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Comparative Studies of Endotoxin Uptake by Isolated Rat Kupffer and Peritoneal Cells

EBEN S. FOX,[1] PETER THOMAS,[2] AND SELWYN A. BROITMAN[1]*

Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts 02118,[1] and Laboratory for Cancer Biology, Department of Surgery, New England Deaconess Hospital, Boston, Massachusetts 02115[2]

Received 16 June 1987/Accepted 10 September 1987

The process of uptake of endotoxin by cells of the reticuloendothelial system is of current interest. Rabbit peritoneal macrophages have been used to study macrophage-endotoxin interactions and have suggested a receptor-mediated process. It is generally believed that the site of in vivo endotoxin clearance is the liver and that this clearance involves the Kupffer cell population. In the current report, the uptake characteristics of iodine-125-labeled *Salmonella minnesota* lipopolysaccharide (LPS) were compared in both isolated rat Kupffer cells and elicited rat peritoneal cells. Both types of cells were isolated from male Sprague-Dawley rats fed a semisynthetic AIN-76 5% saturated-fat diet either by peritoneal lavage for peritoneal cells or by collagenase perfusion followed by purification on a 17.5% metrizamide gradient for Kupffer cells. Hot phenol water-extracted *S. minnesota* LPS was labeled with iodine by the chloramine-T method following a reaction with methyl-*p*-hydroxybenzimidate. The in vitro uptake of [^{125}I]LPS by Kupffer cells was unsaturable up to concentrations of 33.33 μg/ml, while peritoneal cells became saturated at between 16.67 and 25 μg of LPS per ml. Uptake by both types of cells could be inhibited by a 10-fold excess of unlabeled LPS. Kinetic experiments demonstrated that Kupffer cells were unsaturable after 60 min of incubation, while peritoneal cells were saturable after 40 min of incubation. Pretreatment with 75 mM colchicine inhibited uptake by peritoneal cells but not Kupffer cells, while pretreatment with 12 mM 2-deoxyglucose inhibited uptake by Kupffer cells but not peritoneal cells. These results are consistent with a process of receptor-mediated endocytosis for peritoneal cells, while Kupffer cells may internalize endotoxins by absorptive pinocytosis. These results suggest that studies of peritoneal cell-endotoxin interactions do not accurately describe the physiologic process within the liver, the major site for the clearance of gut-derived endotoxins.

Macrophages of the reticuloendothelial system represent a major portion of the body's nonspecific defense mechanism against environmental toxins and invading pathogens. The ability of a macrophage to capture and retain antigens is the first step in the activation of the body's humoral and cellular immune responses. Kupffer cells, tissue-fixed macrophages present in the sinusoids of the liver, serve as a filter for portal vein blood. In fact, over 50% of circulating monocytes take up residence in the liver and differentiate into Kupffer cells (23). Their orientation within the venous return from the gastrointestinal tract results in a constant exposure to nutritional substrates as well as antigens traversing the gastrointestinal mucosa. Their placement within the liver suggests that they represent the next step beyond gut-associated lymphoid tissues and mesenteric lymph nodes in the physiologic mechanisms responsible for dealing with the clearance and detoxification of gut-derived substances.

The liver is by far the largest reticuloendothelial system organ, and its constant exposure to physiologic levels of immunogenic gut-derived antigens gives it a role in the immune response different from that of other reticuloendothelial system organs, such as the spleen. There exists within the liver unique regulatory mechanisms for the disposition of gastrointestinal tract antigens and the sequestration of these antigens from peripheral blood lymphocytes. Previous studies with carcinoembryonic antigen (27) have described a mechanism in which this glycoprotein is bound by Kupffer cells, internalized, modified, and then passed to the hepatocytes for final disposition. The possibility was explored that this represents a general mechanism for the clearance and detoxification of other gastrointestinal tract antigens, and this report focuses upon gut-derived bacterial endotoxins.

