-treated mice was measured at as much as 10^5 U/ml. Without CAR pretreatment, a LPS dose of less than 10 μg did not induce TNF. With CAR pretreatment, however, more than 10^4 U of TNF per ml was induced after an injection of 1 μg of LPS (Fig. 2).

In order to confirm that L929 cell lysis is due to TNF, we treated the samples with anti-murine TNF-α antibody. TNF activity in the serum of CAR-pretreated mice was completely inhibited by antiserum (data not shown).

Kinetics of TNF production in sera. The mice were pretreated i.p. with 5 mg of CAR. After 24 h, they were injected i.v. with 50 μg of LPS and were bled at intervals (Fig. 3). Low levels of TNF were detected in the sera after 30 min of LPS injection. The maximum activity of TNF in sera appeared 1 to 2 h after LPS administration. The level of TNF activity declined sharply thereafter and was hardly detectable after 6 h.

Effect of CAR dose on TNF production and lethality. Various doses (0.01, 0.1, 0.5, 1, 2.5, and 5 mg) of CAR were injected i.p. into mice 24 h before challenging them with 50 μg of LPS. All mice were bled at 2 h after LPS administration (Fig. 4). Without CAR pretreatment, TNF was hardly produced by injection of 50 μg of LPS. Significant production of TNF was observed when mice were pretreated with 0.01 mg of CAR. CAR pretreatment enhanced LPS-induced TNF activity and lethality in a dose-dependent fashion (Fig. 4).

Effect of time interval between CAR pretreatment and LPS administration. LPS (50 μg) was injected i.v. at various intervals after pretreatment with 5 mg of CAR, and TNF production was assayed (Fig. 5). All mice were bled 2 h after LPS administration. The level of TNF activity increased after the 2-h interval, and peaked after a 12-h interval between pretreatment with CAR and LPS injection (Fig. 5). Mortality due to LPS injection also increased from the 2-h interval, and all mice had died when a 12- to 24-h interval

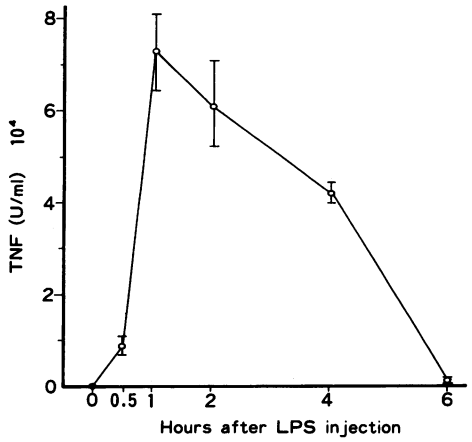

FIG. 3. Kinetics of TNF production in sera. Mice were pretreated i.p. with 5 mg of CAR and injected with 50 μg of LPS 24 h later. After LPS injection, mice were bled at various intervals. Data are expressed as the means ± standard deviations for eight mice.

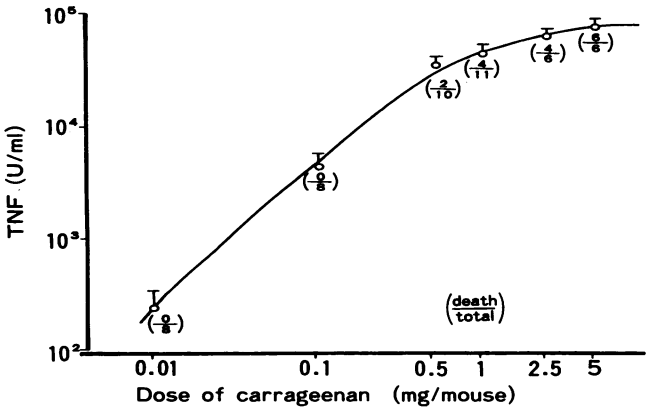

FIG. 4. Dose response of CAR pretreatment on LPS-induced TNF production and lethality. Mice (eight in each group) were injected i.p. with various doses of CAR. Twenty hours later, they were injected i.v. with 50 μg of LPS. Mice were bled 2 h later, and the sera were used for TNF assay. Data are expressed as means ± standard deviations for eight mice. Mice without CAR treatment showed no detectable serum TNF activity. Numbers in parentheses represent numbers of dead mice/total number of mice used obtained from the TNF lethality experiment.

was used. The enhancing effect of CAR pretreatment on TNF activity and lethality continued for at least 3 weeks.

Effect of CAR on LPS-induced TNF activity in C3H/HeJ mice. The effects of CAR pretreatment on LPS-induced TNF production and lethality were investigated by using LPS-resistant C3H/HeJ mice and LPS-sensitive C3H/HeN mice. LPS (50-μg dose) induced a low level of TNF (160 ± 80 U/ml) in C3H/HeN mice but no TNF in C3H/HeJ mice. By CAR pretreatment, the serum in C3H/HeN mice showed remarkably high levels of TNF (8.8×10^4 U/ml), but a low level of TNF (240 ± 56.6 U/ml) appeared in C3H/HeJ mice. In CAR-pretreated C3H/HeN mice, 50 μg of LPS sufficed to cause death of all eight mice. In contrast, CAR-pretreated C3H/HeJ mice showed only 12.5% mortality at 72 h after LPS (50-μg) injection.

