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Subcellular Localization of *Salmonella enteritidis* Endotoxin in Liver and Spleen of Mice and Rats

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Salmonella enteritidis ^{14}C-endotoxin was recovered predominantly from the nuclear and mitochondrial subcellular fractions of livers and spleens of mice and rats, 3.5 hr and 3 days after intravenous administration. Of the recovered radioactivity, 10 to 20% was present in the liver mitochondrial fraction as high-molecular-weight, biologically active material, suggesting the presence of intact endotoxin. Autoradiographic studies demonstrated nuclear and cytoplasmic labeling in the liver and at least nuclear label in spleen cells. The resistance of rats, as compared to mice, to the induction of amyloidosis does not appear to be based on a difference in subcellular localization of endotoxin within the reticuloendothelial system.

It has recently been reported that *Escherichia coli* and *Salmonella* endotoxins can induce murine amyloidosis (1; W. F. Barth et al., Arthritis Rheum, *in press*). Amyloid first appears in the spleen and liver, the primary organs in which endotoxin is found after parenteral injection (2). Further studies have shown that amyloid can be induced in recipient mice after transfer of splenic subcellular fractions from animals with endotoxin-induced amyloid (3). Little information is available regarding subcellular localization of endotoxin in liver and spleen. The present study was undertaken to characterize the cellular and subcellular localization of ^{14}C-labeled *S. enteritidis* endotoxin in these organs in two species: mice, which develop amyloid after endotoxin injection, and Sprague-Dawley rats, in which amyloid has not to date been inducible by endotoxin.

MATERIALS AND METHODS

S. enteritidis endotoxins. Purified ^{14}C *S. enteritidis* endotoxin was prepared from cells labeled by using ^{14}C-glucose as the only carbon source and kindly donated by Jon A. Rudbach (National Institute of Allergy and Infectious Diseases). Unlabeled *S. enteritidis* endotoxin was obtained from the same source (4). The radioactively labeled preparation was diluted with unlabeled endotoxin to a concentration of 2 mg/ml in phosphate-buffered saline (*p*H 7.4). The specific activity of the final endotoxin preparation was 1.5×10^6 counts per min per mg.

Administration of endotoxin. Six-week-old male NIH G.P. (Swiss) mice and Sprague-Dawley rats were obtained from the Laboratory Aids Branch of the National Institutes of Health. All injections were administered intravenously into a lateral tail vein. Needles, syringes, and media were sterile and pyrogen-free.

Sublethal amounts of endotoxin were used. A 75-μg amount of the ^{14}C-labeled *S. enteritidis* endotoxin was given in a volume of 0.5 ml of Eagle's medium to the mice and 180 μg in the same volume to the rats. At 3.5 hr and 3 days later, separate groups of at least three treated animals were sacrificed. Livers and spleens were removed, and subcellular fractions were prepared. Portions of the livers, spleens, and kidneys were taken from the mice for autoradiography. Certain mice and rats were pretreated with unlabeled *S. enteritidis* endotoxin. One intravenous injection of 75 μg was given every third day for 9 days (total of three injections). The ^{14}C-labeled *S. enteritidis* endotoxin (75 μg to mice and 180 μg to rats) was given 24 hr after the last unlabeled injection.

Preparation of subcellular fractions. Liver and spleen fractions were prepared (5), utilizing the technique of equilibrium fractionation (6). Protein determinations were done by the method of Lowry et al. (7).

Liquid scintillation counting. Duplicate 0.1-ml portions of each of the subcellular fractions suspended in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 7.5) were counted in 10 ml of Bray's solution (8) in a liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Channel ratios to study quenching and self-absorption were obtained, and appropriate corrections were made.

Acid precipitation. A 1/10-ml amount of each of the subcellular fractions to be tested was precipitated in 5% trichloroacetic acid. After 15 min in ice, the precipitates were collected on a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.) and washed twice with 5 ml of trichloroacetic acid. The filters were removed and placed in a counting vial containing 10 ml of Liquifluor (New England Nuclear Corp., Boston, Mass.) for scintillation counting.

Extraction of ^{14}C-labeled material from subcellular fractions. Portions of washed liver nuclear and mitochondrial fractions containing radioactivity from ^{14}C-labeled *S. enteritidis* lipopolysaccharide were suspended in 2 ml of water. These were extracted four times with an equal volume of 90% phenol at 65 to 68 C, by the method of Westphal and Jann (9).

