Functional Heterogeneity of the Kupffer Cell Population Is Involved in the Mechanism of Gadolinium Chloride in Rats Administered Endotoxin

Hiroshi Kono, Hideki Fujii, Masami Asakawa, Masayuki Yamamoto,* Akira Maki, Masanori Matsuda, Ivan Rusyn,† and Yoshiro Matsumoto

First Department of Surgery, Yamanashi Medical University, Yamanashi 409-3898, Japan; *Department of Surgery, Shinko Byoin Hospital, Hyogo, Japan; and †Department of Environmental Sciences and Engineering, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

Submitted for publication December 26, 2001; published online July 2, 2002

Background. The purpose of this study was to determine if evidence of functional heterogeneity between subtypes of the Kupffer cell (KC) may be involved in the mechanism of the protective effect of gadolinium chloride (GdCl₃) in endotoxemia.

Methods. Rats pretreated with or without GdCl₃ were administered lipopolysaccharide (LPS) or vehicle. Serum and liver tissues were collected after LPS administration for cytokine measurements and pathological and immunohistochemical evaluation.

Results. After LPS administration, increases in expression of TNF-α and IL-6 mRNA in the liver were blunted significantly by GdCl₃. In control liver tissue, ED2-positive cells were a predominant fraction, with a few ED1-positive cells, and GdCl₃ eliminated only ED2-positive cells. Further, ED2-positive cells were larger in size than ED1-positive ones. Importantly, the number of ED1-positive cells in the liver was increased about threefold in the control group but not in the GdCl₃ group after LPS injection. Intermediate or large KCs isolated by counterflow centrifugal elutriation showed greater capacity for phagocytosis and production of superoxide and TNF-α than small ones. In contrast, IL-6 production was increased to a greater extent in small than in intermediate or large cells. GdCl₃ eliminated the intermediate or large KC subpopulation predominately.

Conclusion. Collectively, functional heterogeneity of the KC population was involved in the mechanism of the protective effects of GdCl₃ in endotoxemia. TNF-α derived from activated intermediate or large KCs may activate small KCs and the latter may be recruited to other organs, such as lungs and kidneys, and produce a large amount of IL-6, leading to multiple organ failure. © 2002 Elsevier Science (USA)

Key Words: Kupffer cell; heterogeneity; IL-6; TNF-α; endotoxemia.

INTRODUCTION

Systemic infection that involves gram-negative bacteria results in development of endotoxin shock. Endotoxin, also known as lipopolysaccharide (LPS), is a component of the outer wall of gram-negative bacteria and is released from the gut or inflammatory foci and is cleared by the reticuloendothelial system. Kupffer cells, the resident macrophages in the liver, remove most of the endotoxin but may become activated in this process [1, 2]. Upon activation, Kupffer cells are known to release pathologically active substances such as eicosanoids, inflammatory cytokines [e.g., interleukin (IL)-6 and tumor necrosis factor (TNF)-α], superoxide anion, and nitric oxide, all of which participate in the process of development of septic shock [3–5]. Collectively, Kupffer cells play an important role in inflammation and immune response [6, 7].

Inhibition of Kupffer cells by gadolinium chloride (GdCl₃), a compound that is toxic to Kupffer cells, prevented liver injury and mortality due to endotoxin nearly completely in both normal [3] and hepatectomized rats [8]. It has been suggested that two morphological subpopulations of Kupffer cells, the “large” and “small” cells, exist in the liver based on these studies with GdCl₃ [9]. Also, GdCl₃ was shown to affect function of Kupffer cells as it inhibits expression of TNF-α.

1 This work is supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

2 Abbreviations used: tumor necrosis factor-alpha, TNF-α; interleukin-6, IL-6; lipopolysaccharide, LPS; alanine aminotransferase, ALT.
mRNA in the liver [10]. Further, basal superoxide production was greater in large Kupffer cells compared to that of the small cell, and the number of small Kupffer cells was shown to increase in the liver following injection of LPS [8, 11]. These results suggest that the response of these two cell subtypes to LPS administration may differ. The purpose of this study was to determine if evidence of functional heterogeneity between subtypes of the Kupffer cell may be involved in the mechanism of the protective effect of GdCl₃ in rats administered LPS.

