Lipopolysaccharide (LPS) Processing by Kupffer Cells Releases a Modified LPS with Increased Hepatocyte Binding and Decreased Tumor Necrosis Factor-α Stimulatory Capacity (43521)

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Abstract. **Normal physiological clearance of gut-derived endotoxin lipopolysaccharide [LPS] has been described previously; initially, there is uptake by Kupffer cells (KC), then release of modified LPS, followed by hepatocyte uptake. Previous work in our laboratories indicated that LPS is structurally modified with loss of carbohydrate prior to its release by KC. In this study, we functionally characterize KC modified LPS. KC-modified ^{125}I-LPS was prepared from primary rat KC. *Escherichia coli* 0127:B8 native ^{125}I-LPS or KC-modified ^{125}I-LPS (40 ng) was incubated for 1 hr with 1 × 10E6 primary hepatocytes. The binding of KC-modified LPS was 4.33-fold higher than native LPS ($P = 0.0024$). Binding analysis studies were conducted to determine the region of KC-modified LPS responsible for enhanced hepatocyte binding. KC-modified *Salmonella minnesota* LPS was competed with 100-fold excess native or mutant (Ra, Rc, Rd, or Re) strains of LPS or Lipid A with no decrease to hepatocyte binding. *S. minnesota*-native ^{125}I-LPS was compared with KC-modified ^{125}I-LPS in a study to assess induction of tumor necrosis factor (TNF)-γ by rat peritoneal macrophages. Native or KC-modified ^{125}I-LPS (100 ng) was presented to 1 × 10E7 peritoneal macrophages for 6 hr. TNF-α was measured in supernatants using the WEHI-164 cytotoxicity assay. Native LPS induced 5.7-fold higher TNF-α levels than KC-modified LPS ($P < 0.0001$). The above data suggest that structural alterations in KC-modified LPS are accompanied by functional alterations resulting in enhanced hepatocyte binding and decreased TNF-α release. The latter result implies that an early step in LPS detoxification occurs in the KC in which LPS is modified to prevent elicitation of biologically active cytokines.** [P.S.E.B.M. 1993, Vol 202]

Previous studies on the clearance of lipopolysaccharides (endotoxins [LPS]) by the liver have shown the involvement of both Kupffer cells, the tissue-fixed macrophages lining hepatic sinusoids, and hepatocytes (1–3). Initially, there is uptake of LPS from the portal blood by Kupffer cells (KC), then release of modified LPS back into the sinusoid, which our laboratories have shown is structurally altered by the loss of carbohydrate (4, 5). The initial involvement of KC in LPS clearance and their role in structurally modifying LPS (4, 5) suggested to us that KC may facilitate hepatocyte uptake of LPS. Such a mechanism is similar to our previous study involving the glycoprotein carcinoembryonic antigen that initially is cleared by KC, structurally modified and released by KC, and then picked up by hepatocytes (6). Therefore, we examined the possibility that KC-mediated structural alterations to LPS allow for enhanced hepatocyte binding by KC-modified LPS. Competitive binding studies aimed at understanding the domain of LPS responsible for hepatocyte binding were also conducted using *Salmonella minnesota* wild-type and mutant strains of LPS, and Lipid A.

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The concept that hepatic LPS clearance represents a "detoxification" process was also investigated. LPS are capable of effecting biological activities *in vivo* due to their ability to stimulate the release of a wide array of cytokines which in turn act as effectors (7–12). Among these cytokines is tumor necrosis factor (TNF)-α, a 17-kDa nonglycosylated protein (13) that is released by macrophages and monocytes (14, 15) in response to LPS. TNF-α is capable of many *in vivo* effects, including the induction of fever (16, 17), hypotensive shock (18–20), cachexia (21), renal tubular necrosis (18), hepatic (20, 22) and pulmonary (18) dysfunction, mesenteric ischemia (18), and the release of acute phase reactants (23). TNF-α also induces the release of the cytokines interleukin (IL) 1 and IL-6. Many of the *in vivo* effects of IL-1 and IL-6 are similar to those of TNF-α (16, 24). IL-1 and IL-6 are also released by monocytes and macrophages in response to LPS (9, 11, 12). TNF-α, though, may in fact be the principal mediator of the LPS response, since passive transfer of anti-TNF-α antibodies protects animals exposed to lethal doses of LPS (25).

