Reduction of Mortality in Endotoxemic Rats Pretreated with Gadolinium Chloride: Relationship to Suppression of Superoxide Production in Liver Macrophages

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Abstract: Possible causes of reductions in mortality in severely endotoxemic rats, brought about by gadolinium chloride (GdCl₃) treatment, was analyzed in terms of the superoxide (O₂⁻-) producing capacity of isolated liver macrophages (Mφs) and in terms of their morphological changes. The addition of GdCl₃ to the culture medium of isolated Mφs from normal rats suppressed their O₂⁻-producing capacity in a dose-dependent manner. The cytoplasm of the Mφs in medium containing more than 1 × 10⁻³ mg/ml of GdCl₃ was not spread or swollen, compared with that of Mφs without GdCl₃. Changes in the O₂⁻-producing capacity of Mφs obtained from endotoxemic rats pretreated with or without GdCl₃ were then analyzed. After two injections of GdCl₃ (5 mg/kg at -24 and -48 hours), sublethal (1 mg/kg) or lethal (10 mg/kg) doses of lipopolysaccharide (LPS) were injected intravenously at 0 h. Without GdCl₃, O₂⁻-production capacity in the sublethal, but not in the lethal dose group, was enhanced. Pretreatment with GdCl₃ reduced the mortality of the endotoxemic rats from 100% to 0%; however, the O₂⁻-production capacity in both the sublethal and lethal groups was suppressed to the same extent. These results suggest that the suppression of O₂⁻-production by GdCl₃ administration in vivo induced reductions in hepatotoxicity and mortality in endotoxemia. Thus, GdCl₃ may be useful for the prevention of severe liver dysfunction induced by postoperative infection.

Key words: Superoxide, Liver macrophage, Gadolinium chloride, Lipopolysaccharide, Endotoxic shock

INTRODUCTION

Macrophages (Mφs) play an important role in the immune system by producing various mediators, such as reactive oxygen intermediates (ROIs), prostaglandins, interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), and others. Hyperactivity of Mφ, however, induces harmful effects in the host and sometimes leads to multiple organ failure (MOF). If the over-activation of Mφs could be controlled, it would be possible to reduce their production of inflammatory cytokines and thus to reduce mortality.

In our preliminary study, we found that pre-administration of gadolinium chloride (GdCl₃), a blocker of phagocytosis in liver Mφs, was effective in reducing the hepatotoxicity of lipopolysaccharide (LPS) and the mortality of lethally endotoxemic rats. However, the endotoxin clearance rate after infusion of a lethal dose of LPS was not disturbed in the GdCl₃-treated animals. Moreover, no significant differences in blood TNF levels were observed between the GdCl₃-treated and the control...
groups, although TNF is considered the prime cytokine in the inflammatory cytokine cascade.\(^8\),\(^9\)

Accordingly, in the present study, we attempted to elucidate the role of GdCl\(_3\) in reducing hepatotoxicity and mortality in lethal endotoxemia. We did this by assessing the superoxide (O\(_2^-\))-producing capacity of isolated liver M\(_\circ\)s obtained from normal and from sublethally and lethally endotoxemic rats pretreated with or without GdCl\(_3\). To determine hepatotoxicity, we examined O\(_2^-\)-anions derived from phagocytic cells, since oxygen-derived radicals generated by phagocytic cells upon activation by soluble and particulate stimuli have been suggested to produce tissue injury during sepsis and endotoxic shock.\(^10\)

**Materials and methods**

**Animals**

Male Sprague-Dawley rats, weighing 210–280 g, were used for the following experiments. Throughout the experimental period, the rats were given food and water ad libitum. The experiment was performed in accordance with Guideline for Animal Experiments, Yamanashi Medical College.

Experiment I: Mortality of lethally endotoxemic rats pretreated with GdCl\(_3\) and analysis of their blood TNF levels.

An intravenous injection of GdCl\(_3\) (gadolinium chloride hexahydrate; Wako Pure Chemicals, Osaka, Japan) (5 mg/ml/kg) or the same amount of saline (for the control rats) was administered at −48 and −24 hours in the tail vein. At 0 hours 10 mg/kg of LPS (lipopolysaccharide from Escherichia coli; 0111: B4 Sigma, St. Louis, Mo, U.S.A.) was injected into the tail vein in both groups, and subsequent mortality was assessed at 24-hour intervals. At 30, 60, 90, 180, and 360 minutes after the injection of LPS, rats were anesthetized with diethyl ether and killed. Blood samples (about 7 ml) were taken from the abdominal aorta for measurement of TNF activity.

