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Fate of ^{51}Cr-Labeled Lipopolysaccharide in Tissue Culture Cells and Livers of Normal Mice[1]

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Livers of normal mice trapped over 80% of intravenously injected ^{51}Cr-labeled lipopolysaccharide after 1 h. Liver fractionation studies showed that nearly 45% of the labeled endotoxin was associated with cell nuclei, 20% with the mitochondrial-lysosomal fraction, and approximately 30% with the cell sap. Analysis of the distribution of ^{51}Cr-labeled lipopolysaccharide among parenchymal and Kupffer cells showed that over 75% of the in vivo-trapped counts were parenchymal cell associated. Cell populations were approximately 65% parenchymal cells and 35% nonparenchymal cells. Further, six non-reticuloendothelial system tissue culture cell lines were tested for their ability to internalize labeled lipopolysaccharide. In all cells studied, 1 to 4% of the labeled lipopolysaccharide was taken up after 3 h, with greater than 80% of the counts localized in the nuclear fraction. The data show that non-reticuloendothelial system cells can sequester endotoxin both in vivo and in vitro and suggest that parenchymal cells as well as Kupffer cells remove circulating endotoxin from the blood.

Numerous metabolic alterations in liver parenchymal cells after endotoxin poisoning (1-4, 12-15) have been implicated as significant contributory factors to the pathophysiology of endotoxemia. Such changes range from depletion of carbohydrate reserves to inhibition of enzyme induction and gluconeogenesis (9, 17, 18, 20, 24). A central question in research on the role of the liver in the pathophysiology of endotoxin shock is whether parenchymal cells are directly affected by endotoxin or whether the metabolic changes observed in endotoxemia are mediated through soluble products released from the macrophage after phagocytosis of endotoxin (L. J. Berry, personal communication). The initial objective of this study was to determine whether liver parenchymal cells in vivo or a variety of non-reticuloendothelial system (RES) cells in tissue culture are able to internalize endotoxin.

MATERIALS AND METHODS

Animals. Female, Spartan HA-DCR mice (Haslett, Mich.), weighing 18 to 22 g, were housed five per cage with wood chips as bedding. Purina Laboratory Chow and water were available ad libitum. Animal care was under direction of the Laboratory Animal Care Services, Michigan State University.

Chemicals. Lyophilized *Salmonella typhimurium* lipopolysaccharide extracted by the Westphal procedure (Difco Laboratories, Detroit, Mich.) served as endotoxin in all experiments. It was stored at 4 C and dissolved in isotonic, sterile saline before use.

Chromium-51 ($Na_2{}^{51}CrO_4$; New England Nuclear Corp., Boston, Mass.) was purchased in 2-ml amounts, 1 mCi/ml, with a specific activity of approximately 375 mCi/mg.

Minimum essential medium (MEM), medium 199 (M-199), and fetal calf serum (FCS) were obtained from GIBCO (Grand Island, N.Y.). MEM and M-199 were stored at 4 C, and the FCS was kept at −20 C.

Preparation of radiolabeled endotoxin. Chromium-51 (1.5 mCi) was added to 100 mg of *S. typhimurium* lipopolysaccharide. The mixture was diluted to 10.0 ml with saline and incubated at 37 C for 48 h with constant stirring. The preparation was dialyzed for 3 to 5 days against frequent changes of deionized water. The labeled endotoxin was centrifuged at 8,000 × *g* for 1 h, and the supernatant was recentrifuged at 100,000 × *g* for 8 h. The pellet from the second centrifugation was resuspended in 4.0 ml of deionized water and stored at −70 C. This material contained toxic, heavily labeled, high-molecular-weight lipopolysaccharide (8) with a specific activity less than 4.5 μCi/mg. Before injection, the ^{51}Cr-labeled lipopolysaccharide was appropriately diluted in sterile saline. ^{14}C-labeled *Salmonella enteritidis* endotoxin was generously supplied by Jon Rudback, University of Montana.

Distribution of ^{51}Cr-labeled endotoxin in experimental animals. Approximately 0.1 mg of ^{51}Cr-labeled endotoxin, in a volume of 0.3 ml of sterile saline, was injected intravenously into mice. After 60 min the mice were killed by cervical dislocation, and selected organs (liver, gall bladder, spleen, gut, kidneys, heart, lungs) and the remaining carcass were counted in the Packard gamma counter for 10 min. The percentage of radioactivity per organ was calculated as follows: [(radioactivity of organ)/(total radioactivity recovered)] × 100.