Impairment of the normal hepatic clearance of LPS may contribute to systemic "TNF-αemia." In a recent study by this laboratory, systemic TNF-αemia was demonstrated in acute viral hepatitis patients (26). In contrast, TNF-αemia was not seen in healthy controls. Interestingly, a correlation between TNF-αemia and endotoxemia could be demonstrated in acute viral hepatitis patients (26); this supports the concept that macrophages and monocytes may be releasing TNF-α in response to LPS. Systemic TNF-αemias have also been shown in other hepatic disorders, including alcoholic hepatitis (27) and fulminant hepatic failures (28), and in volunteers subjected to intravenous administration of *Escherichia coli* LPS (29).

The studies reported here suggest that normal hepatic LPS clearance represents a detoxification process wherein Kupffer cell-mediated structural alterations of LPS allow for enhanced hepatocyte binding while preventing release of the biologically active cytokine TNF-α.

Animals

Male Sprague-Dawley rats (Charles River Laboratories) weighing 500–750 g were used for liver cell isolations. Animals were housed in a temperature- and light-controlled environment and were fed *ad libitum* an AIN-76 semisynthetic diet supplemented with 5% coconut oil (30). Animals were maintained in accordance with standards of care and use recommended by the National Institutes of Health.

Liver Cell Preparations. Rats were fasted overnight and anesthetized with sodium pentobarbital (25 mg/kg), and anesthesia was maintained by ether cone. Upon loss of eye blink reflex, 500 units of sodium heparin (Sigma Chemical Co.) were administered via femoral vein injection. Using aseptic surgical procedures, the peritoneal cavity was exposed and an 18-gauge angiocatheter (Becton Dickinson) was inserted and secured with cotton suture into the portal vein. The vena cava was severed and 100 ml of Ca^{2+} free buffer (0.01 *M* HEPES with 0.83% NaCl and 0.05% KCl at pH 7.4) were perfused via the angiocatheter to exsanguinate the liver (4, 5). With the angiocatheter still secured in the portal vein, the liver was removed from the rat and perfused *ex vivo* with preaerated 0.05% collagenase (Types II and IV) buffer (0.01 *M* HEPES with 0.39% NaCl, 0.05% KCl, and 0.05 *M* CaCl2 at pH 7.6) and incubated for 2 min at 37°C. The liver was minced and incubated at 37°C for 45 min with 0.05% collagenase buffer. The resulting cell slurry was pelleted 10 min at 400*g* and incubated with fresh collagenase buffer for an additional 20 min. The cell suspension was then strained aseptically through 4 × 4 sterile gauze.

Hepatocyte Isolations. Using the cell suspension described above, hepatocytes were isolated by centrifugation at low speed (50*g*) for 10 min. The pellet was resuspended in Williams E media (Gibco) supplemented with penicillin, streptomycin, glutamine and 200 mU/ml of insulin (31). Cell clumps were removed by centrifugation at 50*g* for 1 min. Hepatocytes were collected from supernatant by centrifugation at 50 *g* for 10 min. The pellet was washed three times with Williams E media. Viability of hepatocytes was over 80% as determined by trypan blue staining. Hepatocytes (1 × 10E6) were cultured in 10 ml of Williams E media for 24 hr at 37°C in T25 flasks (Costar), coated with rat tail-derived collagen I (Sigma), and dissolved in acetic acid (31).

Kupffer Cell Isolations. After hepatocyte removal from the whole liver cell suspensions, the supernatants were collected and spun at 400*g* for 10 min. The pellets were resuspended and washed four times in Geys balanced salt solution (Gibco). Kupffer cell purification was achieved by centrifugation at 800*g* for 20 min at 20°C in 12 ml of a 17.5% solution of metrizamide (Nycomed) prepared in Geys balanced salt solution without NaCl, with 2 ml of Geys balanced salt solution with NaCl layered above. KC were collected at the metrizamide solution interface and identified by nonspecific esterase activity in the presence and absence of NaF, and by phagocytosis of 1.1-μm latex beads (4, 5). Viability was over 90%, as determined by a trypan blue exclusion test.

