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Inhibition of a Membrane Complement Regulatory Protein by a Monoclonal Antibody Induces Acute Lethal Shock in Rats Primed with Lipopolysaccharide

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Rats pretreated with traces of LPS developed acute fatal shock syndrome after i.v. administration of a mAb that inhibits the function of a membrane complement regulatory molecule. Such a shock was not observed after the administration of large amounts of LPS instead of the mAb following LPS pretreatment. The lethal response did not occur in rats depleted of either leukocytes or complement, and a C5a receptor antagonist was found to inhibit the reaction. Furthermore, LPS-treated rats did not suffer fatal shock following the injection of cobra venom factor, which activates complement in the fluid phase so extensively as to exhaust complement capacity. Therefore, complement activation on cell membranes is a requirement for this type of acute reaction. *The Journal of Immunology, 1999, 162: 5477–5482.

E ndotoxin shock is characterized by hypotension, hyporeactivity to vasoconstricting agents, inadequate tissue perfusion, vascular damage, and disseminated intravascular coagulation leading to lethal multiple organ failure (1, 2). Many of the pathophysiological events in endotoxemia can be attributed to the Gram-negative bacterial membrane component, LPS. LPS, termed endotoxin, stimulates cells to produce and/or release cytotoxins and inflammatory mediators (3, 4). LPS also has the ability to activate complement (4).

Certain activated complement fragments, particularly C5a, which is known to be a potent anaphylatoxin, are thought to play a role in lowering blood pressure in patients with endotoxemia (4–10). However, fatal shock does not occur via systemic complement activation following the i.v. administration of cobra venom factor (CVF), which forms a stable C3 convertase causing complement activation, resulting in C3 deposition on endothelial cells with certain pathological events (16–19). These events include a mild decrease in blood pressure that was observed following an i.v. injection of the mAb, although the reaction to a single injection of anti-Crry was transient and all rats survived (16). However, when the F(ab′)2 fragment of 5I2 (anti-Crry) was administered to rats pretreated with as little as 0.01 mg/kg LPS (~1/1000 of the lethal dose), the result was acute lethal shock.

Materials and Methods

Animals

Male Wistar rats weighing ~280 g were purchased from Chubu Kagaku Shizai (Nagoya, Japan). The rats were allowed free access to food and water and were maintained on a 12-h light/dark cycle.

Reagents and mAbs

LPS, prepared from phenol extracts of Salmonella typhosa, and cyclophosphamide were obtained from Sigma (St. Louis, MO). Recombinant mouse TNF-α was kindly donated by Drs. Denichi Mizuno and Genichiro Soma (Takano Hospital, Kumamoto, Japan). CVF was purified from lyophilized cobra venom Naja Naja (Sigma) as described previously (16). Nε-2-mercaptomethyl-3-guanidinopropionic acid, a carboxypeptidase inhibitor used to inhibit the inactivation of generated C5a by carboxypeptidases, was obtained from Calbiochem (La Jolla, CA). The production and characterization of 5I2 (anti-rat Crry mAb) and 6D1 (anti-rat CD59 mAb) have been described previously (14, 20). 6D1 was kindly donated by Dr. B. Paul Morgan (University of Wales, Cardiff, U.K.). These mAbs belong to the same subclass (IgG1) and showed similar binding in rat organs (16).

Bacterial endotoxin contamination of the Ab fragments used in the present study was assessed using a specific detection reagent (Pregel-M, Seikagaku Kougyou, Tokyo, Japan) according to the manufacturer’s instructions. Escherichia coli (055:B5 200 pg/1 European unit) was used as a control. The sensitivity of Pregel-M was <50 pg/ml (or 0.25 European units/ml). All stock solutions of F(ab′)2 fragments of 5I2 and 6D1 tested were negative for endotoxins. The levels of endotoxin in serum samples obtained from rats after injection with 0.01 mg/kg LPS were also measured.