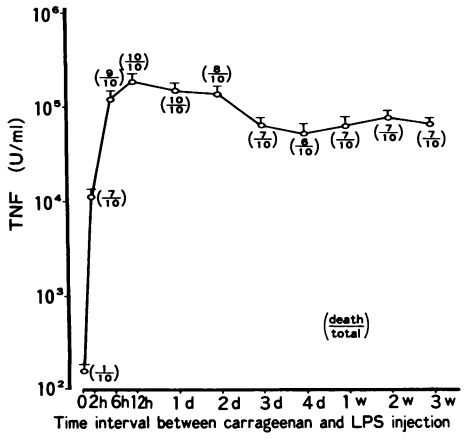

FIG. 5. Effects of time interval between CAR pretreatment and LPS challenge on TNF production and mouse mortality. Data are expressed as the means ± standard deviations for seven mice. Numbers in parentheses represent numbers of dead mice/total number of mice used obtained from the TNF lethality experiment. d, Days; w, Weeks.

DISCUSSION

CAR, a high-molecular-weight sulfated polygalactose obtained from a marine plant, is used as a MP blocker because it destabilizes the lysozomal membrane and MP ingesting CAR result in autolysis (7). We are surprised to find that pretreatment with CAR enhanced LPS-induced TNF production and mouse mortality.

When MP activators such as BCG (21) and *C. parvum* (13) were used, a priming interval of at least 4 days was necessary for LPS-induced lethality and TNF production. In our system, mice injected with CAR 2 h before being challenged by LPS showed 70% mortality and the level of TNF was about 1.1×10^4 U/ml (Fig. 5). Our results suggest that an interval of as short as 2 h was enough to enhance LPS-induced TNF production and lethality and that mechanisms other than those involved in priming by MP activators may be involved in the priming by CAR.

Some possible mechanisms are implied by our findings. It is supposed that inflammation is important for the enhancement of TNF production, because i.p. pretreatment with thioglycolate medium also enhances LPS-induced TNF production (16). CAR is used not only as a MP blocker but also as an inflammatory agent. CAR-induced inflammation is accompanied by a variety of hormone production including prostaglandins (29, 30) in addition to superoxide (20). Lipooxygenase products are involved in the synthesis of TNF, because lipooxygenase inhibitors could suppress the formation of TNF in D-galactosamine-treated mice in vivo and in vitro (27). Superoxide may also contribute in enhancing the secretion of TNF by LPS. Reactive oxygen species facilitate LPS-induced release of TNF in vivo and in vitro (8).

The fact that CAR enhanced LPS-induced TNF production evokes interest in TNF-producing cells. Because it has been reported that CAR is selectively toxic to peritoneal MP and Kupffer cells (26), the reticuloendothelial system except for peritoneal MP and Kupffer cells may be activated by inflammatory mediators. Recent in vitro studies demonstrated that TNF is produced by many kinds of cells including T and B lymphocytes (31, 32), natural killer cells (10), and polymorphonuclear leukocytes (40). Immunocompetent cells other than MP may be involved in producing TNF in vivo.

A number of agents can potentiate the lethal effects of LPS in experimental animals, namely, lead acetate (28), D-galactosamine (17), carbon tetrachloride (11), thorium dioxide (3), silica (37), and CAR (2). In our study, mice injected with CAR were 8 times more sensitive to the lethal effects of LPS than normal mice (Fig. 1).

One of the mechanisms by which CAR potentiates LPS-induced lethality is surely an increased production of TNF in the serum of the mice. The level of TNF in CAR-treated mice was significantly higher than that in nontreated mice at all doses of LPS (Fig. 2). TNF has been shown to mediate endotoxin shock (1, 35, 36), since TNF induces mortality with symptoms typical for endotoxin shock and anti-TNF antibodies prevent lethal toxicity caused by LPS (5). Although TNF production was remarkably enhanced in CAR-treated mice, the kinetics of TNF concentration in the sera was almost the same as that in rats, as previously reported (39). TNF concentration reached its peak within 2 h and it was cleared from serum within 6 h after LPS injection (Fig. 3). The mediating function of TNF is restricted to the very early phase of septic shock. Second, coinjection of recombinant human TNF and LPS synergistically caused hemorrhagic necrosis and lethal shock in mice (22). It has been

reported that inhibition of the reticuloendothelial system depresses the rate at which endotoxin is cleared from plasma (9, 19, 23). Synergism between serum TNF and prolonged retention of LPS in blood may increase mouse mortality. Third, the loss of stability of lysosomal membrane of CAR-ingesting MP may be a crucial condition in the pathogenesis of endotoxin shock (2). The contribution of other factors such as interleukin-1 and platelet-activating factor to mortality is under investigation.

In our study, even endotoxin-resistant C3H/HeJ mice with CAR pretreatment produce a low level of TNF in the serum by inducing LPS. C3H/HeJ mice without CAR pretreatment had no detectable TNF in their serum. It has been shown that gamma interferon permits endotoxin-induced production of TNF by C3H/HeJ macrophages in vitro (6, 14). Vogel et al. (38) demonstrated that pretreatment of C3H/HeJ mice with viable BCG organisms 11 days before LPS administration rendered them sensitive to the lethal effects of phenol-extracted LPS (38). They suggested that the T-cell–macrophage system is the major site of action in BCG enhancement of LPS sensitivity. However, our study showed that pretreatment of C3H/HeJ mice with CAR only 24 h before LPS administration was sufficient for the development of LPS-induced TNF in serum.

In summary, our study demonstrated that LPS-induced TNF production and lethality was increased in CAR-pretreated mice. The results made clear that TNF production is enhanced not only by a MP activator but also by a MP blocker. There is no clear understanding regarding the enhancing mechanism of TNF production and the sensitization phenomenon to the lethal effect of endotoxin. Our findings present a new insight into the nature of the priming mechanism of TNF production and endotoxin-induced shock.

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