The pooled aqueous layer was dialyzed with repeated changes against 0.01 M phosphate buffer (*p*H 7.0) containing 0.1 M NaCl. Since both endotoxin and ribonucleic acid (RNA) are extracted into the aqueous phase by phenol, this dialysis was followed by treatment with bovine pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N.J.) and dialysis against water (10). Samples were treated with cold 5% trichloroacetic acid to determine the amount of high-molecular-weight material present in the ribonuclease-resistant dialyzed extracts.

The material in the phenol layer was examined for lipids after removing phenol either by extraction into ether or by extensive dialysis against water. A portion of the resultant phenol-free material was extracted three times with equal volumes of water-saturated butanol at 0 C or with chloroform: methanol (2:1) at 0 C. The amounts of extracted radioactive material was counted, and portions were then checked for biological activity.

Biological activity studies. Material from the aqueous and phenol layers of the phenol-extracted subcellular fractions were tested in rabbits for pyrogenic activity. The approximate amount of endotoxin in the aqueous layers was estimated from the counts remaining after dialysis and ribonuclease treatment. Samples (1 or 2 ml) of material were given intravenously to New Zealand albino rabbits of both sexes weighing 1.5 to 2.5 kg from the same colony (NIH). Control liver mitochondrial and nuclear fractions from untreated mice and rats were also prepared, phenol extracted, dialyzed, and given to rabbits intravenously. Details of training, temperature recording, and determination of the fever index were as previously reported (11).

Autoradiography. Samples of liver, kidney, and spleen taken from previously untreated mice 3.5 hr and 3 days after the ^{14}C endotoxin injection were fixed in either buffered formaldehyde or in buffered glutaraldehyde followed by osmium tetroxide. The tissues were dehydrated in ethanol and embedded in Araldite (Ciba 6005). Sections 1.5 μm thick were cut on a Porter-Blum MT-2 Microtome, mounted on glass slides, and dip-coated in Kodak NTB-3 Nuclear Track Emulsion. The coated sections were stored with Drierite at room temperature for 3 weeks to 3 months. The autoradiograms were developed in D-19 for 2 min at 18 C and fixed in Kodak Rapid Fix. After thorough washing, the tissues were stained with 0.25% toluidine blue.

RESULTS

Subcellular localization of endotoxin. It was found that 50% of the injected radioactivity was recovered in the liver, spleen, kidneys, lungs, and sera of the experimental animals 3.5 hr after intravenous injection of endotoxin. The liver contained 60% of the recoverable counts (30% of the injected counts), and the spleen and serum contained an additional 25% of the recoverable radioactivity. Of the radioactivity in the liver, 25% was recovered in the prepared subcellular fractions. Most of the unrecovered radioactivity could be accounted for on the walls of containers used to prepare the subcellular fractions.

Label from ^{14}C *S. enteritidis* endotoxin was demonstrated in the nuclear and mitochondrial subcellular fractions of both the liver and spleen (Table 1). The microsomal and supernatant fractions of these organs had a much smaller percentage of recoverable counts. More than 40% of the counts in the subcellular fractions were acid-precipitable, except in the liver supernatant fraction where only 26% were acid-precipitable.

The distribution of label in the four subcellular fractions isolated from liver and spleen was similar for mice and rats, whether or not they were pretreated with endotoxin. The amount of radioactivity recovered in the liver and spleen fractions of mice and rats 3 days after administration of endotoxin was 30 to 50% of the counts present in the same fractions 3.5 hr after administration of endotoxin.

Recovery of labeled material from subcellular fractions. Hot phenol extracted 20 to 40% of the labeled material from the nuclear and mitochondrial fractions (Table 2). Half of these counts remained after extensive dialysis, and 70% of these counts were trichloroacetic acid-insoluble. This high-molecular-weight material had biological activity (*see below*).

To determine whether any of the material in the phenol layer represented lipid liberated from degraded lipopolysaccharide, this layer was rendered phenol-free either by extraction with ether or dialysis against water. The phenol-free material was then extracted with butanol or chloroform-methanol. Only an additional 10 to 15% of the counts was extracted. This phenol-free material did show biological activity (*see below*), indicating the presence of endotoxin.