MATERIALS AND METHODS

Animals and treatments. Male Sprague-Dawley rats (190–200 g body weight) were used in these experiments. The experimental protocol followed the institutional and the National Research Council’s criteria for the care and use of laboratory animals in research. All animals received humane care in compliance with institutional guidelines. Rats were given food and water ad libitum during this study. Animals were divided into four groups (n = 6 in each group): Group I—control (saline vehicle treatment only), Group II—GdCl₃ (10 mg/kg; Wako Pure Chemical Industries, Osaka, Japan), Group III—LPS (10 mg/kg: Escherichia coli serotype 011:B4; Sigma, St. Louis, MO), and Group IV—GdCl₃ + LPS. Both GdCl₃ and LPS were diluted in sterile saline (vehicle) and administered via the tail vein. Control rats and rats given one compound only received an injection of equal volume of the vehicle. GdCl₃ was administered 24 h prior to LPS treatment.

Blood sampling. In some experiments, blood samples were collected from the aorta 9 h after LPS or saline injections and centrifuged at 1200g for 10 min at 4°C (n = 6 in each group). Serum was stored at −80°C for further assays of activity of alanine aminotransferase (ALT). ALT was measured by standard enzymatic procedures using commercially available kits (Nissui Transnase; Nissui Pharmaceutical Co., Tokyo, Japan) [8].

Pathological evaluation and immunohistochemistry. Liver tissue samples were collected from rats sacrificed 9 h after injection of LPS or vehicle, fixed in formalin, embedded in paraffin, and serially sectioned (5 μm thick). Some sections were stained with hematoxylin–eosin to assess inflammation and necrosis. Others were used for the immunohistochemistry using ED1 and ED2 monoclonal antibodies and the Labeled Streptavidin Biotin kit (Dako, Carpinteria, CA). The antibodies used were ED1, specific for blood macrophages/monocytes [9, 12] and dendritic cells but not granulocytes, and ED2, for Kupffer cells [9, 13] (Serotec, Oxford, UK). Immunohistochemistry was performed as follows. Liver sections were fixed in acetone for 5 min and covered with 0.5% hydrogen peroxide in methanol to block endogenous peroxidase. The sections were first incubated with normal goat serum, then with primary diluted antibodies (1:200 for ED1 and 1:100 for ED2), a secondary antibody composed of biotinylated goat anti-rabbit and mouse immunoglobulins, and finally peroxidase-incorporated streptavidin. The sections were developed with 3,3'-diaminobenzidine and counterstained with hematoxylin. The number of ED1- and ED2-positive cells per 100 hepatocytes was counted in five different low-power fields.

Kupffer cell isolation by counterflow centrifugal elutriation. Nonparenchymal cells were isolated from rats given saline or GdCl₃, by collagenase digestion and differential centrifugation using Nycodenz (Nycomed Pharma AS, Oslo, Norway) as described elsewhere [14, 15]. Counterflow centrifugal elutriation (Hitachi CR 21G centrifuge and RSE elutriator rotor) at constant speed (3250 rpm) and different flow rates was used for the separation of the nonparenchymal cells based on cell size and density [15]. Endothelial cells were obtained at 25 ml/min flow rate. Small Kupffer cells (95%) and some endothelial cells (5%) were coelutrated at 32 ml/min flow rate, intermediate Kupffer cells (99% purity) were collected at 40 and 50 ml/min, and large Kupffer cells (99% purity) were collected at 60 and 70 ml/min. Kupffer cell purity and viability were above 95% as determined by peroxidase and Giemsa stains and trypan blue exclusion. Cells were cultured for 24 h and used for measurement of production of superoxide, TNF-α, and IL-6 as well as phagocytotic index.

Phagocytic index of the Kupffer cell. Isolated Kupffer cells (10⁶ cells per culture dish, n = 6 in each group) were plated in 35-mm culture dishes on coverslips in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO Laboratories Life Technology, Grand Island, NY) supplemented with 10% fetal calf serum and antibiotics. Fluorescein isothiocyanate (FITC)-labeled latex beads (1 μm; Polysciences, Warminster, PA) were added to each culture dish and cells were incubated for 5 min at 37°C and washed three times with phosphate-buffered saline and fluorescence was determined in situ with a Universal Imaging Corp. Image-1AT image acquisition and analysis system (Chester, PA) incorporating an Axioskop 50 microscope (Carl Zeiss, Thornwood, NY). The same microscopic field was photographed in normal light or with FITC filter. The number of FITC-labeled beads phagocyotised by each cell was counted in 10 different microscopic fields per dish to assess function of phagocytosis.