**Assay of TNF activity**

TNF activity in the arterial blood was measured with a cytotoxicity assay that employs a TNF-sensitive fibroblast cell line (LP3).\(^10\) Fifty μl of LP3 cell suspension (1 × 10\(^6\) cells/ml) in RPMI 1640 (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) medium supplemented with 10% fetal calf serum (FCS) was plated in 96-well culture plates and incubated overnight to establish a confluent monolayer. On parallel plates, serial dilutions of serum samples were made in complete media containing 2 μg/ml of actinomycin D. Fifty-μg aliquots were transferred to the wells containing the LP3 cells, giving a final concentration of 1 μg/ml of actinomycin D. The microtiter plates were incubated for 14 hours at 37°C in a 5% CO\(_2\)-air mixture. The adherent cell monolayer was stained with crystal violet dissolved in a methanol-water mixture. After incubation, the dye solution was removed by inverting and flicking the plates. The plates were then washed with tap water, and absorbancy was measured with a microplate spectrophotometer equipped with a 570-nm filter (Titertek Multiskan; Flow Laboratories Inc.). TNF units were defined as the reciprocal of the dilution required to kill 50% of the target cells. To confirm that the activity in the samples was indeed TNF-α, we carried out a neutralizing study, using a polyclonal rabbit anti-murine TNF-α antibody. We observed 80% inhibition of TNF activity in this study.

Experiment II: Analysis of the effects of GdCl\(_3\) on O\(_2^-\) production by isolated M\(_\circ\)s, and their morphological changes (Table 1).

Experiment II-1: Analysis of the O\(_2^-\)-producing capacity of isolated liver M\(_\circ\)s from normal rats in relation to the dose of GdCl\(_3\), and their morphological changes.

Liver M\(_\circ\)s were prepared by the Munthe-Kass method with some modification.\(^15\) Rats...
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Table 1. Effects of GdCl₃ on superoxide production by isolated macrophage

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Addition in vitro</th>
<th>Addition in vivo</th>
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<tbody>
<tr>
<td>II-1</td>
<td>GdCl₃</td>
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<tr>
<td>II-2</td>
<td>GdCl₃ and LPS</td>
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<tr>
<td>II-3</td>
<td>GdCl₃</td>
<td>LPS</td>
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<tr>
<td>II-4</td>
<td>GdCl₃ and LPS</td>
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were anesthetized with 5 mg/kg of pentobarbital sodium, and after laparotomy, the liver was perfused through the portal vein with Ca²⁺-Mg²⁺-free Hank’s solution containing 0.5 mM ethylene glycol-bis-N,N,N',N'-tetraacetic acid (EGTA). Subsequently, the liver was perfused with Hank’s solution supplemented with 0.05% collagenase. The liver was then removed and cut into small pieces with scissors. These pieces were incubated at 37°C for 10 minutes with Hank’s solution supplemented with 0.05% collagenase, following which they were passed through stainless steel mesh and then centrifuged for 10 minutes at 1,500 rpm. The supernatant was discarded and the pellet was resuspended in minimal essential medium (MEM) and centrifuged for 2 minutes at 400 rpm. By this procedure, nonparenchymal cells (NPC) remained in suspension. Centrifugation was repeated and the NPC suspension was then recentrifuged for 5 minutes at 1500 rpm. The NPC pellet was suspended in Eagle’s MEM containing 10% FCS, at a concentration of about 5×10⁵ cells/ml. This NPC suspension (1.5 ml) was placed in a 35 mm×10 mm plastic dish and incubated at 37°C under a 5% CO₂ atmosphere for 1 hour. During this period, Mφs adhered to the plastic dish. After 1 hour, the dish was washed with MEM to detach the nonadherent cells, leaving the adherent cells, which were virtually all hepatic Mφs. Next, the NPC were cultured for 24 hours with 1.5 ml MEM (10% FCS) containing GdCl₃·6H₂O (0, 1×10⁻², 1×10⁻³, or 1×10⁻⁴ mg/ml). After 24-hour incubation, the supernatant was removed and the dish was washed with Hank’s solution. These cells were used for measurement of O₂⁻ production and for morphological study. The amount of O₂⁻ released was measured by the method of Babior with some modification. O₂⁻-Dependent cytochrome was determined spectrophotometrically at 550 nm, in the presence or absence of superoxide dismutase (SOD). To activate O₂⁻ production, opsonized zymosan (OZ) was added to the incubating dishes. Final concentrations in Hank’s solution were: cytochrome, 0.06 mM; OZ, 1 mg/ml; and SOD, 0 or 0.03 mg/ml. After 1 hour incubation, the reduced form of cytochrome, ferricytochrome, was measured spectrophotometrically at 550 nm. The production of O₂⁻ was expressed as nm/hour/mg protein. The protein content of the dish was determined by the method of Lowry et al. Morphological changes were estimated under a phase-contrast microscope.

