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Distribution and Degradation of Sublethal Doses of I^{125} Labeled Endotoxin from *Salmonella enteritidis* in Mice* (33907)

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Injection of minute quantities of lipopolysaccharide extracted from cell walls of gram-negative organisms (endotoxin) produces a variety of physiological responses of several target organs. Information about the distribution of endotoxin in the target organs would be helpful in elucidating the mechanism of those effects, and radioactive labeled endotoxins have been employed for this purpose.

Braude and co-workers (1, 2) studied the distribution of lethal doses of ^{51}Cr labeled endotoxin from *E. coli* injected intravenously in rabbits and mice, and attempted to correlate the physiological response with the distribution of radioactivity. Similarly, Starzecki *et al.* (3) studied the plasma clearance and tissue distribution of ^{51}Cr labeled endotoxin in normal and endotoxin-resistant dogs. Other workers (4–6) also studied the distribution of endotoxins from other bacterial species labeled with other isotopes. However, in these studies a lethal dose of endotoxin had to be injected because of the low specific activity of the endotoxin. High specific activity can be obtained by labeling purified endotoxin from *Salmonella enteritidis* with ^{125}I (7). We have investigated the possibility that this preparation might be useful in studying the distribution of small nontoxic amounts of endotoxin, but serum enzymes degrade the endotoxin molecule *in vitro*, removing a large part of the radioiodinated lipid from the bulk of the lipopolysaccharide (8). If similar degradation occurs *in vivo*, interpretation of the results of tracer injection studies would become complex, for part of the radioactivity would be iodinated fatty acids or free iodide. The present experiments on the distribution of radioactivity after injection of ^{125}I labeled endotoxin in mice showed that degradation of the endotoxin complex does indeed occur *in vivo* and the radioactivity in some tissues is due more to iodine freed from the endotoxin than to the localization of the injected endotoxin complex itself. By comparing the distribution of radioactivity after injection of chromium and iodine labeled endotoxin some information can be obtained about the distribution of the different parts of the endotoxin complex.

Methods. Experimental animals. Swiss Webster mice of either sex weighing 20–25 g were housed in cages containing 4–5 animals, given food and water *ad libitum* and kept for 2 hr at 25 or 37° before the intravenous injection of endotoxin and held at the same temperature for the duration of the experiment. At various times after injection the animals were anesthetized with ether, decapitated and the blood was collected directly

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into counting tubes. The organs were dissected and counted separately. The thyroid was included with the head. Blood and clots in the carcass and organs were absorbed as carefully as possible with absorbent paper. The counts of the paper and the counts of the blood obtained by exsanguination were added to give the total radioactivity of blood. No effort was made to collect and count the urine or feces. The samples were counted in a Packard Autogamma scintillation counter. The results were expressed as percentage of the total injected radioactivity recovered in each of the various organs.

Endotoxin. Detailed methods of preparation of the endotoxin and isotope labeling have been described (7). Briefly, the endotoxin was a 68% alcohol precipitate of the aqueous ether extract of the cell wall of *Salmonella enteritidis* grown in a synthetic medium. It was labeled with ^{125}I using chloramine T. Most of the label is on the lipid portion of the complex. More than 95% of the radioactivity of the freshly labeled endotoxin can be co-precipitated with specific antibody by half saturated ammonium sulfate. The LD_{50} of this preparation for this strain of mice kept at 25° was 15 μg and at 37° was 6 μg. One μg of the endotoxin preparation with a ^{125}I count of 45,000 to 50,000/min was injected into each animal. When needed, the labeled endotoxin was diluted with nonlabeled endotoxin to get the desired amount of endotoxin with the desired counts. For labeling with ^{51}Cr the method of Braude *et al.* (9) which labels predominately the carbohydrate portion was used. One μg of ^{51}Cr labeled endotoxin contained 800–1000 cpm.

Immunization of mice. For the study of the distribution of labeled endotoxin in immunized mice, the animals were immunized by intravenous injection of 0.1 μg of endotoxin. In some, 14 days after this primary injection a second injection of 0.5 μg of endotoxin was given. Control animals received saline instead of endotoxin. The distribution of radioactivity was studied on day 14 after the primary or secondary immunization. After counting the radioactivity of whole blood the serum was assayed for antibody by a modification of the Farr procedure (7).