To determine the subcellular distribution of la-

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beled endotoxin in liver, organs were homogenized in glass homogenizing tubes with Teflon pestles in 9.0 ml of saline. Homogenates were centrifuged at 700 × *g* for 20 min (nuclear fraction). Resultant supernatants were centrifuged for 1 h at 15,000 × *g* (mitochondrial-lysosomal fraction), and finally for 1 h at 100,000 × *g* (microsomal fraction). All pellets and the final supernatant were measured for radioactivity. Percentage of radioactivity in each fraction was calculated as follows: [(radioactivity of fraction)/(total radioactivity recovered)] × 100. Over 85% of the injected counts were routinely accounted for.

Separation of liver cell populations. Livers from mice injected 1 h previously with about 40,000 counts/min of ^{51}Cr-labeled lipopolysaccharide were perfused in situ with 5 to 10 ml of M-199 to remove gross blood. The perfused livers were immediately removed, placed in 2 ml of cold calcium-free Hanks balanced salt solution (HBSS), and minced with a razor blade. The liver cubes were transferred to an enzyme digestion solution containing 0.1% hyaluronidase and 0.05% collagenase dissolved in HBSS (Ca^{2+} free) and incubated with occasional mixing at 37 C for 30 to 45 min (pH = 7.45). When isolated cells were clearly visible on microscopic examination, the whole suspension was filtered through nylon mesh (80 μm). Liver tissue retained on the mesh was resuspended in 10 ml of cold M-199 and refiltered. This procedure was repeated several times. All filtrates were pooled, and the cells were washed five times in cold HBSS containing 20 mM HEPES (*N*-2-hydroxyethyl piperazine-*N*'-2-ethanesulfonic acid) buffer (pH = 7.45) at 40 × *g*. By the last wash only negligible radioactive counts were detected in the supernatant. The final pellet was resuspended in HBSS, total and differential cell counts were made, and the total radioactivity in the sample was determined.

Parenchymal and nonparenchymal cells were easily distinguished by size, the parenchymal cells usually being at least four to five times larger than the nonparenchymal cells. In preliminary experiments mice were injected with 5 mg of colloidal carbon (Pelikan special black ink, Günther-Wagner), generously supplied by J. Filkins, Department of Physiology, Loyola University Medical School. Carbon-filled cells were only observed among the smaller, nonparenchymal cell populations. A typical distribution of cell types by this method would yield 65% parenchymal cells and 35% nonparenchymal cells. The latter, composed of approximately 70% carbon-containing cells, were designated Kupffer cells.

To determine the percent distribution of radiolabeled lipopolysaccharide between parenchymal and nonparenchymal cells, the mixed cell populations were incubated with 1% Pronase for 1 h at 37 C with occasional shaking. Such treatment has been shown to destroy all parenchymal cells without causing significant decreases in nonparenchymal cell populations (11, 16). In experiments to determine the percentage of endotoxin associated with parenchymal cells, it was assumed that all counts not sedimentable after Pronase treatment had originally been parenchymal cell associated.

Tissue culture cells. HeLa cells, obtained from Nathaniel Young, National Cancer Institute, were maintained in MEM with 10% FCS. Before experimentation, the cells were washed three times in M-199 without serum and resuspended to a concentration of approximately 10^6 cells/ml. Hepatoma cells were kindly supplied by Brad Thompson (National Institutes of Health) and were maintained in MEM with 10% FCS. Three myeloma cell lines (S194, XC-1, MOPC-21) and a lymphocytic sarcoma line (S49.1) were supplied by Ronald Patterson, Department of Microbiology and Public Health, Michigan State University. These latter cells were grown in disposable tissue culture flasks and maintained in Dulbecco modified Eagle medium containing 10% FCS and supplemented with streptomycin, penicillin, and nystatin.

Uptake and distribution of ^{51}Cr-labeled endotoxin in tissue culture cells. For HeLa cells, approximately 4 × 10^5 counts/min of ^{51}Cr-labeled endotoxin was added to 10 ml of cell suspension containing 10^6 cells/ml. The mixture was incubated at 37 C, with constant stirring, for 3 to 4 h. At appropriate intervals, duplicate 2.0-ml aliquots were removed, washed six times with Tris buffer [0.04 M tris(hydroxymethyl)aminomethane-hydrochloride, 0.1 M KCl, 0.001 M $MgCl_2$; pH 7.2] and counted in the gamma counter for 10 min. The counted cells were homogenized with glass and Teflon tissue grinders and centrifuged at 700 × *g* for 20 min. Counts per minute in the supernatant (cytosol) and pellet (nuclei) was determined. Experiments with all other cell lines followed the same procedure, except that the nuclear and cytoplasmic fractions were obtained after lysing cells with the detergent Nonidet P-40. This detergent lyses cells, leaving only nuclei intact and free of gross cytoplasmic membrane contamination as judged by microscopic examination (R. Patterson, personal communication). Control experiments show that the two lysing procedures gave similar results.