Real-time reverse transcription polymerase chain reaction for TNF-α and IL-6 mRNA in liver tissue. Messenger RNAs (mRNAs) were quantified by a real-time reverse transcription polymerase chain reaction (RT-PCR) procedure (TaqMan; Applied Biosystems, Foster City, CA). Real-time PCR was performed by the GeneAmp 5700 Sequence Detection System (PE Biosystem, Foster City, CA). The sequences of the specific primers for TNF-α and IL-6 (Pre-Designed TaqMan Assay Reagents; Applied Biosystems) were TNF-α 5’; CACAGTTCTCTTCAAGGGACAA, and 3’; ATGGCAAGAGGAGCTGACT; IL-6 5’; CCA AACTCAATGCTCTCTATG, and 3’; TTTCTGCGATAGACTCATTAGA. Ribosomal RNA (18S) was used as an internal control.

Liver tissue samples were collected from rats sacrificed at 1 h for TNF-α or 4 h for IL-6 mRNA assay after injection of LPS or vehicle. Total RNA was isolated from pieces of about 25 mg of liver tissue by the use of a RNA purification kit (Qiagen GmbH, Hilden, Germany). Reverse transcription of total RNA (2 μg) was performed in a final volume of 100 μl containing 1× TaqMan RT buffer, 5.5 mM MgCl₂, 500 μM each dNTP, 2.5 μM random hexamers, 0.4 U/μl RNase inhibitor, and 1.25 U/μl Multiscribe reverse transcriptase from the GeneAmp PCR System 9700 (PE Biosystem). Two microtits of cDNA sample was used for quantitative RT-PCR (a 10-min step at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C) in the presence or absence of bovine erythrocyte superoxide dismutase reduction was determined by measuring absorbance at 366 nm in the presence of specific forward and reverse primers and TaqMan Universal PCR Master M1x (PE Biosystem). Messenger RNA levels were calculated using the comparative Cᵗ method [16] and normalization to 18S ribosomal RNA. To confirm amplification specificity, PCR products were subjected to a melting-curve analysis. Quantification is reported as the x-fold difference relative to a calibrator cDNA from saline-treated rats. Because inflammatory cytokines were not detectable in liver tissues from saline-treated animals, signals that exceeded a 1Ct of 15 were arbitrarily set to 1-fold to enable a calibration. The relation to this arbitrary 1-fold is represented by the data described as "x-fold increase in mRNA.”

Superoxide production by isolated Kupffer cells. Superoxide production was measured as reduction of cytochrome c as described elsewhere [17]. Superoxide-dependent cytochrome c (type IV; Sigma) reduction was determined by measuring absorbance at 366 nm in the presence or absence of bovine erythrocyte superoxide dismutase (SOD; Wako). After 24 h incubation, culture dishes were washed with Hanks’ buffer. LPS (10 μg; Sigma) was added to each culture dish. The final concentrations were cytochrome c—0.06 mM, LPS—1 μg/ml, and SOD—0 or 0.03 mg/ml. After incubation for 1 h at 37°C in 5% CO₂, reduced cytochrome c was measured spectrophotometrically by measuring absorbance at 550 nm in triplicate. Protein con-
tent in culture dishes was determined by the method of Lowry et al. [18]. Superoxide production was presented as nmol/5 \times 10^5 \text{cells/h}.

TNF-\alpha and IL-6 production by isolated Kupffer cells. Kupffer cells were seeded onto 24-well plates and cultured in DMEM supplemented with 10% FBS and antibiotics at 37°C in the presence of 5% CO2. Cells were incubated with fresh medium containing LPS (10 \mu g/ml) or rat recombinant TNF-\alpha (0.1 ng/ml; Cosmo Bio, Tokyo, Japan) supplemented with 5% rat serum for an additional 4 h. Medium was collected and kept at 80°C until assay. TNF-\alpha and IL-6 were measured in the culture medium using an enzyme-linked immunosorbent assay kit (Cosmo Bio) and data were corrected for dilution according to the manufacturer's instructions.