Preparation and fractionation of stomach extract. Stomach extract was prepared by macerating the stomachs of the experimental animals in 0.15 *M* phosphate buffer, pH 7.8, centrifuging off the supernatant, and re-extracting the residue three times in a similar fashion. More than 90% of the radioactivity was recovered. Extracts were pooled and concentrated. Two ml (equivalent to the extract from the stomachs of 3 mice) were passed through a 40 × 2-cm Sephadex G-200 column and eluted by Tris–NaCl buffer, pH 7.8, and the radioactivity of each tube was determined. The column was calibrated with blue dextran 2000, bovine serum albumin, and $Na^{125}I$.

TABLE I. Distribution of Radioactivity after Injection of 1 μg ^{125}I Labeled Endotoxin from *S. enteritidis* into Mice Kept at 25 and 37°.[d]

Time after injection (hr)	Temp (°)	Percentage of injected radioactivity in									
		Heart, lung, thymus	Liver	Spleen	Stomach	Intestine	Kidney	Blood	Head	Body	Total
1	25 (10)	1.4	18.0	3.5	17.9	5.7	1.7	5.6	9.4	23.3	86.7
	37 (10)	2.0	18.4	3.3	18.0	7.1	2.0	5.9	10.1	23.2	90.0
5	25 (11)	0.9	11.3	2.1	27.6	5.9	1.0	5.0	9.9	14.4	78.1
	37 (10)	1.1	12.8	1.8	25.9	8.2	1.3	4.8	11.3[c]	17.3[a]	85.3
24	25 (10)	0.3	6.2	1.1	0.9	1.0	0.3	0.4	8.9	2.6	21.7
	37 (11)	0.3	6.1	0.8	6.8[b]	2.7[a]	0.5	0.9	13.0[b]	6.9[c]	38.0[c]

Difference between the two temperatures significant [a] $p < .05$; [b] $p < .02$; and [c] $p < .01$.

[d] Figures in parentheses are the number of animals in the group; values are the means of these animals.

Results. The distribution of radioactivity was measured in mice kept at 25 and at 37°C (Table I). One hr after injection the liver, stomach (mostly the stomach wall), head, and body contained most of the injected radioactivity. Part, but not all of the radioactivity of the head was in the thyroid and salivary glands. Only 6% remained in the blood. Radioactivity in the liver and body decreased to a considerable extent after 5 hr and increased in the stomach. At this time slightly more radioactivity was found in the liver, head, and body of the animals kept at 37° than those at 25°. After 24 hr most of the radioactivity had been excreted with only the liver, head, and body retaining appreciable amounts of ^{125}I. After 24 hr considerably more radioactivity was found in the stomach, head, and body of the animals kept at 37° than those at 25°.

TABLE II. Comparative Distribution of Radioactivity after Injection of ^{125}I and ^{51}Cr Labeled Endotoxin and ^{125}I and ^{51}Cr Isotopes Only.[a]

Substance injected	Time after injection (hr)	Percentage of injected radioactivity in								
		Heart, lung, thymus	Liver	Spleen	Stomach	Intestine	Kidney	Blood	Head	Body	Total
^{125}I labeled endotoxin	5 (11)	0.9	11.3	2.1	27.6	5.9	1.0	5.0	9.9	14.4	77.9
	24 (10)	0.3	6.2	1.1	0.9	1.0	0.3	0.4	8.9	2.6	21.7
^{51}Cr labeled endotoxin	5 (6)	0.3	58.4	4.8	0.8	4.7	1.4	3.2	3.0	13.3	89.9
	24 (5)	1.4	56.4	4.9	1.1	4.3	2.0	0.3	3.5	10.5	84.4
$Na^{125}I$ (40,000 cpm)	5 (5)	0.3	1.4	0.1	8.7	2.0	0.2	1.0	6.2	12.1	32.8
	24 (6)	0.0	0.6	0.6	0.4	0	0.1	0.2	3.4	2.8	7.9
$Na_2{}^{51}CrO_4$ (8000 counts/10 min)	5 (5)	2.1	8.8	0.5	3.4	5.4	3.3	7.4	7.4	22.8	61.7
	24 (6)	1.6	7.5	0.3	1.2	2.3	3.1	5.6	6.7	21.1	49.9

[a] Figures in parentheses are the number of animals in the group; values are the means of these animals.

The distribution of two endotoxin preparations, one labeled with ^{51}Cr and the other with ^{125}I was compared (Table II). The ^{51}Cr was excreted slowly localizing in the liver and to a lesser extent in the body. Little ^{51}Cr was deposited in the stomach or head. When a doubly labeled endotoxin preparation was used, the distribution of ^{125}I was similar to single labeled ^{125}I labeled endotoxin, and most of ^{51}Cr was found in the liver (unpublished data). Table II also lists the distribution of radioactivity after injection of $Na^{125}I$ and $Na_2{}^{51}CrO_4$. Both iodide and chromate were excreted more rapidly than when they were combined with endotoxin. With $Na^{125}I$ after 5 hr there was no radioactivity in the liver and blood, but most of the radioactivity was in the stomach, head, and body. The ^{51}Cr on the other hand, was found both in blood and liver even 24 hr after injection, and a considerable amount was retained in the body.