Iodination of LPS. LPS was iodinated by the chloramine T method of Greenwood *et al.* (32) as modified by Ulevitch (33). In brief, hot-phenol-water-extracted *E. coli* 0127:B8 or *S. minnesota* LPS (Sigma) was coupled to methyl *p*-hydroxybenzimidate (Sigma). A 20-m*M* benizimidate solution was added to a 1-mg/ml solution of LPS dissolved in 0.5 *M* sodium borate buffer

(pH 8.5) and incubated for 8 hr at 37°C. The reaction mixture was dialyzed against a 20 m*M* benzimidate solution overnight at 37°C. Unbound benzimidate was removed by extensive dialysis against 0.1 *M* phosphate buffer (pH 7.0) at 4°C. The phosphate buffer (250 ml) was changed every 2 hr for 6 hr and followed by overnight dialysis (4, 5). Benzimidate-coupled LPS was labeled with Na ^{125}I (New England Nuclear). In this process, 50 μl of 0.5 *M* phosphate buffer (pH 7.2) and 500 μl of 1-mg/ml stock benzimidate-coupled LPS were added to 1 mCi of Na ^{125}I. Chloramine T (15 μl, 4 mg/ml in 0.05 *M* phosphate buffer) was added. After 1 min, sodium metabisulfite (15 μl, 4 mg/ml in 0.05 *M* phosphate buffer) was added and followed 1 min later by KI (100 μl, 20 mg/ml in 0.05 *M* phosphate buffer). Labeled LPS was separated from free Na ^{125}I by fractionation on a Sephadex G-50 column, which was washed extensively with RPMI 1640 plus 5% fetal bovine serum (FBS). The specific activity of the labeled LPS was approximately 1.0 μCi/μg. The resultant iodinated LPS molecule is unchanged in antigenicity, toxicity, and biological effect (33).

Preparation of Kupffer Cell-Modified LPS. ^{125}I-LPS (33.3 μg) was incubated with 1 × 10E7 KC in 10 ml of RPMI 1640 supplemented with penicillin and streptomycin for 1 hr at 37°C. Unbound ^{125}I-LPS was removed by washing KC three times with RPMI 1640. KC were incubated for 18 hr at 37°C in RPMI 1640 supplemented with 5% FBS, penicillin, and streptomycin. KC-modified LPS was collected in the supernatant after centrifugation of cell suspensions at 400*g* for 20 min. The KC-modified ^{125}I-LPS was concentrated by either filtration through an Amicon model 8050 ultrafiltration cell with a YM5 (5,000 mol wt cutoff) membrane or by centrifugation at 1,500*g* through a Millipore ultrafree 60 polysulfone cell with a 10,000 mol wt cutoff. Chromatographic analysis on a Biogel A1.5M column was performed in these and prior studies (4, 5) to confirm release of KC-modified ^{125}I-LPS.

Comparative Binding Studies of Native ^{125}I-LPS and KC-Modified ^{125}I-LPS to Hepatocytes. Native or KC-modified *E. coli* 0127:B8 ^{125}I-LPS (40 ng) was incubated in triplicate sets for 1 hr with 1 × 10E6 hepatocytes cultured on collagen I-coated Costar T25 flasks in 10 ml of Williams E media. The cells were washed three times with phosphate-buffered saline (PBS) and trypsinized, and the suspension was filtered through Whatman GF/C filters with repeated washings of ice-cold PBS. The filters were preincubated in 5% bovine serum albumin at 37°C for 30 min before use to reduce nonspecific binding (34). After filtration, the filters were vacuum-dried on a Millipore manifold and dried filters were counted for ^{125}I-LPS with a LKB 1275 gamma counter. In hepatocyte binding studies, KC-modified *S. minnesota* LPS was competed with 100-fold excess cold *S. minnesota* native and O-specific side chain or core mutant strains (Ra, Rc, Re, Rd) of LPS, and Lipid A (List Biologicals).

Peritoneal Macrophage Isolation. Peritoneal macrophages (PM) were elicited by intraperitoneal injection of a 10% proteose peptone (Difco) solution into nonfasting male Sprague-Dawley rats 4 days before harvesting. Peritoneal cells were collected from sodium pentobarbital-anesthetized rats by peritoneal lavage with Hanks' balanced salt solution. The cells were incubated in RPMI 1640 plus 5% FBS, penicillin, and streptomycin for 3 hr in collagen I-coated Costar T75 flasks. Nonadherent cells were removed by washing with PBS. Adherent cells were removed by trypsinization, washed three times with RPMI 1640, and counted by hemocytometer. Identification of peritoneal macrophages was made by α-naphthyl acetate esterase staining in the presence and absence of NaF, and by phagocytosis of 1.1-μm latex beads. PM were over 90% viable as determined by the trypan blue exclusion test.