Endotoxin administered intravenously is cleared from the systemic circulation principally by the liver (5, 15), although small percentages can be found in the spleen, lungs, kidneys, adrenal glands, and skeletal muscle (15). Absorption of endotoxin from the gut has been demonstrated experimentally with everted gut sacs and radiolabeled endotoxin (19). Both gelation and chromogenic *Limulus* amoebocyte lysate assays have revealed the chronic presence of endotoxin within the portal veins of normal subjects and in the systemic circulation of patients with hepatic disease, suggesting the chronic passage of gut-derived endotoxins and clearance by the normal liver (1, 11, 22, 28). Ruiter et al. (24) and Praaning-van Dalen et al. (21) demonstrated that in rats, Kupffer cells, rather than endothelial or parenchymal cells, are responsible for the clearance of intravenously administered ^{51}Cr-endotoxin. In situ immunoperoxidase staining of sections of rat organs (5, 6) supports this pathway, revealing that endotoxin first is associated with Kupffer cells and after 2 days appears to be redistributed to hepatocytes.

The process of uptake of endotoxin by cells of the reticuloendothelial system is of current interest. Rabbit peritoneal exudate cells have been used to study macrophage-lipopolysaccharide (LPS) interactions. Evidence has been provided that the uptake of ^{3}H-*Bordetella pertussis* endotoxin by elicited peritoneal macrophages is a receptor-mediated process which exhibits classic saturation kinetics (10). In the current report, the uptake characteristics of

* Corresponding author.

iodine-125-labeled *Salmonella minnesota* LPS were compared in both isolated rat Kupffer cells and elicited rat peritoneal cells. The data imply that studies of peritoneal cell-endotoxin interactions do not accurately describe the physiologic process within the liver, the major site for the clearance of gut-derived endotoxins.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc.) weighing 250 to 300 g were used in all experiments. Animals were housed in a temperature- and light-controlled room and fed an AIN-76 semisynthetic diet supplemented with 5% coconut oil ad libitum. The diet was prepared in accordance with established guidelines (J. Nutrition **107**:1340, 1977).

LPS. Hot phenol water-extracted *S. minnesota* wild-type LPS was obtained from List Biochemicals or Sigma Chemical Co. These preparations were essentially protein free, as determined by analysis by the suppliers.

Iodination. Iodination was carried out by the procedure of Ulevitch (29). Iodinated endotoxins are unchanged by this procedure in their toxicity, antigenicity, and ability to stimulate biologic responses (29). A 20 mM solution of methyl-*p*-hydroxybenzimidate (Sigma) was prepared in a 1-mg/ml solution of *S. minnesota* LPS in 0.5 M sodium borate buffer (pH 8.5). This mixture was incubated in a 37°C water bath for 8 h and then dialyzed against 20 mM benzimidate overnight at 37°C. Uncoupled benzimidate was removed by extensive dialysis against 0.1 M phosphate buffer (pH 7.0) at 4°C. The dialysate was changed every 2 h for a minimum of 6 h, followed by overnight dialysis. The modified LPS was then iodinated by the chloramine-T method (8). High-concentration sodium iodide in a 0.1 N sodium hydroxide solution was obtained from New England Nuclear Corp. Sequentially, 50 μl of 0.5 M phosphate buffer (pH 7.2) and 450 μl of the modified LPS solution (1 mg/ml) was added to a reaction vial containing 1 mCi of $Na^{125}I$. Chloramine-T (15 μl, 4 mg/ml) in 0.05 M phosphate buffer was added. After 1 min, 15 μl of sodium metabisulfate solution (4 mg/ml) in 0.05 M phosphate buffer was added. After an additional minute, 50 μl of potassium iodide (20 mg/ml) in 0.05 M phosphate buffer was added, followed by 0.75 ml of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered RPMI 1640 medium with 5% fetal bovine serum. Labeled LPS was separated from the sodium iodide by fractionation on a Sephadex G-50 column which had been washed extensively with 20 mM HEPES-buffered RPMI 1640 medium with 5% fetal bovine serum. The specific activity of the labeled LPS was approximately 1.0 μCi/μg.