Biological activity of the recovered subcellular material. Material extracted by phenol from the liver mitochondrial fractions of pretreated and previously untreated mice and rats produced a

TABLE 1. *Subcellular localization in the livers and spleens of mice and rats 3.5 hr after one injection of ^{14}C-labeled S. enteritidis endotoxin*[a]

Tissue fraction	Mice[b]			Rats[c]
	Total counts per min recovered	Counts per min per mg of protein	Trichloroacetic acid-precipitable	Total counts per min recovered
			%	
Liver (nuclear fraction)	2,121	359	50	2,800
Liver (mitochondrial fraction)	3,270	386	50	8,600
Liver (microsomal fraction)	135	23	100	100
Liver (supernatant fraction)	348	128	26	200
Spleen (nuclear fraction)	486	121	43	100
Spleen (mitochondrial fraction)	147	35	42	260
Spleen (microsomal fraction)	0			0
Spleen (supernatant fraction)	0			0

[a] Dosage: mice received 75 μg (112,500 counts/min); rats received 180 μg (270,000 counts/min).
[b] A 1,000-μg amount of liver or 125 mg of spleen.
[c] Data obtained from counting 300 mg of sample of liver and spleen and extrapolating to total organ weight.

TABLE 2. *Phenol extraction of subcellular liver fractions from mice and rats treated with S. enteritidis endotoxin*

Subcellular fraction	Time	Total counts	Phenol extracted counts before dialysis	Counts after dialysis and ribonuclease treatment	Counts extracted	Acid-precipitable (per cent of extracted counts)	Approximate amt of endotoxin[a]	FI (cm^2)[b]
					%			
Expt. 1[c]								
Mice nuclear	3.5 hr	3,160[d]	780	318	11	62	0.04	9
Mice nuclear	3 days	1,920	639	364	18	74	0.05	4, 5
Mice mitochondrial	3.5 hr	9,480	2,208	1,525	19	79	0.20	26, 28
Mice mitochondrial	3 days	3,040	864	715	22	70	0.09	16, 15
Expt. 2[c]								
Mice mitochondrial (P)	3.5 hr	6,311[e]	—	1,683	27	—	0.44	59
Rats mitochondrial (N)	3.5 hr	3,101	—	345	11	—	0.10	22, 16
Rats mitochondrial (P)	3.5 hr	7,091	—	1,696	24	—	0.43	51

[a] Expressed in micrograms.
[b] FI, individual fever indexes; temperature (C) during a 5-hr period in individual recipient rabbits. Rabbits given control liver mitochondrial samples from mice and rats had FI of 9.
[c] Experiment 1, mice were not previously given endotoxin; experiment 2, P, previously given endotoxin; N, not previously given endotoxin.
[d] 1,500 mg of tissue.
[e] 1,000 mg of tissue.

fever spike suggestive of varying amounts of endotoxin when given to rabbits (Fig. 1; Table 2). The material obtained from control subcellular fractions was not pyrogenic. A lower but still definite fever spike was obtained from material extracted from the liver mitochondrial subcellular fractions as long as 3 days after the labeled endotoxin injection (Table 2).

Autoradiography. At 3.5 hr after the intravenous administration of ^{14}C-labeled *S. enteritidis* endotoxin, the autoradiographs of the spleen showed label predominantly over the red pulp. A variety of different cell types within the red pulp were labeled, but a small mononuclear cell with an intensely basophilic nucleus 4 to 6 μm in diameter was particularly heavily labeled (Fig. 2). This small mononuclear cell is a prominent cellular element in the red pulp and at the light microscopic level appears to have only a narrow rim of cytoplasm. In the kidney, the greatest concentration of label was found over the glomeruli and the interlobular vessels. Renal

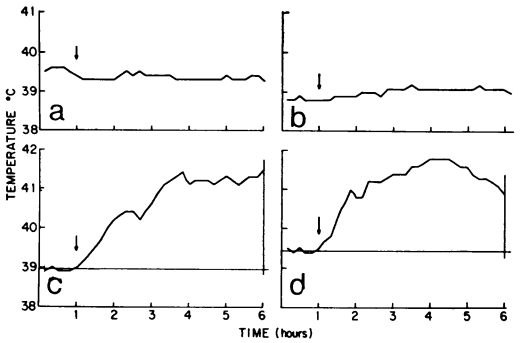


FIG. 1. *Febrile responses of rabbits injected with material phenol-extracted from the mitochondrial fractions from (a) control rat liver; (b) rat liver 3.5 hr after endotoxin administration; (c) control mouse liver (right upper graph); and (d) mouse liver 3.5 hr after endotoxin administration (right lower graph). The injections were given at 1 hour (arrow).*

tubercular cells were only slightly labeled. In the liver, label was present over the hepatic cells, Kupffer cells, and vessels in the portal spaces (Fig. 3). The relative density of labeling over Kupffer and hepatic cells varied considerably. In some regions, little or no radioactive material was present over Kupffer cells, whereas adjacent hepatic cells showed prominent uptake over both the cytoplasm and nucleus. In other regions, the Kupffer cells showed quite prominent labeling and adjacent hepatic cells were only slightly labeled. In general, the density and extent of labeling over the Kupffer cells was greater than that of the hepatic cells.