In view of the similarity in the distribution of ^{125}I in the stomach and head after injection of either Na ^{125}I or endotoxin-^{125}I it seemed that the radioactivity in these two organs was more likely to be free iodide than iodine still bound to endotoxin. The stomach of 3 animals sacrificed 5 hr after injection of ^{125}I labeled endotoxin were extracted with isotonic saline. Unlike the radioactivity in the injected endotoxin, the radioactivity of stomach extracts had become dialyzable and could not be co-precipitated as antigen–anti-

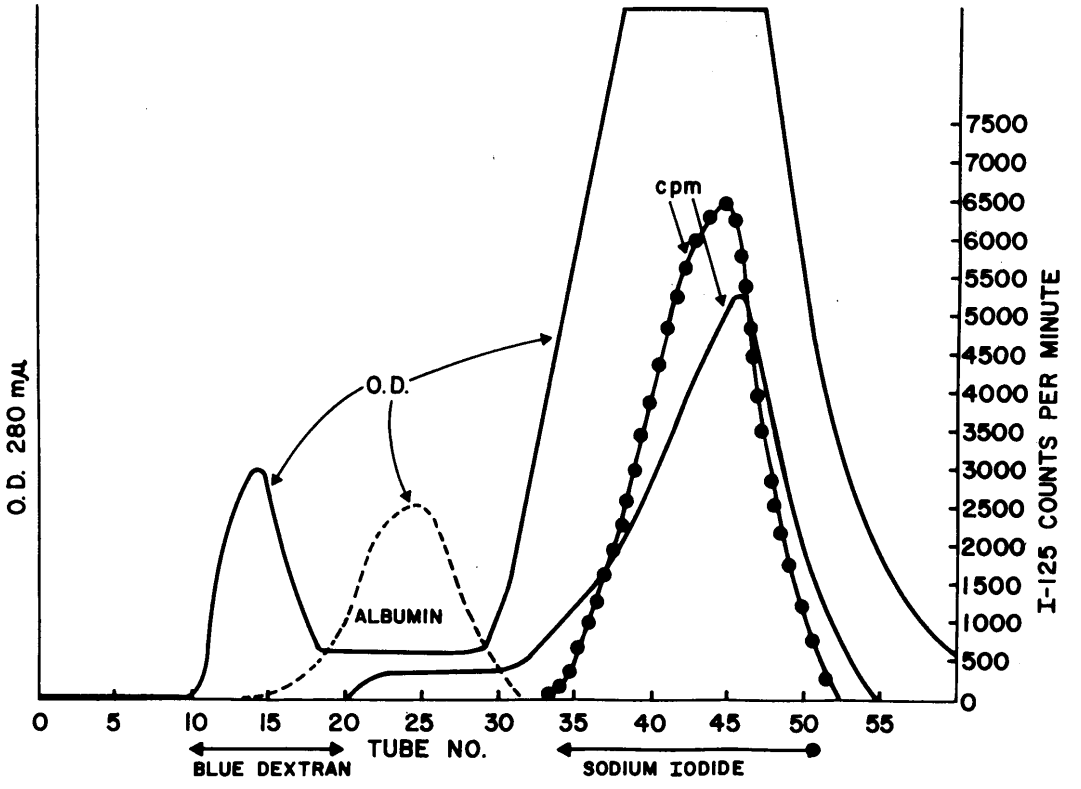

FIG. 1. Sephadex G-200 separation of stomach extract: (—), the optical density and counts per minute of the stomach extract; - - - optical density of albumin; ●-●-● counts per minute of $Na^{125}I$/ double arrows at the bottom indicate where blue dextran and carrier NaI were eluted from the column.

body complexes by half saturated ammonium sulfate. On the other hand, after addition of carrier NaI almost all of the radioactivity could be precipitated by silver nitrate. The stomach extract was passed through a Sephadex G-200 column (Fig. 1). The peak of radioactivity of the stomach extract corresponded with the peak of the radioactivity of $Na^{125}I$. Thus, it seems that the radioactivity in the stomach is free iodide split off from the endotoxin complex.

