Distribution of Radiolabeled Endotoxin with Particular Reference to the Eye: Concise Communication

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A single systemic injection of endotoxin (lipopolysaccharide or LPS) reproducibly induces a cellular infiltrate in the uveal tract of the rat eye within 24 hr. Other organs are not comparably sensitive to systemic endotoxin. One hypothesis to explain this unique sensitivity is that endotoxin is preferentially bound by ocular tissue. We tested this hypothesis by studying the distribution in the rat of intravenously injected endotoxin that had been radiolabeled with Tc-99m or P-32. With either radionuclide the concentration of endotoxin per gram of tissue at a variety of times after injection ranging from 5 min to 3 hr and 45 min, was markedly less in the eye than in liver, kidney, or spleen. A study with radiolabeled albumin indicated that these differences could not be ascribed solely to the organ's blood volume. They demonstrate, therefore, that the eye does not preferentially bind endotoxin, and they are compatible with the hypothesis that endotoxin's ocular effects are indirectly mediated.

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There is increasing clinical evidence that in some patients Reiter's syndrome is secondary to a bacterial infection of the gut, in particular by Gram-negative rods. One of the features of Reiter's syndrome, uveitis, has been produced in rats by the systemic injection of endotoxin from Gram-negative bacilli (*1*). The dose of endotoxin required to induce eye disease did not alter the light-microscopic appearance of the heart, lung, liver, kidney, spleen, or colon (*1*). In order to determine why the eye should be uniquely predisposed, we have radiolabeled endotoxin and studied its distribution after intravenous injection in rats. Despite studies with radiolabeling endotoxin in vivo (*2–11*) no prior study has included the eye as an organ of interest. In order to minimize the possibility that radiolabeling might preferentially label a biologically inactive portion of the endotoxin molecule, we have assessed the intraocular distribution with two nuclides, P-32 and Tc-99m, the former because it allows internal labeling and, therefore, minimal alteration of the labeled molecule; the latter because of its availability, relative safety, and utility for dynamic imaging.

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METHODS

The endotoxin used for the technetium-labeled studies was either *E. coli* lipopolysaccharide (LPS) 055:B5* or *Salmonella typhimurium.*† LPS labeled with P-32 was derived from *Shigella flexneri* 2a. Previous work had indicated that LSP from all of these sources was capable of inducing uveitis (*1*). LPS was labeled with Tc-99m by the method of Hamilton and Walker (*2*). In brief, 3 mCi of pertechnetate were added to 10 mg of LPS in the presence of 1 ml saline and 1 ml 1.0 m*M* stannous chloride. After incubating the mixture for 10 min at room temperature, the solution was passed through a Sephadex G25 column. One-ml aliquots were collected and a discrete separation shown between free Tc-99m and labeled LPS.

For P-32 labeling, a *S. flexneri* Type 2a strain, iso-

lated from an epidemic of enteric dysentery, was grown up in 1 l of brain-heart broth* containing 20 mCi of P-32 as phosphate. Bacteria were grown for 20 hr at 37°, harvested and washed three times with each of the following: sterile pyrogen-free water, 95% ethanol, acetone, and ether. Dried bacteria were suspended in 700 ml of 50% aqueous phenol (v/v), shaken at 68° for 15 min, cooled at 10°, and centrifuged to separate the layers. The phenol layer was extracted with water at 68°, then centrifuged. Aqueous layers were pooled, dialyzed, and lyophilized. Crude LPS was dissolved in 150 ml of water, treated with 2% Cetavalon, and centrifuged to remove precipitated RNA. The supernatant was lyophilized, dissolved in 50 ml of 0.5 *M* NaCl, poured into 500 ml of 95% ethanol at 4°, precipitated over a 2-hr period, and centrifuged. The precipitated LPS was redissolved, dialyzed, and lyophilized. The yield was 50 mg of LPS and the specific activity was ~0.3 μCi/mg. *Limulus* amebacyte assay kit for endotoxin was purchased commercially (*12*) and the test was performed in the standard manner (*12*) with inclusion of positive and negative controls.

Male Lewis rats, 3 to 6 mo of age and ranging in weight from 225 to 300 g, were used for the Tc-99m LPS studies, and similar Sprague-Dawley rats for the P-32 studies. After light ether or pentobarbital anesthesia, rats were injected with radionuclides (0.1–0.2 mCi Tc-99m intravenously through the dorsal vein of the penis and were killed with additional anesthetic at specific times. Organs were removed immediately, weighed, and Tc-99m and I-125 (see below) radioactivity counted in an auto gamma counter. For P-32, a portion of each organ was solubilized with Protosol and counted in a beta scintillation counter. Results for each radionuclide were calculated as a percentage of injected radioactivity per organ and per gram of organ.

Eyes were fixed in hematoxylin and eosin as previously described (*1*). The definition of uveitis was the presence of at least five inflammatory cells in the uveal tract.

Iodine-125 albumin‡ was injected intravenously in a volume of 0.1 ml (1 mCi) into three rats that simultaneously received Tc-99m LPS, and into three that received only $^{99m}TcO_4^-$.