At 3 days after the endotoxin, the autoradiographs of the liver demonstrated less striking Kupffer cell labeling as compared to the 3.5-hr specimens. In the spleen, the pattern was essentially the same as had been noted in the 3.5 hr tissue.

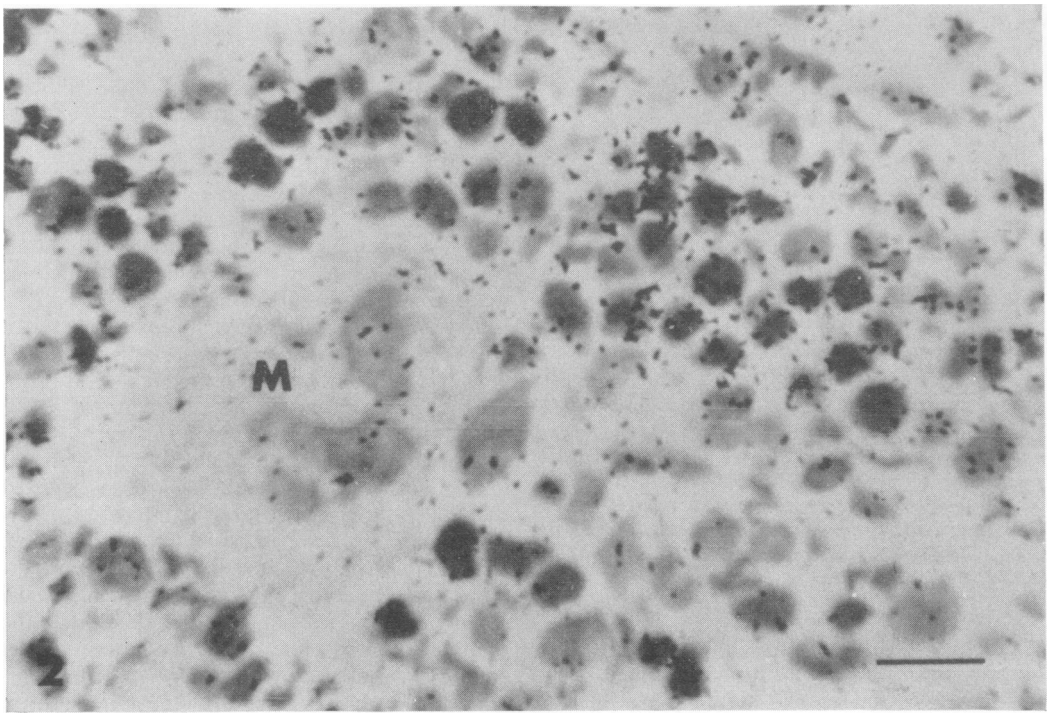
DISCUSSION

Intravenously administered ^{14}C-labeled *S. enteritidis* endotoxin was recovered predominantly from the nuclear and mitochondrial fractions prepared from livers and spleens of mice and rats. Some of the initially administered endotoxin could be recovered, up to 3 days after its administration, as high-molecular-weight, biologically active material. The autoradiographic data support the cell fractionation studies. Label is clearly seen in the nucleus of both liver and spleen cells, confirming that endotoxin or a part of the endotoxin molecule localizes in the nucleus. The cytoplasmic localization, evident but not defined by autoradiography, was studied through the fractionation procedures. Results indicate localization of endotoxin in an organelle found in the mitochondrial fraction: leading possibilities are mitochondria themselves or lysosomes, which are found in this fraction (12).

Approximately 20 to 40% of the recovered label was phenol extractable, and 50% of these counts remained after dialysis. Ribonuclease treatment released no further counts. Pyrogenic activity characteristic of endotoxin remained after the chemical procedures, indicating that at least a portion of the recovered label was probably intact endotoxin. A large portion of radioactivity was not extracted by phenol (60 to 80%). If these counts represented lipid from degraded lipopolysaccharide, it would have been expected that most should have been extracted with organic solvents. However, less than 15% of these counts could be extracted in this way. Furthermore, the remaining material retained biological activity. It appears possible, therefore, that the non-phenol-extractable material is grossly intact endotoxin, possibly bound to protein.