Kupffer cell isolation. Kupffer cells were harvested from the livers of anesthetized fasting male Sprague-Dawley rats by using the isolation buffers described by Seglen (25). After intracardiac administration of sodium heparin (500 U), the livers were exsanguinated by hepatic artery perfusion with Ca^{2+}-free buffer (0.01 M HEPES with 0.83% NaCl–0.05% KCl, pH 7.4). Livers were excised, minced, and subsequently incubated with 0.05% collagenase in buffer (0.1 M HEPES with 0.39% NaCl–0.05% KCl–0.05 M $CaCl_2$, pH 7.6) at 37°C for 60 min. The resulting cell suspension was pelleted and incubated with fresh collagenase buffer for an additional 30 min. The cell suspension was strained, and hepatocytes and cell clumps were removed from the suspension by low-speed centrifugation (50 × *g* for 2 min). Remaining cells were washed several times with Gey balanced salt solution. Final purification was achieved by centrifugation (1,400 × *g*) for 15 min at room temperature in a 17.5% solution of metrizamide in Gey balanced salt solution.

Peritoneal cell isolation. Peritoneal cells were elicited with Proteose Peptone (Difco Laboratories) from nonfasting male Sprague-Dawley rats and harvested by peritoneal lavage with cold sterile phosphate-buffered saline.

Viability and identification of cells. The viability of the cell preparations was greater than 90%, as determined by the trypan blue exclusion test. The identity of the Kupffer cells was established by positive cytochemical staining for nonspecific esterase activity (27). Determination of the percentages of Kupffer and endothelial cells in the preparations was based on the number of cells reacting positively or negatively for nonspecific esterase activity in the presence of 33 mM sodium fluoride. Cell counts were performed with a hemacytometer.

Determination of in vitro uptake of [^{125}I]LPS by isolated cells. Cell suspensions, adjusted to 3×10^6 cells per ml, were incubated with the radiolabeled LPS in 20 mM HEPES-buffered RPMI 1640 with 5% fetal bovine serum. In some experiments, cell suspensions were pretreated with 75 mM colchicine or 12 mM 2-deoxyglucose for 1 h prior to incubation with [^{125}I]LPS. The reaction mixture was incubated at 37°C, and triplicate samples were filtered through Whatman GF/C filters. Nonspecific binding was reduced by preincubating the filters in 5% bovine serum albumin for 30 min before the assay (2). Nevertheless, the filters still absorbed minute quantities of radiolabeled LPS, and this absorption was assumed to represent nonspecific binding. These quantities were estimated by filtering similar reaction mixtures without cells and subtracting the counts from those of experimental samples. Cell-associated activity was converted to micrograms of LPS by including 0.1-μg samples of the labeled preparation for analysis.

Statistical analysis. Statistical significance was determined by the Student *t* test. *P* values with a level of significance of less than 0.05 were assumed to represent statistical significance.

RESULTS

The uptake of [^{125}I]LPS by isolated rat Kupffer cells and elicited rat peritoneal cells was studied in a suspension assay. In dose-dependent experiments (Fig. 1) in which concentrations varied from 8.83 to 33.33 μg of LPS per ml, uptake by Kupffer cells was unsaturable, while peritoneal exudate cells became saturated at concentrations between 16.67 and 25 μg/ml. Kupffer cells were unsaturable in repeat studies carried out with LPS at 0.0883 to 0.3333 μg/ml (Fig. 1, insert) to ascertain whether a high-affinity binding site that was detectable only at low LPS concentrations was present. However, kinetic experiments performed with 16.67 μg/ml and Kupffer cells demonstrated that uptake could be inhibited by a 10-fold excess of unlabeled LPS (Fig. 2), suggesting a finite number of binding sites on the cell membrane. Similar experiments performed with isolated peritoneal cells were consistent with the idea that this macrophage population uses a receptor-mediated process for bacterial endotoxin uptake.