Effects of C5a receptor (C5aR) antagonist

The C5aR antagonist used was a hexapeptide, NEpHe-Lys-Pro-dCHe-Trp-dArg (synthesized by the Peptide Institute, Osaka, Japan). This hexapeptide was originally reported to be an antagonist of C5aR on human peripheral neutrophils (21). The effect of this peptide on rat C5aR was...
confirmed by antagonized myeloperoxidase (MPO) release from rat polymorphonuclear leukocytes (PMNs) by zymosan-activated rat serum using a previously described MPO assay (21, 22). Briefly, zymosan-activated serum, which was used as a source of rat C5a, was obtained by incubating fresh rat serum and zymosan A (Sigma) with 1 mg/ml of rt-2-mercaptopropyl-3-guanidinoethylthiopropanoic acid at 37°C for 2 h and then pooling at −75°C until used. Rat PMNs were obtained from normal rats using Polymorphprep (Nyncomed Pharma AS, Oslo, Norway). Rat PMNs were incubated with cytochalasin B (Sigma) for 5 min at 37°C, followed by the addition of the C5aR antagonist or vehicle and 5 min later by either rat zymosan-activated serum or FMLP (Sigma). Inhibition assays were performed at a concentration of zymosan-activated serum or FMLP that caused 60% MPO release from rat PMNs compared with the release from PMNs treated with Triton X-100 (Sigma). Measurements were made of triplicate samples for each concentration of C5aR antagonist used. Inhibition assays were repeated five times using different samples of rat PMNs. MPO release was calculated according to the following formula: MPO release (%) = (OD value at the selected dose of C5aR antagonist/OD value without C5aR antagonist) × 100.

The C5aR antagonist inhibited MPO release from rat PMNs stimulated with zymosan-activated serum in a dose-dependent manner, but did not inhibit release from rat PMNs stimulated with FMLP (see Fig. 5).

Leukocyte counts and serum complement activity

Samples of peripheral blood from the tail vein of rats were collected in tubes containing EDTA. Total leukocytes were counted using a hemocytometer. For the determination of serum complement activity, CH50 (50% complement hemolytic unit) was measured according to the manufacturer’s instructions using sensitized SRBCs (Ishizu Pharmaceutical, Osaka, Japan).

Arterial blood pressure

Animals were anesthetized by an i.p. injection of 50 mg/kg pentobarbital sodium (Abbott Laboratories, North Chicago, IL). Before insertion of a catheter, the baseline of each radiotransmitter (TA11PA-C40, Data Sciences, St. Paul, MN) was verified to be 4 mm Hg. A fluid-filled sensor catheter was placed into the left femoral artery slightly above the aortic bifurcation and was used for the measurement of arterial pressure, which was monitored with a transducer for 60 min after the injection of anti-Crry. Data were expressed as the percent change in mean arterial blood pressure (MBP) compared with the pretreatment value. Values at selected time-points were calculated using the following formula: MBP (%) = ([MBP value at a selected timepoint – MBP value before anti-Crry injection]/ [value of MBP before anti-Crry injection]) × 100. The treatments administered to animals in experimental groups are shown in Tables I and II.

lp;24Histology and immunohistology

Samples of heart, lung, and liver were obtained from selected rats from groups I-1, I-24, II-1, II-24, IV, V, VI, and VII at 5 min after the injection of the F(ab’)2 fragments of S12 (anti-Crry) or 6D1 (anti-CD59). The organs of group III rats were obtained 5 min after the injection of CVF instead of mAb. For histological analysis, the samples were fixed in methacarn and embedded in paraffin. Sections of 3 μm thickness were stained with hematoxylin and eosin. The binding of injected mAbs, deposition of C3, and leukocyte common Ag-positive cells were detected by direct immunofluorescence. Briefly, samples were snap-frozen in liquid nitrogen. Sections of 2 μm thickness were cut on a cryostat and fixed in acetone at room temperature for 10 min. They were then incubated with fluorescein-labeled rabbit Anti mouse IgG1 (Zymed Laboratories, San Francisco, CA) or fluorescein-labeled goat Ab against rat C3. To detect leukocyte infiltration, sections were stained with fluorescein-labeled mAb against rat leukocyte common Ag (OX-1, Dainippon Pharmaceutical, Osaka, Japan).

Statistical analysis

Statistical analysis was performed in a nonparametric manner using the Mann-Whitney U test for unpaired data. A difference between two groups was considered significant when the p value was <0.05 (5%).