RESULTS

Distribution of radioactive sodium chromate and ^{51}Cr-labeled endotoxin among organs and tissues of normal mice. Table 1 shows the distribution of unconjugated ^{51}Cr among tissues. Only 15% of the counts were found in the liver, with over 60% of the counts in the carcass and intestines. By contrast, greater than 85% of the ^{51}Cr conjugated to lipopolysaccharide was found in the liver. In all studies, an average of greater than 85% of the injected counts were accounted for in the tissues studied.

Subcellular distribution of ^{51}Cr-labeled lipopolysaccharide in livers of mice exposed in vivo and in vitro. Table 2 shows the distribution of labeled endotoxin among the nuclear (700 × *g*), mitochondrial-lysosomal (15,000 × *g*), microsomal (100,000 × *g*), and soluble fractions of liver 1 h after intravenous injection of labeled endotoxin. In all instances more endo-

toxin was associated with the nuclear fraction than with any other single fraction. To establish that the nuclear association of endotoxin was not an artifact of mechanical homogenization, approximately 0.1 mg (40,000 counts/min) of labeled endotoxin was added to excised livers either immediately before or after homogenization. Conditions of temperature and ionic strength were similar to experimental groups. Less than 13 and 10%, respectively, of the labeled fraction was associated with nuclei (Table 2). In both cases between 70 to 80% of the endotoxin was found in the 100,000 × *g* pellet, suggesting it was not bound to any ultrastructural component since the labeled lipopolysaccharide alone sedimented under these conditions.

Localization of endotoxin in the parenchymal and nonparenchymal cells of liver. Mixed populations of parenchymal and nonparenchymal cells were prepared as outlined in Materials and Methods. Final preparations contained an average of 12.6×10^7 total cells, with 8.5×10^7 (65.1%) of these being parenchymal cells and the balance nonparenchymal cells (Table 3). Before Pronase treatment, all radioactive counts were sedimentable. After Pronase treatment for 1 h at 37 C, only nonparenchymal cells could be sedimented. All nonparenchymal cells could be quantitatively recovered, indicating no loss in this cell type after Pronase treatment. By contrast, no parenchymal cells were observed and over 75% of the originally sedimentable counts were recovered in the supernatant (Table 3). These data strongly suggest that parenchymal as well as Kupffer cells internalized intravenously injected endotoxin.

Uptake and subcellular distribution of ^{51}Cr- and ^{14}C-labeled endotoxin in nonphagocytic cells in tissue culture. Six non-RES tissue culture cell lines were studied in all. Hepatoma and HeLa cells were used most extensively. When either hepatoma or HeLa cells were incubated with 4×10^4 counts/min of ^{51}Cr-labeled lipopolysaccharide at 37 C with constant stirring, the amount of cell-associated endotoxin increased linearly with time. After 3 h, approximately 1 to 4% of the original counts became cell associated (Fig. 1A). Both cell types also internalized chromium alone (Fig. 1B), but, as can be seen in Table 4, the subcellular distribution was very different.

To establish that the cell-associated counts were not just passively bound to cell surfaces but actually internalized, the cells were lysed after 3 h of incubation and subjected to differential centrifugation. HeLa cells were lysed by mechanical disruption and hepatoma cells by detergent (Nonidet P-40). Both methods yielded numerous intact nuclei on microscopic examination and gave similar quantitative results. Once internalized, the labeled endotoxin was predominantly associated with the nuclear fraction (Table 4). With HeLa cells, 79.5% of the counts were in the nuclei compared with only 20.5% in cytoplasmic fractions. With hepatoma cells the nuclear-cytoplasmic ratio was even greater. When $Na_2{}^{51}CrO_4$ alone was incu-

TABLE 1. *Tissue distribution of chromium-51 and ^{51}Cr-labeled endotoxin in normal mice*

Expt	Percent uptake[a]						
	Liver	Spleen	Carcass	Lungs	Intestine	Kidneys	Other
Chromium-51	15.6 ± 3.9	0.4 ± 0.5	41.3 ± 5.6	2.4 ± 0.5	21.3 ± 4.4	11.2 ± 3.2	8.2
^{51}Cr-labeled endotoxin . .	88.4 ± 4.0	1.7 ± 0.5	5.3 ± 3.5	0.4 ± 0.2	1.3 ± 0.4	0.1 ± 0.02	2.8

[a] Average of at least six separate experimental determinations. Mean ± standard deviation.