Experiment II-2: Analysis of O₂⁻-producing capacity of isolated liver Mφs stimulated by LPS after 24-hour incubation in culture medium with or without GdCl₃.

Liver Mφs were isolated in the same manner as in experiment II-1. After 24-hour incubation of the Mφs in the culture medium (MEM with 10% FCS) without GdCl₃ (Group A) or with GdCl₃ (Group B), 1 or 1×10⁵ ng/ml of LPS was added to the culture medium in each group (Groups A-1 and A-2, and B-1 and B-2,
Table 2. Incubation conditions of superoxide production assay in experiment II-2

<table>
<thead>
<tr>
<th>Group</th>
<th>Conditions</th>
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<tbody>
<tr>
<td>A: Without GdCl₃</td>
<td>+ LPS (1 ng/ml) (Group A-1) + LPS (1×10⁵ ng/ml) (Group A-2)</td>
</tr>
<tr>
<td>B: With GdCl₃ (1×10⁻³ mg/ml)</td>
<td>+ LPS (1 ng/ml) (Group B-1) + LPS (1×10⁵ ng/ml) (Group B-2)</td>
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Table 3. Incubation conditions of superoxide production assay in experiment II-3

<table>
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<tr>
<th>Group</th>
<th>Conditions</th>
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<tbody>
<tr>
<td>C-1: Without GdCl₃</td>
<td></td>
</tr>
<tr>
<td>C-2: With GdCl₃ (1×10⁻³ mg/ml)</td>
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</tr>
<tr>
<td>D-1: Without GdCl₃</td>
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</tr>
<tr>
<td>D-2: With GdCl₃ (1×10⁻³ mg/ml)</td>
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respectively, and the cells were further incubated for 24 hours (Table 2). Their O₂⁻-producing capacity was analyzed in the same manner as in experiment II-1.

Experiment II-3: Analysis of O₂⁻-producing capacity in isolated liver Møs from endotoxemic rats, incubated with or without GdCl₃, and their morphological changes.

One mg/kg of LPS, for the sublethal endotoxemic model (Group C), or 10 mg/kg of endotoxin for the lethal endotoxemic model (Group D) was injected into the tail vein. Twelve hours later, liver Møs from the models were isolated in the same manner as in experiment II-1. After 24-hour incubation in culture medium with GdCl₃ (1×10⁻³ mg/ml) (Groups C-2 and D-2) or without GdCl₃ (Groups C-1 and D-1), the O₂⁻-producing capacity of and morphological changes in the Møs were analyzed in the same manner as in experiment II-1 (Table 3).

Experiment II-4: Analysis of O₂⁻-producing capacity in isolated liver Møs from GdCl₃-pretreated endotoxemic rats, and their morphological changes.

Intravenous injections of GdCl₃ (5 mg/kg) were administered at −48 and −24 hours, and at 0 hour, 1 mg/kg of LPS (Group E), or 10 mg/kg of LPS (Group F) was injected into the tail vein. Saline was injected into the tail vein for control rats (Group G). Twenty-four hours later, Møs from each model were isolated in the same manner as in experiment II-1. After 24-hour incubation of Møs in MEM with 10% FCS, their O₂⁻-producing capacity and morphological changes were analyzed in the same manner as in experiment II-1.
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Statistical Analysis

Data were expressed as mean±standard deviation (SD). Statistically significant differences between groups were assessed by Student’s t-test and by analysis of variance (ANOVA), and differences were considered significant if p values were less than 0.05.

RESULTS

Experiment I

Mortality

All endotoxemic rats treated with a lethal dose of LPS died within 24 hours. However, all GdCl₃-pretreated rats survived.

TNF activity

There were no significant differences in blood TNF levels between GdCl₃-pretreated and non-treated rats. TNF activity was significantly elevated at 30 minutes and reached its highest level at 60 minutes in both groups, decreasing thereafter (Fig. 1).