To demonstrate that the uptake of [^{125}I]LPS might be energy dependent, we performed kinetic experiments with the metabolic inhibitor 2-deoxyglucose, which depletes cellular ATP levels. A 1-h pretreatment with 12 mM 2-deoxyglucose inhibited Kupffer cell uptake of [^{125}I]LPS by approximately 25% (Fig. 3), while peritoneal cells were not inhibited at this concentration. Experiments with Kupffer

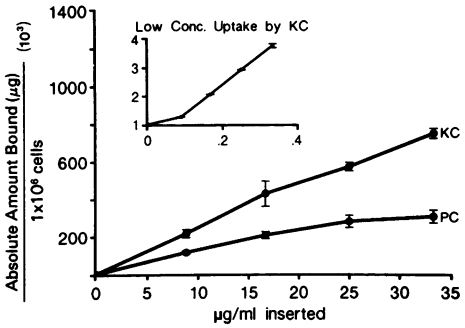

FIG. 1. Uptake of [^{125}I]LPS by isolated rat Kupffer cells (KC) and peritoneal cells (PC). Kupffer cells or peritoneal macrophages were isolated and suspended at 3×10^6 cells per ml. [^{125}I]LPS was added to suspension cultures to achieve final concentrations of 8.83, 16.67, 25, and 33.33 μg of LPS per ml or, in separate experiments, 100-fold-lower concentrations (Conc.) (insert). After 30 min of incubation at 37°C, triplicate samples were filtered through albumin-soaked Whatman GF/C filters and washed with 10 ml of ice-cold saline. There was no statistically significant difference between the 25- and 33.33-μg/ml points for peritoneal cells ($P > 0.05$), while for Kupffer cells there was a statistically significant difference at the same points ($P < 0.001$). The data represent the mean ± standard error for nine samples from nine different animals corrected for appropriate controls.

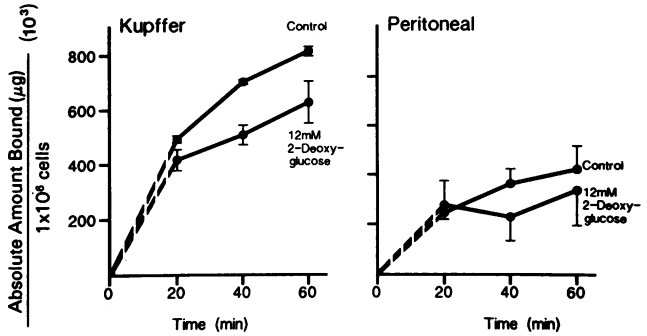

FIG. 3. Effect of a metabolic inhibitor on the kinetics of uptake. Kupffer cells or peritoneal cells were isolated, suspended at 3×10^6 cells per ml, and pretreated with 12 mM 2-deoxyglucose for 1 h. [^{125}I]LPS was added to suspension cultures to achieve a final concentration of 16.67 μg of LPS per ml. At the indicated times, triplicate samples were filtered through albumin-soaked Whatman GF/C filters and washed with 10 ml of ice-cold saline. Treated peritoneal cells were not statistically different from untreated peritoneal cells ($P > 0.05$), while after the 20-min time point, treated Kupffer cells were statistically different from untreated Kupffer cells ($P < 0.02$). The data represent the mean ± standard error for six samples from six different animals corrected for appropriate controls.

cells and 1 mM 2-deoxyglucose produced similar data (data not shown). These results suggest that peritoneal cells are not dependent on a chemical energy source for the uptake of LPS or that there is a difference in the ability of these cells to transport glucose.

Pretreatment of peritoneal macrophages in vitro with colchicine inhibits microtubule formation and partially inhibits receptor-mediated endocytosis of lysosomal enzymes (14). Previous data have demonstrated that colchicine inhibits the uptake of carcinoembryonic antigen by isolated rat Kupffer cells (27). Pretreatment of Kupffer cells with colchicine (75 mM) for 1 h did not affect the uptake of [^{125}I]LPS, suggesting that an intact cytoskeleton is not a prerequisite for binding and internalization by these cells (Fig. 4). Colchicine pretreatment of isolated peritoneal cells, however, resulted in a 30% inhibition of uptake, consistent with a process of receptor-mediated endocytosis. The resistance of LPS uptake by Kupffer cells to colchicine implies that this mechanism for uptake is unrelated to that for carcinoembryonic antigen or to that for lysosomal enzymes, such as β-*N*-acetylhexosaminidase (14, 27).