TABLE 2. *Subcellular distribution of ^{51}Cr-labeled lipopolysaccharide in livers of normal mice exposed to endotoxin either in vivo or in vitro*

Expt	Percent uptake[a]			
	Nuclear	Lysosomal-mitochondrial	Microsomal	Soluble
In vivo	41.2 ± 8.8	21.7 ± 8.1	20.4 ± 7.3	9.5 ± 2.7
In vitro before homogenization	12.9 ± 2.3	5.9 ± 1.4	70.9 ± 4.4	10.3 ± 2.7
In vitro after homogenization	9.1 ± 1.8	5.5 ± 2.1	80.5 ± 1.5	4.9 ± 1.7

[a] Average of at least six separate experimental determinations. Mean ± standard deviation.

TABLE 3. *Effects of Pronase on cell numbers and sedimentable radioactivity in freshly prepared suspensions of liver cells*

Expt	Cell no. (10^7)			Counts/min (%)[a]	
	Parenchymal	Nonparenchmal			
		Carbon	No carbon	Sedimentable	Supernatant
Before Pronase	8.5 ± 3.5	2.9n± .72	1.2	100	0
After Pronase	0	2.9 ± .66	1.2	23.9 ± 4.7	76.1 ± 4.7

[a] Average of at least six separate experimental determinations. Mean ± standard deviation.

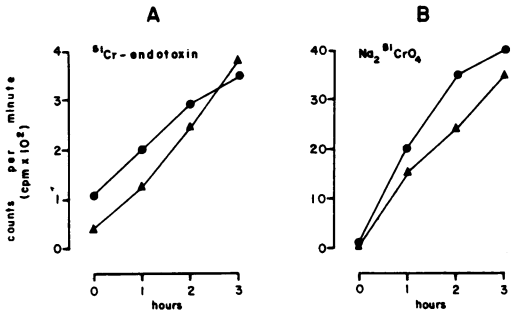

FIG. 1. *Uptake of ^{51}Cr-labeled endotoxin (A) and $Na_2{}^{51}CrO_4$ (B) by HeLa (●) or hepatoma (▲) cells over a 3-h incubation period.*

TABLE 4. *Subcellular localization of chromium-51 and either ^{51}Cr- or ^{14}C-labeled lipopolysaccharide after 3 h of incubation with HeLa or hepatoma cells*

Cell	Percent uptake[a]	
	Nuclear fraction	Cytoplasmic fraction
HeLa		
^{51}Cr-labeled lipopolysaccharide	79.5 ± 1.0	20.5 ± 1.0
Chromium-51	20.0 ± 2.5	80.0 ± 2.5
Hepatoma		
^{51}Cr-labeled lipopolysaccharide	88.0 ± 7.4	12.0 ± 7.4
Chromium-51	31.0 ± 2.9	69.0 ± 2.9
^{14}C-labeled lipopolysaccharide	60.3	39.7

[a] Average of at least six separate experimental determinations. Mean ± standard deviation.

bated with the cells, the nuclear-cytoplasmic ratio was exactly the opposite (Table 4). HeLa cells had only 20% of the cell-associated counts in the nuclear fraction compared with 85% in the cytoplasmic fraction. The same was true for the hepatoma cells.

To further confirm that the nuclear-cytoplasmic ratios were not an artifact of ^{51}Cr-labeled endotoxin, ^{14}C-labeled lipopolysaccharide uptake was investigated in hepatoma cells (Table 4). Once again the majority of the cell-associated counts were in the nucleus, although in this instance the nuclear-cytoplasmic ratio was closer to 60:40 rather than 80:20.

TABLE 5. *Subcellular localization of ^{51}Cr-labeled endotoxin in lymphocytic sarcoma and myeloma cells*

Cell line	Percent uptake[a]	
	Nuclear fraction	Cytoplasmic fraction
S49.1	79.0 ± 15.0	21.0 ± 15.0
S194	87.0 ± 9.4	13.0 ± 9.4
XC-1	83.0 ± 9.9	17.0 ± 9.9
MOPC-21	84.0 ± 10.2	16.0 ± 10.2

[a] Average of at least six separate experimental determinations. Mean ± standard deviation.

Table 5 shows similar subcellular distribution studies using four B-cell tissue culture lines. In all instances, the nuclear-cytoplasmic ratios were approximately 80:20.

DISCUSSION

Preparation of ^{51}Cr-labeled lipopolysaccharide followed the methods of Braude et al. (6) and Chedid et al. (8). Incubation of the isotope with lipopolysaccharide was followed by exhaustive dialysis and ultracentrifugation to insure that the final product was free of unbound $Na_2{}^{51}CrO_4$. Repeated dialysis of the labeled endotoxin over a period of weeks indicated that, once the chromium was attached to the lipopolysaccharide, it remained firmly bound.