Results

Acute lethal shock induced by a mAb in LPS-sensitized rats

The MBP of rats injected with 10 mg/kg LPS dropped by >40% within 1 h of injection, and all of the animals died between 24 and 48 h postinjection. Rats injected i.v. with 0.01 or 1.0 mg/kg LPS showed a minimal change in MBP, and all survived. In the following experiments, rats were sensitized with an i.v. injection of 0.01 mg/kg LPS. This was followed by an i.v. injection 0, 0.5, 1, 4, 24, or 48 h later of 1 mg/kg of the F(ab’)2 fraction of anti-Crry. When rats were injected with anti-Crry 1 h (group I-1), 4 h (group I-4), or 24 h (group I-24) after LPS treatment, MBP progressively decreased; all rats died within 20 min (Table I and Fig. 1A). However, the change in the MBP of rats injected with anti-Crry immediately (group I-0) after LPS administration, was practically the same as that of rats injected with anti-Crry alone (16) (both showing only a transient decrease without any fatal reaction) (Table I and Fig. 1A). The rats of group I-48, which were challenged with anti-Crry 48 h after LPS treatment, showed a prolonged reduction in MBP, although all rats survived (Table I). Among group I-0.5 rats, three of eight survived, and the changes in their MBP were similar to those of group I-0 rats (Table I). An injection of 1 mg/kg of the F(ab’)2 fraction of mAb 6D1 (anti-CD59), which suppresses the function of CD59, did not affect the MBP of rats pretreated with LPS (groups II-1 and II-24; Fig. 1B).

When anti-Crry or anti-CD59 was injected into rats pretreated with LPS, binding was observed mainly along the capillaries of the heart, lung, and liver and was similar to that seen for untreated rats as described previously (16). Deposition of C3 was seen along the heart, lung, and liver and was similar to that seen for untreated rats.
organs were well preserved in all groups of rats. Mild accumulations of inflammatory cells were observed by immunofluorescence using the mAb OX-1, and the inflammatory cells were localized in capillaries of the lung and liver.

To determine the effect of acute complement activation in the fluid phase, as opposed to on cell membranes, the rats of group III were injected i.v. with a bolus of 25 U of CVF at 1 h after the administration of LPS, instead of with anti-Crry. The MBP of the group III rats decreased immediately following the CVF injection and was restored to baseline level within 15 min (Fig. 2). Rats simultaneously injected with 100,000 U/rat of recombinant mouse TNF-α and anti-Crry showed only a mild and transient decrease in MBP (group IV; Fig. 3).

**Effects of systemic complement depletion**

A previous study showed that when rats were injected i.v. with 25 U of CVF, complement activity, as determined by the CH50 level, was undetectable from 24 to 48 h postinjection; no reduction in the number of peripheral leukocytes was observed (23). CVF-treated, complement-depleted rats were injected with 0.01 mg/kg LPS at 1 and 24 h before the i.v. injection of 1 mg/kg anti-Cry. The MBP of these rats did not change after the anti-Cr-γ injection, and all rats survived (group V; Table II and Fig. 4). Deposition of C3 was undetectable in the heart, lung, or liver of the complement-depleted rats, although binding of anti-Crry to these tissues was evident.

**FIGURE 1.** Lethal shock induced by functional suppression of Crry in rats primed with trace amounts of LPS. A. Percent change in MBP when the function of Crry, a membrane complement regulatory protein, was suppressed by the F(ab’)2 fraction of mAb 5I2 in rats injected i.v. with 0.01 mg/kg of LPS before mAb administration. When rats were injected with LPS followed immediately by 5I2 administration (group I-0), transient decreases in MBP were observed and the animals recovered within 60 min. However, all rats pretreated with LPS at 1, 4, or 24 h before 5I2 injection developed severe shock and died within 20 min following administration of the mAb (groups I-1, I-4, and I-24, respectively). B. Percent change in MBP of rats injected with the F(ab’)2 fraction of 6D1 (anti-CD59) after LPS sensitization. Changes in MBP were minimal following anti-CD59 administration at 1 and 24 h after the LPS injection. ■, group II-1; □, group II-24. Data are expressed as means ± SE.