Experiment II

Morphology

The cytoplasm of the Møs at concentrations of GdCl₃ higher than 1×10⁻³ mg/ml were not spread or swollen compared with that of controls. Møs incubated with GdCl₃ adhered to plastic dishes to a lesser extent than controls (Fig. 2-A and 2-B). The protein content per culture-dish of the GdCl₃-treated cells decreased to 70% of the control value after 24-hour incubation (data not shown).

The cytoplasm of Møs from sublethally endotoxemic rats was spread significantly. However, the cytoplasm of Møs from lethally endotoxemic animals was spread less to a lesser extent (Fig. 3).

In Møs from endotoxemic rats pretreated with GdCl₃ in vivo, the same morphological changes were observed as in Møs incubated with GdCl₃ in vitro. The cytoplasm of Møs treated with GdCl₃ was not spread or swollen compared with that in non-treated groups. It seemed that these Møs were morphologically inactive as a rest of GdCl₃ treatment (Fig. 4-A, 4-B, and 4-C).

Superoxide production

Experiment II-1

GdCl₃ suppressed the O₂⁻-producing capacity of isolated liver Møs. Significant differences, compared with the control, were observed at concentrations of GdCl₃ higher than 1×10⁻³ mg/ml (p<0.05) (Fig. 5).

Experiment II-2 (Table 2)

The addition of LPS, at concentrations of 1 ng/ml, to the culture medium did not enhance O₂⁻ production compared with the control (Group A-1). On the other hand, the O₂⁻-producing capacity was suppressed by the addition of LPS at concentrations of 1×10⁻³ ng/ml (Group A-2) (p<0.05). The O₂⁻-producing capacity of Møs incubated with GdCl₃ (Groups B-1 and B-2) for 24 hours was suppressed to the same extent as that in Møs without GdCl₃ (Groups A-1 and A-2) (p<0.01) (Fig. 6).

Experiment II-3 (Table 3)

Compared with Møs from control rats, O₂⁻...
Fig. 2. Morphological changes in liver macrophages isolated from normal rats in relation to dose of GdCl₃ ($\times$ 40).
(A) Macrophages from normal rats.
(B) Macrophages from normal rats incubated with GdCl₃ ($1 \times 10^{-3}$ mg/ml).
Fig. 3. Morphological changes in liver macrophages isolated from sublethal endotoxemic rats (× 40).
Fig. 4. Morphological changes in liver macrophages isolated from GdCl₃-pretreated rats (× 40).  
(A) Macrophages from sublethally endotoxemic rats pretreated with GdCl₃ (Group E).  
(B) Macrophages from lethally endotoxemic rats pretreated with GdCl₃ (Group F).  
(C) Morphological changes in liver macrophages isolated from GdCl₃-pretreated rats (Group G).
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Fig. 5. Superoxide-producing capacity of liver macrophages isolated from normal rats in relation to dose of GdCl₃.

Fig. 6. Superoxide-producing capacity of isolated liver macrophages stimulated by LPS after incubation in culture medium with or without GdCl₃.

Control: macrophages from normal rats

Fig. 7. Superoxide-producing capacity of liver macrophages isolated from endotoxemic rats, incubated with or without GdCl₃.

Control: macrophages from normal rats.

production in the sublethally endotoxemic rats (Group C-1) was accelerated, and that in the lethally endotoxemic rats (Group D-1) was suppressed (p<0.05). After 24-hour incubation with GdCl₃ at a concentration of 1×10⁻³ g/ml, O₂⁻ production was suppressed. However, there were no significant differences between GdCl₃-treated and non-treated groups (Groups C-1 versus C-2, and Groups D-1 versus D-2) (Fig. 7).

Experiment II-4 (Table 4)

The O₂⁻ production in isolated liver Mφs from GdCl₃-pretreated endotoxemic rats (Groups E and F) was suppressed compared with that in control rats (Group C) (p<0.05). In groups pretreated with a sublethal dose of LPS (Groups C-1 and E), there was a significant difference between GdCl₃-treated and non-treated rats (p<0.05). However, in the groups pretreated with a lethal dose of LPS,
Fig. 8. Superoxide-producing capacity of liver macrophages isolated from GdCl₃-pre-
treated endotoxemic rats. Control: macrophages from normal rats.

there was no significant difference between the
GdCl₃-treated and non-treated rats (Groups
D-1 and F). The O₂⁻ production in all GdCl₃-
treated groups (Groups E, F, and G) was
similar (Fig. 8).