Induction of TNF-α Release by Peritoneal Macrophages. Native or KC-modified *S. minnesota* ^{125}I-LPS (100 ng) was incubated for 6 hr with 1 × 10E7 PM cultured on collagen I-coated Costar flasks in 5 ml of RPMI 1640. TNF-α release was measured in the supernatants by the WEHI-164–13 cytotoxicity assay.

TNF-α Assay. WEHI-164–13 fibrosarcoma cells maintained in RPMI 1640 plus 5% FBS were incubated in 96-well Costar dishes (30,000 cells/well) with 50-μl samples or standards prepared with recombinant human TNF-α (Sigma) for 18 hr in the presence of actinomycin D (35). After this, media were removed and 50 μl of a 1-mg/ml solution of dimethylthiazol-diphenyltetrazolium bromide (Sigma) in PBS were incubated with cells for 3 hr. Crystals produced by mitochondrial oxidation of dimethylthiazol-diphenytetrazolium bromide were dissolved in acid isopropyl alcohol and the solution in wells was read at 550 nm on an enzyme-linked immunosorbent assay microreader spectrophotometer.

Statistical Analysis. The results of the binding studies and TNF-α release experiments were analyzed statistically by analysis of variance. *P*-values of less than 0.05 were considered to be statistically significant.

Results and Discussion

In comparative binding studies, KC-modified *E. coli* 0127:B8 LPS was seen to bind to hepatocytes in preference to native *E. coli* LPS (Fig. 1) by a 4.33-fold higher factor ($P = 0.0024$). To determine the region of KC-modified LPS that allows for enhanced hepatocyte binding, KC-modified *S. minnesota* LPS was competed with either 100-fold excess cold *S. minnesota* native or mutant (Ra, Rc, Rd, Re) strains of LPS, or Lipid A. No decrease in hepatocyte binding of KC-modified *S. minnesota* LPS could be demonstrated by these studies

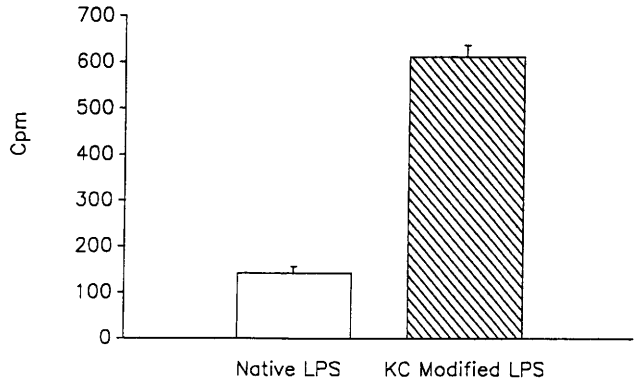

Figure 1. Native ^{125}I-LPS or Kupffer cell-modified *E. coli* 0127:B8 LPS (40 ng) was presented to 1 × 10E6 rat hepatocytes for 1 hr. Competition binding experiments were carried out with 100-fold excess (100×) cold native *E. coli* 0127:B8 LPS. Duplicate samples were used to calculate each value. Background was subtracted from all values. Differences in results among groups were analyzed statistically by analysis of variance and found to be significant ($P = 0.0024$).

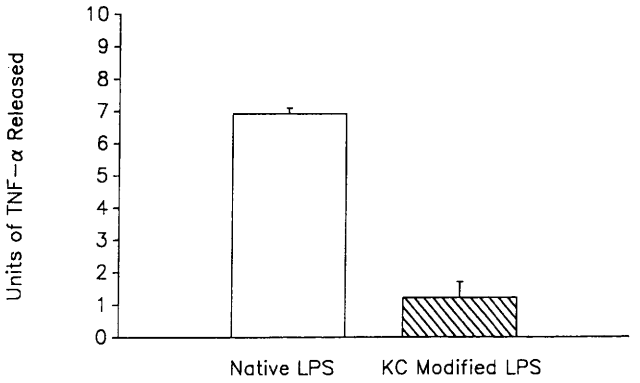

Figure 3. Native or Kupffer cell-modified *S. minnesota* LPS (100 ng) was presented to 1.3 × 10E7 rat peritoneal macrophages. TNF-α release was evaluated by cytotoxicity assay using the TNF-α-sensitive WEHI-164–13 fibrosarcoma line. One TNF-α unit is equal to LD50 of 3 × 10E4 WEHI-164 cells. Triplicate samples were used to calculate each set of TNF-α values. The difference in the results of the two groups was analyzed statistically by analysis of variance and found to be significant ($P < 0.0001$).