Preexisting antibody alters the distribution of ^{51}Cr labeled endotoxin (2). We therefore studied the distribution of radioactivity after injection of ^{125}I labeled endotoxin in immunized and nonimmunized animals (Table III). Radioactivity in each of the various organs was highest in the nonimmunized animals and lowest in those given two immunizing injections. There was a 5-fold increase in antibody after one immunizing injection and about a 75-fold increase after the second. When the results of individual animals given two immunizing injections were compared it was noted that the animals with the most antibody retained the least radioactivity in each organ.

To determine which organ might split the iodine from endotoxin, ^{125}I labeled endotoxin was incubated at 37° with slices of liver, spleen, and stomach in a Dubnoff shaker. At intervals, aliquots were drawn off and centrifuged. The supernatant was assayed for undergraded ^{125}I labeled endotoxin by reaction with antibody (Table IV). Soluble components from each of the organs leached into the incubation fluid and bound some endotoxin. Stomach slices quickly degraded the labeled endotoxin, whereas spleen and liver did not. The breakdown of endotoxin by the stomach slices is not due to acid since incubation in 0.1 *N* HCl for 1 hr had no effect on

the subsequent reaction of endotoxin with antibody.

Discussion. The distribution of radioactivity was quite different with ^{125}I labeled and ^{51}Cr labeled endotoxins. Since ^{51}Cr appears to label chiefly the polysaccharide (10) and ^{125}I chiefly lipid A (although about 20% of the ^{125}I is also bound to the core polysaccharide) (8) the distribution of the two isotopes can shed light on the fate of the two parts of the endotoxin molecular complex. With ^{51}Cr labeled endotoxin, as others have observed, we found the greater part of the radioactivity in the liver. With ^{125}I labeled endotoxin, however, the radioactivity appeared in the stomach, body, and head, although a small part of the radioactivity appeared in the liver also. The distribution of the ^{125}I injected in the form of iodide may explain to some extent the fate of the ^{125}I label. Five hr after injection, the Na ^{125}I was mainly in the stomach, head, and body, similar to the radioactivity of the labeled endotoxin. No radioactivity appeared in the liver or spleen. Radioactivity in the stomach, head, and body is most probably free iodide or iodinated fatty acids released by breakdown of lipid A, rather than iodine still bound to polysaccharide.

This conclusion is based not only on the similarity of distribution of ^{125}I injected either as labeled endotoxin or free Na ^{125}I but also on the recovery of in organic iodide from extracts of the stomach. We presume the iodide was split from the endotoxin by a series of enzyme reactions. The site and mode of action of these reactions is uncertain, but the initial stage of the degradation may be the action of serum lipase on the lipid moiety of the endotoxin (8). The stomach is likely to be one site of the degradation since stomach slices were active *in vitro*. After lipid is removed from endotoxin the iodinated fatty acids appear to be rapidly metabolized and the ^{125}I excreted as iodide along with chloride in the urine, saliva, and gastric secretions.

Similar considerations of the distribution of radioactivity after injection of radioactive chromate and chromate labeled endotoxin are complicated by the *in vivo* reaction of the chromate ion with red cells, serum proteins, and other body constituents. It is clear though, that radioactivity was not found in the liver after injection of sodium chromate, so that the radioactivity in the liver seems to

TABLE III. Difference in Distribution of Radioactivity of ^{125}I Labeled Endotoxin from *S. enteritidis* in Organs of Immunized and Non-immunized Mice Kept at 25° and Sacrificed 5 hr after Injection.[a]

	Antigen binding capacity (μg/ml)	Percentage of injected radioactivity in									
		Heart, lung, thymus	Liver	Spleen	Stomach	Intestine	Kidney	Blood	Head	Body	Total
Nonimmunized animals	0.03	1.4	10.1	2.0	21.6	5.7	1.0	4.0	9.3	14.0	69.2
Animals immunized with 1 injection	1.4	1.1	9.8	1.2	14.2	6.0	0.7	4.1	7.3	14.2	58.7
Animals immunized with 2 injections	2.2	0.7	8.4	0.6	10.3	3.2	0.5	1.9	5.6	10.6	42.5
p Value Nonimmunized vs primary		NS[b]	NS	<.05	<.05	NS	NS	NS	<.02	NS	<.01
Normal vs secondary		NS	<.05	<.01	<.01	<.05	NS	<.02	<.01	<.05	<.01
Primary vs secondary		NS	NS	<.05	<.02	<.05	NS	NS	<.05	NS	<.02

[a] Values are the means of 8 animals.
[b] NS = not significant.

TABLE IV. Effect of Incubation with Tissue Slices on ^{125}I Labeled Endotoxin.