Statistical analysis. ANOVA or Student's t test was used for determination of statistical significance where appropriate. A P value less than 0.05 was selected before the study as the level of significance.

RESULTS

Effect of LPS and GdCl3 on ALT Levels and Mortality

All rats that had not received GdCl3 but were administered 10 mg/kg LPS appeared moribund 9 h after LPS injection and died within 24 h after injection (Table 1). Mortality was prevented completely by GdCl3. Serum ALT levels were about 30 IU/L before LPS administration and GdCl3 alone had no effect. In contrast, ALT levels were increased to about 2000 IU/L 9 h after LPS administration in the control group. Importantly, GdCl3 pretreatment blunted this increase by about 90%.

Pathological Evaluation in the Liver

No pathological changes were observed in liver tissues before LPS administration (Figs. 1A and 1B). In contrast, inflammation, focal necrosis, and hemorrhagic change were observed 9 h after LPS administration in the control group (Fig. 1C). GdCl3 prevented these pathological changes nearly completely (Fig. 1D).

Expression of TNF-\alpha and IL-6 mRNA in the Liver

Expression of TNF-\alpha and IL-6 mRNA was not detectable in the liver before LPS administration (data not shown). In contrast, LPS administration significantly increased these expressions in both groups; however, inductions were about four times greater in rats given saline vehicle than in those given GdCl3 (Fig. 2).

Immunohistochemical Localization of ED1- and ED2-Positive Cells

In further experiments, we assessed the effect of GdCl3 and LPS on the Kupffer cell population by immunohistochemistry with ED1 and ED2 monoclonal antibodies.
antibodies (Fig. 3). Both ED1- and ED2-positive cells were detected in livers from rats given vehicle. ED2-positive cells were located mainly around the periporal area in control rats; however, no ED2-positive cells were detected in rats pretreated with GdCl3 (Table 1). Further, ED2-positive cells were larger in size than ED1-positive ones. Importantly, GdCl3 did not affect the number of ED1-positive cells. Administration of LPS did not increase the number of ED2-positive cells. In contrast, the number of ED1-positive cells was increased about threefold in liver tissues from rats given vehicle but not GdCl3 after LPS administration (Table 1).

Photographs of Isolated Kupffer Cells and Phagocytotic Index

On normal-light photographs (No filter in Fig. 4), Kupffer cells that were isolated from control rats were mainly intermediate or large Kupffer cells, with a few small Kupffer cells; small Kupffer cells were present mainly in isolates from rats given GdCl3 (Fig. 4B). Furthermore, Kupffer cells from control rats took up more fluorescent beads than cells from rats given GdCl3 (FITC filter).

To study this in greater detail, Kupffer cells were isolated from the control group by counterflow centrifugal elutriation. Isolated Kupffer cells were divided into three subtypes based on cell size and density: small, intermediate, and large. On normal-light photographs, the smallest Kupffer cell subtype was observed in the fraction at 32 ml/min flow rate as expected (Fig. 4C). Cell size increased in a flow-rate-dependent manner. Furthermore, small Kupffer cells took up few fluorescent beads (Figs. 4C). Although intermediate or large cells took up a lot of fluorescent beads, intermediate Kupffer cells took about three times more beads than large ones (Figs. 4D and 4E). Thus, intermediate or large Kupffer cells have greater phagocytotic capacity than small ones.

Effect of GdCl3 on the Number of Isolated Kupffer Cells

The numbers of isolated Kupffer cells from control rats were $10.8 \pm 0.8 \times 10^6$ in the small Kupffer cell
fraction, $30.3 \pm 4.8 \times 10^6$ in the intermediate Kupffer cell fraction, and $3.9 \pm 1.0 \times 10^6$ in the large Kupffer cell fraction (Fig. 5). GdCl$_3$ significantly reduced the number of intermediate or large Kupffer cells but not small Kupffer cells.