External imaging was done with a portable gamma camera with high-resolution collimator (*5*). Statistical analysis used Student's t-test.

RESULTS

Table 1 shows the distribution of Tc-99m-labeled endotoxin at 5, 15, and 60 min after injection. A very small percentage of the injected endotoxin was present in the eye (0.01–0.02%) and remained essentially constant over one hour. Calculated as the amount of radioactivity in the eye per unit organ weight, far less endotoxin was present per gram than in any other organ measured. For the liver, kidney, and spleen, the concentration per gram was approximately 25 to 100 times that of the eye for each of the times of determination.

In order to show that the Tc-99m-labeled LPS was still biologically active, eye histology was assessed in a rat 24 hr after injection of labeled endotoxin. The typical picture of endotoxin-induced uveitis—the presence of polymorphonuclear leukocytes in the anterior uveal tract—was evident. In addition, the Tc-99m-labeled material was capable of gelling the *Limulus* amebacyte lysate whereas the free technetium was endotoxin-free according to this assay.

Table 2 shows the distribution of free technetium as pertechnetate 1 hr after i.v. injection. Technetium-99m was concentrated by the stomach, and did not show the pronounced uptake in the reticuloendothelial system seen with Tc-99m-labeled endotoxin. The distribution was statistically different from that of Tc-99m endotoxin for all organs except the eye.

Table 3 shows the distribution of the internally labeled P-32 endotoxin. The results are comparable to the results with Tc-99m endotoxin except that the values for the

TABLE 1. DISTRIBUTION OF Tc-99m-LABELED ENDOTOXIN*

	5 min			15 min			60 min		
Organ	% LPS organ	Range	% LPS g tissue	% LPS organ	Range	% LPS g tissue	% LPS organ	Range	% LPS g tissue
Liver	28.4	(26.2–29.2)	2.7	30.6	(22.3–43.7)	2.9	32.4	(24.0–39.0)	2.5
Spleen	2.0	(1.7–2.3)	2.0	2.0	(1.9–2.1)	3.3	2.0	(1.6–2.3)	4.4
Kidneys	5.5	(4.3–7.1)	2.1	9.9	(7.8–11.9)	3.8	5.3	(4.6–5.8)	2.1
Heart	0.5	(0.4–0.5)	0.5	0.3	(0.2–0.5)	0.3	0.2	(0.16–0.26)	0.2
Lung	3.3	(2.7–4.2)	2.1	1.4	(0.5–2.1)	0.8	1.9	(1.7–2.1)	0.8
Stomach	0.5	(0.4–0.6)	0.2	0.4	(0.3–0.5)	0.3	0.6	(0.4–0.7)	0.4
Eyes	0.01	(0.005–0.01)	0.03	0.01	(0.002–0.02)	0.03	0.016	(0.008–0.02)	0.0
Blood			2.7			1.1			0.6

* Mean uptake of Tc-99m endotoxin at 5, 15, and 60 min after intravenous injection. Each point is average of three results.

TABLE 2. DISTRIBUTION OF Tc-99m (as $^{99m}TcO_4^-$)*

Organ	Mean % dose/organ	Range	% Dose/ g tissue
Liver	4.8†	4.1–5.5	0.4†
Spleen	0.07†	0.07–0.08	0.16†
Kidneys	1.0†	0.9–1.0	0.8†
Heart	0.1†	0.1	0.2‡
Lung	0.6§	0.5–0.7	0.3§
Stomach	2.7§	1.9–3.9	1.8§
Eyes	0.01NS¶	0.01–0.02	0.09NS

* Data represent mean values for three Lewis rats, killed 1 hr after i.v. injection of radionuclide.
† $p < 0.01$ (compared with Tc-99m toxin).
‡ $p < 0.02$ (compared with Tc-99m toxin).
§ $p < 0.05$ (compared with Tc-99m toxin).
¶ NS = Not statistically different from Tc-99m toxin result.

liver, while greater than for any other organ by 3 hr and 45 min, tended to be lower than those obtained with Tc-99m-labeled LPS. The values for the eye were slightly greater than those obtained with Tc-99m-labeled endotoxin, but still showed minimal radioactivity within the eye.

Table 4 shows the distribution of I-125 albumin at 1 hr after injection into endotoxin-treated animals and controls. This provides an estimate of the blood volume within any given organ, since most albumin remains intravascular over this time. The lower values for blood, heart, and kidney in the animals that received endotoxin were compatible with either an expansion of the intravascular volume after endotoxin and/or permeability changes that permit leakage of albumin at other sites. The percentage of injected radioactive albumin in the eye per gram of tissue was comparable to the percentage of radioactive endotoxin found within the eye, whereas certain organs such as liver and spleen clearly showed enhanced uptake of endotoxin relative to albumin.