**FIGURE 2.** Effects of complement activation in the fluid phase in rats primed with trace amounts of LPS. The percent change in MBP when CVF was injected as a bolus 1 h after pretreatment with LPS (group III) is shown. MBP decreased immediately following the injection of CVF but was restored to baseline level within 15 min.

**FIGURE 3.** The effects of administration of a large dose of TNF-α in exchange for LPS pretreatment. The percent change in MBP when 100,000 U/rat of TNF-α (rather than LPS) and 5I2 were simultaneously administered (group IV) is shown. The change in MBP was similar to that seen for group I-0 rats. Data are expressed as means ± SE.
Effects of systemic leukocyte depletion

At 4–5 days after an i.p. injection of 200 mg/kg cyclophosphamide, the total number of peripheral leukocytes dropped to <25/mm³, with no change in complement activity (19, 23). When leukocyte-depleted rats received the same LPS and anti-Cry injections as the rats of group I-I, they showed only a mild and transient decrease in MBP after anti-Cry injection, and all survived (group VI; Table II, Fig. 4A). The binding of the injected anti-Cry and deposition of C3 in the leukocyte-depleted animals appeared similar to the results obtained with normal rats (group I-I). However, when the interval between LPS and anti-Cry administration was 24 h, five of six leukocyte-depleted rats died. Four of the rats died within 20 min after anti-Cry injection, one died within 1 h, and the last rat recovered from a severe decrease in MBP and survived.

Effects of C5aR antagonist in acute lethal shock

To determine a possible involvement of C5a in the anti-Cry-induced lethal shock in LPS-treated rats, 3 mg/kg of C5aR antagonist was administered i.v. 30 min before the anti-Cry challenge. When the interval between the administration of LPS and anti-Cry was 1 h, one-forth of the rats were rescued from lethal shock by C5aR antagonist (group VII; Table II). Furthermore, when the interval was 24 h, the change in the MBP was slight and all rats survived (Fig. 5A), although C3 deposition in the heart, lung, and liver was evident.

Discussion

Endotoxin was undetectable in serum samples collected 1 h after an i.v. injection of 0.01 mg/kg LPS. However, an acute lethal reaction with a severe decrease in blood pressure was induced in the LPS-pretreated rats by the administration of the F(ab')² fragment of mAb 5I2 (anti-Cry), which induces transient complement deposition on endothelial cells and blood cells in vivo by suppressing the function of a membrane complement regulatory protein (14, 15). Although this phenomenon reminded us of Schwartzman’s reaction (24), such an acute lethal shock was not induced by administering 0.1, 1, or 10 mg/kg LPS as the challenge injection instead of anti-Cry (data not shown). Although TNF-α production could be induced by 0.01 mg/kg LPS, serum TNF-α levels were only detectable at 30 min, peaked at 60 min, and were again undetectable at 4 h (data not shown). Furthermore, rats injected simultaneously with a large amount of recombinant mouse TNF-α together with anti-Cry showed only a mild and transient decrease in MBP. Therefore, the serum level of TNF-α at the time of anti-Cry injection might not be directly correlated with the severity of the decrease in MBP. We were interested in determining whether pretreatment with TNF-α would sensitize an animal, making it susceptible to lethal shock by the mAb. However, this experiment could not be performed because all of the TNF-α preparations available were slightly contaminated with LPS, which would interfere with the results.

Because there was no MBP change in complement-depleted rats (group V) following anti-Cry injection after priming with LPS, complement activation was considered indispensable for inducing the lethal reaction. When LPS-primed rats were challenged with CVF instead of anti-Cry (group III), only a mild and transient decrease in MBP was observed. Although the complement activation induced by CVF is so profound as to exhaust complement capacity in a body, the reaction occurs in the fluid phase; in contrast, anti-Cry blocks the function of Cry on cell membranes, resulting in complement activation on cell surfaces. Therefore, for induction of the acute lethal reaction, complement activation on cell membranes must be essential. Activation of complement on cell membranes in vivo results in the deposition of complement components such as C3 (16). The deposition of C3 may facilitate cell adhesion to or aggregation of PMNs and macrophages as well as platelets, which have C3 receptors such as CR1, CR2, CR3, and/or CR4 on their cell membranes. This type of cell aggregation might facilitate the release of the factors responsible for the lethal shock reaction.