Production of Superoxide, TNF-$\alpha$, and IL-6 by Isolated Kupffer Cells

To test the functional capacity of different Kupffer cell subpopulations, cells were isolated from control rats and superoxide, TNF-$\alpha$, and IL-6 production was measured. This production was not detected without LPS or TNF-$\alpha$ administration (data not shown). In contrast, addition of LPS resulted in increased superoxide and TNF-$\alpha$ among the groups studied; however, values were about two to five times greater in the intermediate or the large Kupffer cells compared to the small Kupffer cells (Fig. 6). Furthermore, addition of TNF-$\alpha$ led to increased IL-6 production in all groups. Significantly, values were about two to three times greater in the small Kupffer cell fraction compared to the others.

DISCUSSION

Mechanism of Elimination of Kupffer Cells by GdCl$_3$

The exact mechanism of the effect of GdCl$_3$ on the Kupffer cell remains unclear. Koudstaal et al. have suggested that GdCl$_3$ injected intravenously forms colloidal aggregates in the systemic circulation at pH above 6.0 [19]. The presence of GdCl$_3$ in vacuoles of some remaining liver macrophages was detected 1 day after GdCl$_3$ injection. Because the colloidal GdCl$_3$ aggregates will dissolve again at pH below 6.0, GdCl$_3$ ions originating from the complex at the acidic pH of the endosomal-lysosomal compartment of the cell will attach to components of this compartment. Recycling of endosomes to the plasma membrane may gradually change this membrane, finally resulting in cellular disintegration. Plasma membrane changes such as loss of glycosyl receptors and ED2 positivity occur in all hepatic macrophage fractions. However, aggregates of gadolinium are taken up mainly by large or intermediate Kupffer cells and to a lesser extent by the small Kupffer cell subpopulation. Because of their phagocytic capacity, large Kupffer cells are more vulnerable.
FIG. 5. Effect of GdCl₃ on the number of isolated Kupffer cells. Kupffer cells were isolated from rats given saline or GdCl₃ by counterflow centrifugal elutriation as described under Materials and Methods. The number of isolated Kupffer cells in each fraction was counted (n = 5). Vehicle, VEH. Small, small Kupffer cells; Intermediate, intermediate Kupffer cells; Large, large Kupffer cells. *P < 0.05 compared with VEH by ANOVA with Bonferroni’s post hoc test.

Indeed, in this study we observed that the number of fluorescent beads phagocytosed was greater in intermediate or large Kupffer cells than in small ones (Fig. 4). Further, GdCl₃ treatment reduced the number of isolated Kupffer cells in the intermediate or large cell fractions predominantly (Fig. 5). Thus, these data support the hypothesis that intermediate or large Kupffer cells take colloidal forms of GdCl₃ more freely than small Kupffer cells, which leads to the predominant elimination of the former.

Heterogeneity of the Kupffer Cell Population

Morphological heterogeneity of the Kupffer cell population, large and small cells, was reported first by Hardonk et al. [9]. In this study, immunohistochemical analysis revealed that both ED1- and ED2-positive cells are normally present in the rat liver and that ED2-positive cells are larger in size than ED1-positive ones (Fig. 3). Furthermore, Kupffer cells isolated from control rats existed as small, intermediate, and large cells (Figs. 4 and 5). Collectively, our data support the fact of morphological heterogeneity in Kupffer cell populations. Importantly, functional heterogeneity between these subtypes was also reported in several studies [20–22]. To study this in greater detail, production of superoxide, TNF-α, and IL-6 was determined in vitro. Production of superoxide and TNF-α was greater in intermediate or large Kupffer cells than in small ones, confirming previous work (Figs. 6A and 6B) [20, 21]. In contrast, production of IL-6 was greater in small cells (Fig. 6C). Furthermore, intermediate or large Kupffer cells showed greater phagocytic capacity than small cells (Fig. 4). This heterogeneity could be involved in the pathophysiology of endotoxemia.