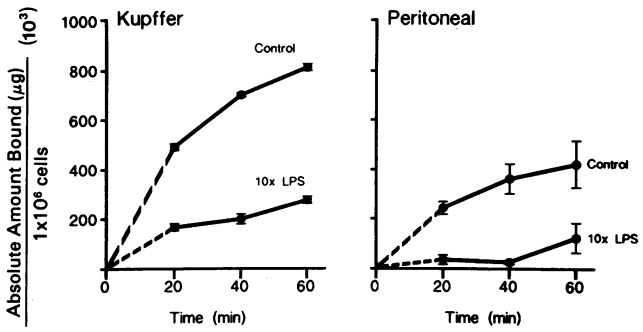

FIG. 2. Effect of 10-fold excess of LPS on the kinetics of uptake. Kupffer cells or peritoneal cells were isolated and suspended at 3×10^6 cells per ml. [^{125}I]LPS was added to suspension cultures to achieve a final concentration of 16.67 μg of labeled LPS per ml with or without a 10-fold excess of unlabeled LPS. At the indicated times, triplicate samples were filtered through albumin-soaked Whatman GF/C filters and washed with 10 ml of ice-cold saline. There were statistically significant differences between treated and untreated cells for both cell populations ($P < 0.001$). The data represent the mean ± standard error for six samples from six different animals corrected for appropriate controls.

DISCUSSION

The results of this study suggest that Kupffer cells and peritoneal cells, both members of the mononuclear phagocyte system and arising from common bone marrow progenitor cells (7), possess different mechanisms for the uptake

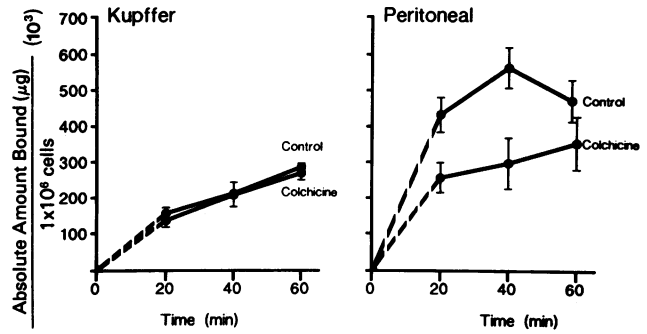

FIG. 4. Effect of colchicine pretreatment on the kinetics of uptake. Kupffer cells or peritoneal cells were isolated, suspended at 3×10^6 cells per ml, and pretreated with 75 mM colchicine for 1 h. [^{125}I]LPS was added to suspension cultures to achieve a final concentration of 16.67 μg of LPS per ml. At the indicated times, triplicate samples were filtered through albumin-soaked Whatman GF/C filters and washed with 10 ml of ice-cold saline. Treated Kupffer cells were not statistically different from untreated Kupffer cells ($P > 0.05$). Treated peritoneal cells were significantly different from untreated peritoneal cells at 20 and 40 min ($P < 0.02$) and at 60 min were not significantly different ($P > 0.05$). The data represent the mean ± standard error for six samples from six different animals corrected for appropriate controls.

and internalization of bacterial endotoxin. Peritoneal cells have previously been shown to possess a receptor for endotoxins (10), a finding confirmed by the present study. Kupffer cells behave quite differently; they do not bind LPS saturably, nor do their binding kinetics conform to those of classic receptor-mediated processes.