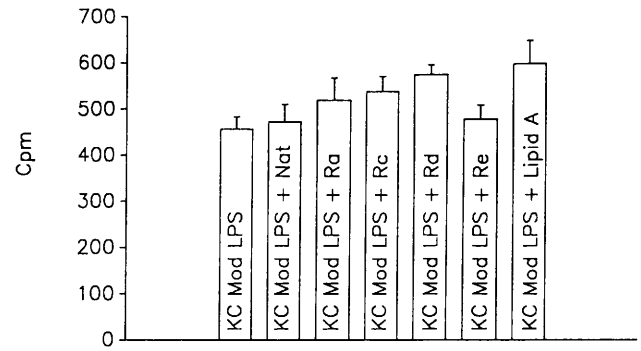

Figure 2. Kupffer cell-modified *S. minnesota* ^{125}I-LPS (100 ng) was presented to 1 × 10E6 rat hepatocytes for 1 hr. Competition binding experiments were carried out with 100-fold excess cold native or mutant strain LPS or Lipid A. Duplicate samples were used to calculate each value. Background (121 cpm) was subtracted from all values.

(Fig. 2). Native *S. minnesota* LPS was next compared with its KC-modified LPS for the ability to induce TNF-α release by PM (Fig. 3). Native *S. minnesota* LPS induced 5.7-fold higher TNF-α levels than KC-modified LPS ($P < 0.0001$).

Previous work from our laboratories had demonstrated alterations in the structure of LPS that accompany uptake and release by Kupffer cells (4, 5). Those studies indicated that LPS is structurally modified by loss of carbohydrate prior to its release by KC.

In these studies, we functionally characterized KC-modified LPS. KC-modified *E. coli* LPS showed a 4.33-fold enhanced binding to hepatocytes over the unaltered form. This suggests that KC-mediated structural changes in LPS may allow for, or enhance, a subsequent step(s) in the clearance of LPS by hepatocytes. Such a scheme is consistent with prior studies that localized LPS initially to KC followed by redistribution 2–3 days later to hepatocytes (2, 3). Interestingly, when native (S-form) LPS was compared with Re mutant strain (R-form) LPS for distribution to the liver, the S-form localized to KC for the first 3 days and was followed by redistribution to hepatocytes. In contrast, Re mutant (R-form) LPS localized initially to both KC and hepatocytes, suggesting that loss of O-specific side chains or core regions does, in fact, promote hepatocyte binding (3). These results are in contrast to the study by Parent (36), which demonstrated binding of both native (S-form) and mutant (R-form) LPS (except the Re mutant LPS) to hepatocytes. He showed that binding of either S- or R-forms of LPS could be competed with each other, and by heptose, a component of the core region of LPS, but not by Lipid A.

Through competition binding analysis studies, attempts were made to identify the region of LPS responsible for enhanced binding of KC-modified LPS to hepatocytes. Native and mutant strains of LPS, along with Lipid A from *S. minnesota*, were used to compete hepatocyte binding of KC-modified *S. minnesota* LPS. These studies demonstrated no decrease in KC-modified LPS binding by competition with excess native LPS, mutant strains of LPS, or Lipid A. These results argue for structural alterations occurring within the Lipid A region of KC-modified LPS that allow for enhanced binding of KC-modified LPS to hepatocytes. This is in view of our finding that PM exhibit decreased TNF-α release when stimulated with KC-modified LPS versus native LPS, and that TNF-α release is mediated by the Lipid A portion of LPS (37). Modifications to the Lipid A region of KC-modified LPS may in fact accompany alterations to the polysaccharide portions of KC-modified LPS, which we reported previously (4, 5).

The concept that normal hepatic LPS clearance represents a detoxification process was also investi-

gated. LPS are capable of eliciting biologically active cytokines, among them TNF-α. The results reported here suggest that clearance of LPS by KC represents a detoxification process wherein KC-modified LPS has a decreased ability to elicit TNF-α release by macrophages. This decreased ability of KC-modified LPS to elicit cytokine release by macrophages may be secondary to altered binding or signal transduction characteristics, although other mechanisms may also be possible and are the subject of future studies.

The studies reported here extend our understanding of the role Kupffer cells play in the hepatic clearance of LPS. The results suggest that Kupffer cells are important participants in normal hepatic LPS clearance where KC-mediated structural alterations to LPS allow for enhanced hepatocyte binding. In addition, these results suggest that Kupffer cells contribute to the detoxification of LPS by diminishing their ability to elicit biologically active cytokines.

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