Discussion

GdCl₃, the chloride of a rare earth metal,
inhibits the attachment phase of Kupffer cells
(KC) and their phagocytosis. All our
endotoxemic rats pretreated with GdCl₃ sur-
vived after the injection of a lethal dose of LPS,
and no hepatocyte necrosis was observed micro-
scopically, while extensive necrosis was
observed in the midzonal area of the lobule in
endotoxemic rats not treated with GdCl₃. We
considered that one cause of the necrosis could
have been TNF, a cytokine which is released by
Mφs in an early inflammatory phase. In these
models, however, there was no differences in
peripheral blood TNF levels between GdCl₃-
treated and non-treated groups. In light of
this result, we are unable to address the
relationship between mortality and circulating
TNF activity during a severe infection, since
we speculate that the presence of circulating
cytokines does not necessarily parallel cytokine
activity. A possible interaction between the
cytokines and their corresponding inhibitors
should be considered; we believe that the
absence of detectable circulating cytokines
does not indicate that cytokines are not being
produced by activated cells. It is possible that
TNF activity in the liver could differ in these
two groups (i.e., gadolinium-treated and non-
treated). Further, we postulated that O₂⁻
anions derived from activated phagocytic cells
also played a role in the hepatocyte necrosis.
Oxygen-derived radicals generated by pha-
gocytic cells that are activated by soluble and
particulate stimuli, have been suggested to
cause tissue injuries during sepsis and
endotoxemia.

Accordingly, the relationship between the
O₂⁻-producing capacity of isolated liver Mφs,
their morphological changes, and the dose of
GdCl₃ were analyzed (experiment II-1). The
addition of GdCl₃ to the culture medium
suppressed O₂⁻ production. It seemed that
Mφs incubated with GdCl₃ were morphologi-
cally inactivated, particularly at concentrations
higher than 1×10⁻⁵ mg/ml since the cytoplasm
of Mφs in GdCl₃-containing medium was not
spread or swollen, compared with that in
medium without GdCl₃.

We studied the O₂⁻-producing capacity of
Mφs that were incubated for 24 hours with
GdCl₃ (1×10⁻³ mg/ml) and stimulated with
LPS for a further 24 hours (experiment II-2).
We found that, although the concentrations of
LPS in the culture medium differed, the
O₂⁻-producing capacity of Mφs treated with
GdCl₃ was suppressed equally, compared with
that of Mφs without GdCl₃. The results of
these experiments in which GdCl₃ was added
in vitro (experiments II-1 and II-2) suggest
that the action of GdCl₃ in reducing mortality is due to its suppressive effect on O₂⁻ production by liver Møs in vivo.

We then examined the O₂⁻-producing capacity of isolated liver Møs from endotoxemic rats pretreated with GdCl₃ (experiment II-4). Compared with the O₂⁻-producing capacity in the control rats, that in the sublethal endotoxemic model (Group C-1) was accelerated. However, in the sublethally endotoxemic rats pretreated with GdCl₃ (Group E), O₂⁻ production was suppressed. Sublethal doses of LPS lead to immigration of phagocytic cells into the liver and activate Møs. These activated Møs release large amounts of O₂⁻. In the lethal model O₂⁻ production was suppressed to same extent with and without GdCl₃ pretreatment. We hypothesized that the Møs of these lethally endotoxemic rats had been so severely damaged by LPS, that they could not release as much as the Møs from the sublethally endotoxemic animals. The O₂⁻ production in all GdCl₃-pretreated rats was suppressed; there were no significant differences in the O₂⁻ production in these groups. It is conceivable that GdCl₃ might also suppress the O₂⁻ production of Møs in vivo; this suppression might be related to the blocking of receptor-mediated phagocytosis and the suppression of phagocytic cell function.

The reduced number of cells adhering to the culture dish and the protein content in the dish after 24-hour incubation with GdCl₃ indicate that GdCl₃ may be cytotoxic. Liver Møs have been classified into several types, all of which appeared to be present in the Møs prepared by our method. It has been reported that large KC are more vulnerable than small KC and spleen Møs. Thus, we speculated that the reduced number of phagocytic cells adhering to the dish was due to the elimination of large Møs by GdCl₃. When rats were treated with GdCl₃, large KC in the liver disappeared at an early stage after GdCl₃ injection. We considered that GdCl₃ temporarily suppressed the negative effects of the over-activation of Møs both by reducing the total number of Møs in the liver and by suppressing their function; hepatocyte functions would thus be protected during severe infection. Therefore, GdCl₃ may be useful of value in preventing severe liver dysfunction following infection.

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