The uptake of [^{125}I]LPS by isolated rat peritoneal cells is both saturable and sensitive to a 10-fold excess of unlabeled LPS. These data are consistent with those from previously published studies with isolated rabbit peritoneal macrophages and ^{3}H-*B. pertussis* endotoxin (9, 10). Our kinetic studies demonstrated saturability and colchicine sensitivity but insensitivity to 12 mM 2-deoxyglucose. Previous studies have shown that 50 mM 2-deoxyglucose inhibits Fc and complement receptor-mediated phagocytosis (opsonin dependent) by mouse peritoneal macrophages but not phagocytosis of latex or zymosan (opsonin independent) (16, 17). It is possible that a higher concentration of 2-deoxyglucose might inhibit peritoneal cell uptake of LPS; however, in the present study we found that the inhibition at 12 mM is similar to that observed at 1 mM for isolated Kupffer cells.

Isolated Kupffer cells take up LPS nonsaturably at concentrations well beyond those encountered physiologically. This uptake is sensitive to a 10-fold excess of unlabeled LPS, suggesting a finite number of binding sites on the cell membrane. Kinetic experiments demonstrated that with respect to time, these cells are not saturable at 16.67 μg of LPS per ml. To demonstrate a role for the cellular cytoskeleton or cellular metabolism in LPS uptake, we performed kinetic experiments in the presence of 75 mM colchicine or 12 mM 2-deoxyglucose. While colchicine pretreatment had no demonstrable effect on the kinetics of uptake, the depletion of cellular ATP by deoxyglucose resulted in a 25% decrease in total uptake while the kinetics remained the same. These results suggest either different mechanisms for energizing uptake or perhaps different abilities to transport the analog in these two macrophage populations. The present study does not confirm the report of Yamaguchi et al. (30) that Kupffer cells possess a specific cellular receptor for endotoxins. Kupffer cells are the reticuloendothelial cells of the liver situated in the liver sinusoids such that the cell membrane is oriented into the blood flow of the portal vein. As such, these cells are exposed to the low-level but chronic endotoxemia of the portal vein and do not require a high-affinity receptor-mediated system to remove endotoxins from blood. The present data suggest that the process of internalization by Kupffer cells is by absorptive pinocytosis.

The abilities of these cell populations to handle endotoxin extends beyond the process of uptake. Recent studies of endotoxin modification by murine peritoneal cells (18) have indicated that this macrophage population deacylates the fatty acids of the lipid component. Recent data from our laboratories with ^{3}H, ^{14}C-*Escherichia coli* J5 endotoxin have shown that isolated rat Kupffer cells modify endotoxin by cleaving the sugar side chains to a uniform length (unpublished data). Presumably, Kupffer cells modify the endotoxin molecule such that it can be passed to hepatocytes for final disposition; this scheme would agree with previously published data (5).

The differences between peritoneal and Kupffer cells could be explained by their relative states of activation and maturation as well as the influence of the unique microenvironment of the liver. It is generally believed that Kupffer cells represent a terminally differentiated macrophage population and as such cannot respond to antigens in a classic immunologic sense (23). Recent in vitro data have shown that Kupffer cells can express classic macrophage functions, in some cases without prior in vitro stimulation with endotoxin (12, 13, 23, 26). In all cases, the response of Kupffer cells, with or without endotoxin stimulation, is not of the same order as that of peritoneal cells. This response may be a result of the low percentage of cells in the population able to express the class II histocompatibility antigen Ia (T. M. Rogoff, E. S. Vitetta, and P. E. Lipsky, Gastroenterology **77**:A35, 1979). The percentage of Ia-positive cells in the population is considered to be an indication of its functional state. The relatively low level of expression of Ia in the Kupffer cell population would tend to explain why immune responses do not occur appreciably within the normal liver. Recent studies have identified two naturally occurring liver proteins in both humans and rats which suppress immune responses in vitro (3, 4, 20). It is conceivable that the liver exerts a unique suppressive effect on its resident macrophage population such that it cannot respond to the chronic stimulation received through the clearance of endotoxins and possibly other gut-derived antigens. In this sense, the physiologic mechanisms for the clearance of gut-derived endotoxins represent biochemical processes of the liver. Sequestration of gut-derived antigens within the liver spares the body the consequences of a chronically activated macrophage population constantly presenting gut-derived antigens to peripheral blood lymphocytes and influencing local inflammatory responses within the liver.

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