TABLE 3. DISTRIBUTION OF P-32-LABELED ENDOTOXIN

	10 min			3 hr, 15 min		
Organ	% LPS organ	Range	% LPS g tissue	% LPS organ	Range	% LPS g tissue
Liver	10.8	(6.6–14.2)	0.8	17.7	(16.8–18.4)	1.3
Spleen	1.2	(0.7–1.7)	0.8	1.4	(1.2–1.7)	1.1
Kidneys	8.1	(7.8–8.5)	3.4	2.2	(2.1–2.4)	0.9
Heart	0.6	(0.5–0.6)	0.5	0.7	(0.6–0.8)	0.6
Lung	1.1	(0.8–1.5)	0.7	0.8	(0.6–1.2)	0.5
Stomach	1.0	(0.5–1.4)	0.4	0.5	(0.1–0.9)	0.3
Eyes	0.04	(0.04)	0.1	0.03	(0.03	0.1

Uptake of P-32 LPS (average of three measurements) after intravenous injection of ~300 μg P-32 endotoxin.

TABLE 4. DISTRIBUTION OF I-125 ALBUMIN

Organ	LPS-treated animals % dose/g	Range	Control animals % dose/g	Range
Liver	0.3	(0.2–0.4)	0.4	(0.3–0.6)
Spleen	0.2	(0.1–0.2)	0.2	(0.2)
Kidneys	0.6	(0.5–0.7)	0.9	(0.8–1.1)
Heart	0.2	(0.2)	0.2	(0.2–0.3)
Lung	0.6	(0.4–0.8)	0.7	(0.6–0.9)
Stomach	0.2	(0.2)	0.4	(0.1–0.9)
Eye	0.03	(0.02–0.03)	0.09	(0.04–0.2)
Blood	0.7	(0.6–0.8)	1.3*	(1.0–1.6)

Labeled albumin and either radiolabeled endotoxin or free technetium (control) were injected i.v. 1 hr before animals were killed. To avoid interference from Tc-99m emissions, I-125 radioactivity was determined 72 hr (12 half-lives) after the Tc-99m injection. Results are means for three rats and range.

* The difference between the control animals and endotoxin treated animals is statistically significant, $p < 0.05$.

There was no uptake noted in the eyes on the gamma-camera images, although other organs such as liver and spleen could be clearly visualized.

DISCUSSION

The radiolabeling of endotoxin has been a valuable method for studying the in vivo distribution of LPS (*2–11*). These studies have utilized a variety of species, doses, and tracers, including Tc-99m (*2*), I-125 (*3*), Cr-51 (*4–7*), and P-32 (*8–11*). The eye has been omitted from these investigations because its unique susceptibility to endotoxin has only recently received emphasis. Phosphorus-32 is a valuable nuclide for radiolabeled LPS studies since bacteria can be grown in radioactive media and can therefore incorporate the tracer into the lipopolysaccharide portion of the cell wall. Technetium offers the advantage of availability, high count rates, and imaging by gamma camera. Using both of these radionuclides, we were unable to demonstrate a preferential uptake of endotoxin in the eye. Our experience with the two tracers yielded essentially concordant results. With either P-32 or Tc-99m, liver, kidney, and spleen show an enhanced binding of endotoxin, whereas the distribution of endotoxin in organs such as heart, stomach, and eye can be ascribed largely to the relative blood volumes.

Several explanations could account for the inability to demonstrate an increased uptake of endotoxin in the eye.

1. We have failed to label the active moiety of the endotoxin molecule. Preferential binding might then be occurring but would not be reflected by the observed counts. This possibility is remote, since the active moiety of LPS is lipid A (*13*), which is rich in phosphorus and therefore should be readily labeled by P-32.

2. Radiolabeling destroys the activity of endotoxin. The results of eye histology, *Limulus* gelation, and internal labeling argue against this.

3. We have not assessed increased binding at the proper time. Since endotoxin induces changes of ocular permeability in the rat (*14*) and rabbit (*15*) within one hour of injection, it seems unlikely that a preferential binding would be delayed beyond this time.

4. Preferential binding occurs in a portion of the eye (for example the uveal tract), but this binding is obscured when radioactivity is averaged for the entire organ. This possibility cannot be completely ruled out by the present data but essentially all of the radioactivity detected within the eye can be accounted for by the intraocular vascular volume as estimated from the study with labeled albumin.

The data then are most compatible with the hypothesis that the effects of endotoxin on the eye are not mediated by preferential binding of LPS to ocular tissue. In both the rat (*16*) and rabbit (*17,18*), prostaglandins, thromboxane, and other inflammatory mediators appear to mediate endotoxin's ocular effects. The data presented here are certainly in accord with the possibility that these eye effects are indirectly determined by local production of arachidonic acid metabolites, or perhaps by platelet aggregation (*19*) and mediator release.

A secondary benefit of this study is the verification of the utility of technetium-labeled endotoxin studies. By examining eye histology and *Limulus* gelation after Tc-99m labeling, we have shown that the radiolabeled molecule retains its biologic activity and, therefore, have extended the work of previous investigators (*2*). Since dynamic imaging is feasible with this radionuclide, our method should prove useful in producing dynamic pictures of phenomena that require more than one LPS injection, such as the Shwartzman reaction and endotoxin tolerance.

FOOTNOTES

* Difco.
† Sigma.
‡ Mallinckrodt, St. Louis.

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