The tissue distribution of endotoxin (cf. Table 1) reaffirms previous work indicating the major role played by the liver in clearance of lipopolysaccharide from the blood. Over 85% of the endotoxin was trapped in the liver within 15 min after intravenous injection. The dose of endotoxin (about 0.1 mg) was approximately one-third the 50% lethal dose of endotoxin. The

distribution was not altered when analyzed up to 8 h postinjection (data not shown).

The finding of large amounts of ^{51}Cr-labeled lipopolysaccharide associated with the cell nuclei of liver homogenates was surprising. Willerson et al. (23) had demonstrated localization of ^{14}C-labeled endotoxin from *S. enteriditis* in cell nuclei obtained from livers and spleens of mice injected intravenously. Autoradiographic studies confirmed these data and also delineated label in hepatic cells other than Kupffer cells. Bona (5), using fluorescent and ^{14}C-labeled lipopolysaccharide from *S. typhimurium*, showed pinocytosis of endotoxin by peritoneal macrophages. After attachment to cell membranes and penetration, there was no detectable association with cell nuclei. We had presumed that Kupffer cell phagocytosis of lipopolysaccharide would be the major clearance mechanism in the liver and would result in concentration of label in the mitochondrial-lysosomal fraction. Instead, the greatest quantities of lipopolysaccharide were associated with the nuclear fraction. Nuclear binding was not an artifact of homogenization since in vitro incubation of liver suspension with ^{51}Cr-labeled lipopolysaccharide both before and after homogenization (cf. Table 2) showed much less nucleus-associated lipopolysaccharide with a corresponding increase in the microsomal fraction. ^{51}Cr-labeled lipopolysaccharide alone sedimented at 100,000 $\times$ *g*.

Treatment of liver cell populations with Pronase selectively destroyed parenchymal cells, leaving nonparenchymal cells intact and viable (11, 16). This principle was used to determine whether ^{51}Cr-labeled lipopolysaccharide was trapped in vivo in parenchymal as well as nonparenchymal cells. Nonparenchymal cells could be distinguished from parenchymal cells by size and carbon uptake and were monitored for cell counts and viability (trypan blue stain) both before and after Pronase treatment. Pronase treatment had no detectable effect on the nonparenchymal cell numbers (Table 3). Initial cell counts showed approximately a 65:35 ratio of parenchymal cells to nonparenchymal cells, a ratio consistent with previously reported results (7). Parenchymal cells were completely destroyed by Pronase, and with their lysis over 70% of the previously cell-associated and sedimentable radioactivity was in the supernatant. To our knowledge this is the first quantitative data showing that non-RES cells of the liver are involved in endotoxin clearance. Since non-RES cells (parenchymal cells) can take up endotoxin in vivo, it was of interest to determine whether the same was true in tissue culture cells in vitro. If so, tissue culture could provide a much less expensive and more controllable environment for studying endotoxin-cell interactions. The demonstration that hepatoma cells as well as five other non-RES cell lines could concentrate endotoxin in their nuclei (cf. Fig. 1 and Tables 4 and 5) confirms our in vivo findings. The ability of ^{14}C-labeled lipopolysaccharide to concentrate in nuclei further showed that this observation is not an artifact of chromium labeling. Uptake of lipopolysaccharide was linear with time over the 3-h test period. Serum was omitted from the present uptake studies to avoid potential interactions with the complement system (21) or serum esterases (19) that may complex to or inactivate endotoxin. It may be, however, that serum could enhance uptake, and experiments to analyze the effects of serum on tissue culture cell uptake are currently under way.

Our ability to routinely demonstrate endotoxin in eukaryotic cell nuclei could be highly significant to understanding why the adaptive liver enzymes tryptophan oxygenase and phosphoenolpyruvate carboxykinase fail to be inducible by cortisone in endotoxin-poisoned mice. It has been shown in recent years that cortisone binds to cytoplasmic receptors that transport the steroid to cell nuclei before stimulation of deoxyribonucleic acid-dependent ribonucleic acid synthesis (10, 22). If the association of endotoxin with eukaryotic chromatin blocks the ability of steroid hormones to act, then this effect would be highly significant in understanding the molecular basis of the pathophysiology of endotoxemia. We have been able to demonstrate in preliminary experiments that endotoxin does bind to isolated chromatin in vitro, and the effects of this interaction on steroid binding are currently under active investigation in our laboratory.

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