Mechanisms of Effects of GdCl₃ in Endotoxemia

It has been observed here that intermediate or large Kupffer cells produced greater amounts of superoxide (Fig. 6A). Superoxide may act as a signaling molecule to activate transcription factor NF-κB and increase TNF-α production [23] that ultimately leads to LPS-induced liver injury [8, 24, 25]. Indeed, GdCl₃ reduced production of superoxide and TNF-α derived from intermediate or large Kupffer cells (Fig. 6B). Further, GdCl₃ also blunted increases in TNF-α mRNA expression in the liver caused by LPS administration (Fig. 2). This further suggests that intermediate and large Kupffer cells are the major subpopulations that are involved in LPS-induced liver injury. TNF-α signals further increase expression of adhesion molecules such as ICAM-1 and chemokines such as MIP, which leads to an increase in the number of infiltrating inflammatory cells into the liver [26]. Indeed, the number of ED-1-positive Kupffer cells was increased about threefold in the liver after LPS administration (Table 1). Since small Kupffer cells produced greater amounts of IL-6 in response to LPS than intermediate or large Kupffer cells (Fig. 6C), infiltrating hepatic macrophages, which are identified as small Kupffer cells, are also involved in manifestation of liver injury. Thus, the protective effect of GdCl₃ is mainly an elimination of intermediate or large Kupffer cell populations, which leads to decreases in cytokine production in endotoxemia.

Possible Mechanisms of Multiple Organ Failure in Endotoxemia

A variety of pathophysiological responses in various tissues and organ systems occur during endotoxemia. In particular, circulatory failure, leukocyte-induced tissue injury, and activation of coagulation systems appear to be critical determinants in the development of sequential organ failure [27]. A number of mediators derived from host cells such as neutrophils, monocytes, and macrophages are responsible for most of the manifestations of endotoxemia. Indeed, proinflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-8, IL-12, and interferon-γ, play a critical role in the inflammatory responses [28]. Further, lipid mediators, such as platelet activating factor, prostaglandins, thromboxanes, and leukotrienes, also exert a variety of effects in endotoxemia. On the other hand, the enhanced generation of free radicals also induces tissue injury directly and indirectly. The production of superoxide (O₂⁻) is the most prominent precursor of all reactive oxygen species (ROSs). The production of O₂⁻ by neutrophils and
Macrophages is mainly attributed to membrane-associated NADPH oxidase. Most $O_2$ is rapidly converted to $H_2O_2$, which can be a source of hydroxyl radicals. Importantly, these ROSs can activate transcriptional factors, such as nuclear factor-κB and activator protein 1, which promote gene expression of the proinflammatory mediators mentioned above [23]. Furthermore, nitric oxide is now known to induce a variety of responses in addition to hypotension and subsequent tissue hypoxia [29]. Collectively, a very complex condition comprising a large variety of local and systemic inflammatory responses is involved in the mechanisms of multiple organ failure in endotoxemia.

It is known that IL-6 levels are increased continuously in patients with septic shock or multiple organ failure [30, 31]. This suggests that IL-6 is involved in pathophysiology of endotoxemia. Since GdCl$_3$ pretreatment diminished an increase in IL-6 mRNA expression in the liver (Fig. 2) and serum IL-6 levels (data not shown) following LPS injection, it appears that IL-6 levels correlate with a degree of pathophysiology in endotoxemia.
the inflammatory response of the immune system. TNF-α has local pathophysiological effects in the early phase of endotoxemia and, on the other hand, IL-6 acts as a systemic inflammatory cytokine causing multiple organ failure.

**REFERENCES**


Small Kupffer cells have a much greater capacity to produce IL-6. Furthermore, the number of the ED-1-positive Kupffer cells was increased significantly in the lung (data not shown) after LPS injection. Importantly, these increases and organ injuries such as the lung and the intestine were all prevented by elimination of intermediate or large Kupffer cells (data not shown), suggesting that interaction between the intermediate or large Kupffer cell and the small Kupffer cell is a pivotal event in developing multiple organ failures. Abbreviations: KC, Kupffer cells; MOF, multiple organ failure.

**FIG. 7.** Working hypothesis. TNF-α derived from intermediate or large KCs could activate small KCs and the latter may be recruited to other organs such as lungs and the intestine and produce large amounts of IL-6, leading to organ injury [6] (see Fig. 7). Collectively, we suggest that the liver is the central organ in the inflammatory response of the immune system. TNF-α has local pathophysiological effects in the early phase of endotoxemia and, on the other hand, IL-6 acts as a systemic inflammatory cytokine causing multiple organ failure.