Certain endotoxins rapidly induce amyloid in mice (1; W. F. Barth et al., Arthritis Rheum., *in press*), suggesting that the endotoxin molecule or some portion of it stimulates the production of the amyloid protein. Furthermore, endotoxin is preferentially localized in those organs which show early signs of amyloid. Localization was studied in a strain in which amyloid was inducible (mice) and not inducible (rats). Significant differences between mice and rats were not seen. The apparent resistance of the rat to experimental induction of amyloidosis, therefore, does not appear explicable on the basis of a difference in subcellular localization of endotoxin but may result from differences in events after subcellular localization.

Amyloidosis can be transferred by subcellular fractions from an appropriately treated donor. The mitochondrial subcellular fraction is the one most potent in the transfer (3) and, from these present studies, also appears to be a prominent fraction in which endotoxin localizes. We have not been able to implicate endotoxin per se in the transfer phenomenon, but the evidence presently available suggests that endotoxin may initiate directly or indirectly a series of events that lead to the production of amyloid. It may be that this series of events, from endotoxin entrance into a cell to production of amyloid, occurs within the same cells. Although interesting and provocative, the relationship of the localization of endotoxin to amyloid formation is presently not clear.



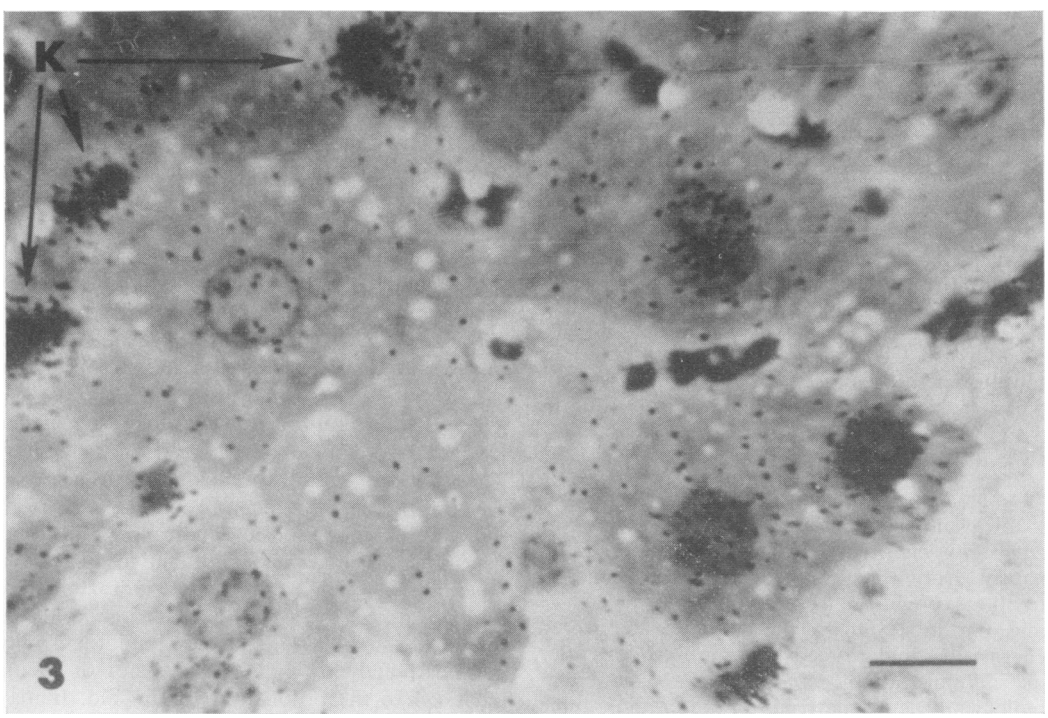


FIG. 2. *Autoradiogram of the red pulp from a mouse spleen 3.5 hr after administration of* ^{14}C*-endotoxin. A variety of different cell types are illustrated, including a megakarocyte (M). Label is most prominent over small round cells with basophilic nuclei.* × *1,420. Bar, 10 μm.*

FIG. 3. *Autoradiogram of mouse liver 3.5 hr after administration of* ^{14}C*-endotoxin. Label is prominent over both Kupffer cells (K) and hepatocytes. In the liver hepatocytes, label is present over both nucleus and cytoplasm.* × *1,420. Bar, 10 μm.*

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