	Duration of incubation (hr)	Radioactivity (%) bound by tissue constituents[a]	Unaltered endotoxin (%) remaining in supernate[b]	Endotoxin (%) degraded[c]
Buffer	0.25	0	100	0
	1	0	100	0
	4	0	100	0
	24	0	100	0
Liver	0.25	18	82	0
	1	21	79	0
	4	36	67	0
	24	49	51	0
Spleen	0.25	8	92	0
	1	11	89	0
	4	14	85	0
	24	16	84	0
Stomach	0.25	30	18	52
	1	36	21	45
	4	40	15	45
	24	12	9	79
0.1 *N* HCl	1	0	100	0

[a] Value is obtained by subtracting the radioactivity precipitated by half saturated ammonium sulfate in the aliquots drawn from the incubation mixture of endotoxin and buffer from radioactivity precipitated in the aliquots drawn from the incubation mixture of endotoxin and tissue slices.

[b] Value obtained by subtracting the value of the previous column from the value obtained after reacting an aliquot with excess antibody and precipitating it with 50% saturated ammonium sulfate.

[c] Value is the difference obtained by subtracting the sum of the first two columns from 100.

represent deposition of endotoxin. The results of studies with endotoxin labeled with the two isotopes are consistent with the view that the lipid portion of endotoxin is rapidly split off and metabolized, perhaps even before phagocytosis, while the core polysaccharide is more stable remaining more or less intact for many hours after it is cleared from the circulation by the RES.

Endotoxin is more lethal when the mice are kept at 37° than at 25° after the injection. While the uptake by the liver was not affected by temperature, more radioactivity was retained in the other organs by the animals kept at 37°. The precise significance of this finding is not clear, but may reflect a difference in blood flow to various organs at different temperatures. It seems unlikely that the greater lethality at 37° is related to differences in degradation or distribution of endotoxin.

Less radioactivity of ^{125}I labeled endotoxin was retained by immune than nonimmune mice, with amount of radioactivity recovered being inversely proportional to the amount of antibody, but immunization did not alter the relative distribution of radioactivity among the various organs. It appears that antibody-bound endotoxin is degraded at a faster rate than the unbound antigen, and that most of the iodide from antibody-bound endotoxin is excreted in the urine rather than the stomach which is a major route of excretion of the iodide removed from nonantibody-bound endotoxin.

Summary. The distribution of radioactivity after injection of endotoxin labeled with ^{51}Cr and ^{125}I was quite different. The ^{51}Cr, label-

ing chiefly the core polysaccharide, was retained by the liver while ^{125}I, labeling chiefly lipid A, was rapidly excreted by the kidney, stomach, and salivary glands probably as free iodide. Stomach slices, but not liver or spleen slices, split ^{125}I from labeled endotoxin *in vitro* suggesting that the stomach may play a role in endotoxin degradation. The radioactivity of ^{125}I labeled endotoxin was excreted more rapidly by immunized than nonimmunized animals.

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Studies on Early-Appearing Interferon *in Vitro*
I. Production of Endotoxin-Induced Interferon by Mouse Spleen Cells Cultured *in Vitro* (33908)

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It has been demonstrated that at least two kinds of interferon (IF) appear in plasma of intact animals by injections of endotoxin (ET) or virus (1). The ET produces peak levels of plasma IF about 2 hr after injection. In contrast, virus-induced IF reaches its maximum titers at 6–12 hr after injection of the inducer. The former and the latter are called the early-appearing IF (early-IF) (2, 3) and the late-appearing IF (late-IF) or IF (4, 5), respectively.

It is well known that the late-IF is synthesized within the cells in response to the inducer. By using metabolic inhibitors, the above fact was confirmed both *in vivo* and *in vitro* (6–9). On the other hand, in the case of early-IF, it has been observed that its production in rabbit plasma was not affected by the treatments of actinomycin D (10) or puromycin (11). These results have been confirmed in mice (12).

Recently, Smith and Wagner (13) reported that rabbit macrophages cultures *in vitro* could produce ET-induced IF and spontaneous IF without inducer. Nagano *et al.* (14) also demonstrated rapid "sponatneous" release of IF by similar cells *in vitro*.

However, the production of macrophage-IF was inhibited by actinomycin D or puromycin. More recently, Finkelstein *et al.* (15) reported that the production of ET-induced IF by mouse macrophages cultures *in vitro* was relatively resistant to inhibition by actinomycin D. This result was of much interest. However, the molecular basis for its production mechanism is still unclear.

The present paper describes the kinetics and cellular conditions for producing the ET-

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