With a 1-h interval between LPS sensitization and anti-Cry administration, the decrease in the MBP of leukocyte-depleted rats (group VI) was mild and transient after an injection of anti-Cry (Fig. 4A); the value obtained was essentially the same as that for
rats injected with anti-Crry alone. These results indicate that the involvement of LPS-sensitized leukocytes is required for severe MBP decrease and for acute death in addition to complement activation on cell membranes. Only one of six rats pretreated with cyclophosphamide survived lethal shock when the interval between LPS sensitization and anti-Crry administration was 24 h, and significant MBP decrease was observed in all six animals (Fig. 4B). It has been reported that a trace amount of LPS primes and causes an increase in chemoattractant-induced cell surface expression (25, 26). Therefore, it may be possible that during the 24 h, LPS may have stimulated the few remaining leukocytes that survived cyclophosphamide treatment, and these cells may have generated granules in sufficient amounts to cause the lethal shock after anti-Crry administration. Among this group, four rats died within 20 min after anti-Crry injection, one died within 1 h, and one survived. This variation might be a reflection of the amount and/or character of the leukocytes remaining after cyclophosphamide treatment.

C5a has been shown to participate in the mediation of endotoxin shock (5, 6), and its receptors are known to be mainly expressed on leukocytes (22, 27). Because a C5aR antagonist administered 30 min before anti-Crry administration prevented the lethal reaction in rats that had been pretreated for 24 h with LPS (Table II, Fig. 5A), the C5a generated by complement activation on cell membranes by anti-Crry may be playing a role in the lethal shock. The requirement of a ≥1-h interval between LPS sensitization and anti-Crry administration for the induction of acute lethal shock may be related to the up-regulation of C5aR by LPS (28–30). The binding of C5a to circulating neutrophils is enhanced on leukocytes primed with LPS and certain cytokines (27, 31, 32). However, the binding of C5a to the up-regulated C5aR should not be sufficient to induce lethal shock because CVF, which strongly activates complement in vivo, could not induce such a lethal response in the LPS-treated rats. In addition to the generation of C5a through complement activation, the deposition of complement on the membranes of blood cells and endothelium would likely facilitate cell adhesion and aggregation, and would consequently appear to play an essential role in the lethal reaction. Therefore, we further speculate that the following mechanism may be involved in the various types of serious shock syndromes often observed in clinical practice. LPS of bacterial origin may sensitize patients, resulting in the up-regulation of inflammation-related receptors including C5aR (28, 29). Serious shock, such as that involving disseminated intravascular coagulation, could be induced when complement is activated on cell membranes, causing cell adhesion and aggregation via complement receptors on leukocytes and platelets, which could then release inflammatory mediators such as IL-1, TNF-α, IL-12, superoxides, and nitric oxide. Impairment of the membrane complement regulatory proteins demonstrated here by the neutralizing mAb may possibly occur in vivo by some other mechanisms, and may allow for significant complement activity to take place on cell membranes. For example, a transient down-regulation of complement regulatory membrane proteins has been found to occur as a result of viral infection (33–36). Complement activation on membranes could also be induced by LPS adsorbed onto cell surfaces or by excess deposition of immune complexes on cell membranes of the capillary endothelium and blood cells, overpowering the function of membrane inhibitors of complement.

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FIGURE 5. Effects of C5aR antagonist in rat PMNs and in lethal shock. A, Percent change in MBP reflecting the protective effect of C5aR antagonist in the lethal reaction. Transient decreases in MBP (<20%) were observed after the injection of anti-Crry when rats were injected i.v. with 3 mg/kg of C5aR antagonist 23.5 h after the administration of LPS and 30 min before 5I2 injection. All rats survived after the anti-Crry injection. B, Percent change in the release of MPO from 1 × 10⁵ rat PMNs pretreated for 5 min with different concentrations of C5aR antagonist and incubated for 5 min at 37°C with 1 × 10⁻⁸ M fMLP or with 8-fold-diluted zymosan-activated serum. C5aR antagonist suppresses MPO release by rat PMNs stimulated with zymosan-activated serum but does not suppress release by rat PMNs stimulated with fMLP. Triplicate samples for each timepoint were assayed, and each determination was repeated five times. Values are expressed as means ± SE.


