CHARACTERIZATION OF A Cr^{51}-LABELED ENDOTOXIN AND ITS IDENTIFICATION IN PLASMA AND URINE AFTER PARENTERAL ADMINISTRATION

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Radioisotopes such as P^{32}, $Na_2Cr^{51}O_4$, and $Cr^{51}Cl_3$ have been utilised for marking endotoxin in efforts to determine *in vivo* uptake and distribution of the latter material (3, 7–9, 11, 12, 14). These reports are in fair agreement concerning the quantitative distribution of the endotoxin as based on subsequent counting of plasma and organ samples. Until recently, evidence for the presence of endotoxin itself in tissues and fluids has been presumed on the basis of the following two observations; (*a*) differences in *in vivo* distribution of the free label as contrasted to that of the label bound to endotoxin and (*b*) the increased rate of plasma clearance of radioactivity in animals rendered tolerant to endotoxins. Current studies have shown that a correlation exists between the toxicity and the radioactivity present in plasma samples collected at various times after administration of a chromate-labeled endotoxin (6). Furthermore, the presence of endotoxin in such plasma samples was demonstrated immunologically by means of ring test precipitation (5). However, evidence has been lacking that the endotoxin moiety *per se* bears the radioactive label either before injection or after recovery from the tissues and fluids of recipient animals. It is the purpose of this report to show that the labeling of endotoxin with hexavalent Cr^{51} results in a specifically tagged product, a part of which retains both label and toxicity in circulating plasma for several hours after administration. Furthermore, it will be shown that endotoxin is demonstrable in urine but in a non-toxic and non-labeled state.

Materials and Methods

Endotoxin.—The source of endotoxin was the Danysz strain of *Salmonella enteritidis* prepared by the method of Boivin (1). The product was labeled by incubating 100 mg with 1.5 mc of $Na_2Cr^{51}O_4$ (specific activity = 73 mc/mg), during 48 hours according to a method of Braude, *et al.* (2). After labeling, the specific activity of the endotoxin was 1.5 μc/mg which

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was equivalent to 110,000 counts/minute. This material was not precipitated with alcohol but was dialyzed for 5 days against weakly buffered water (pH 7.3) to remove free chromium and designated preparation A. Aliquots of this product were subjected to centrifugation in a Spinco model L instrument with a swinging bucket rotor. Preparations B_s (supernatant) and B_p (pellet) were obtained by centrifugation at 10,000 RPM (8000 *G*) for 1 hour. Preparations C_s (supernatant) and C_p (pellet) were obtained after ultracentrifugation at 40,000 RPM (125,000 *G*) during 8 hours. The upper 3 to 4 ml of supernatant in each tube were carefully drawn off and the pellet was washed once with cold distilled water and resuspended to the original volume of 5 ml. The proportions of endotoxin sedimented in preparations B_p and C_p were determined by lyophilizing and weighing the pellets, and the proportions of endotoxin in the supernatants were obtained by subtraction. The radioactivity of the various preparations was measured in a scintillation well counter and, with the exception of C_s, 1 mg of endotoxin averaged approximately 100,000 counts/minute.

Plasma and Urine.—Blood was taken from the abdominal aorta of mice in heparinized syringes and then centrifuged to separate the plasma. Pooled urine samples were obtained by placing groups of 10 mice over funnels covered by fine wire mesh. Collections were taken during periods of 0 to 6 hours and 6 to 12 or 24 hours into small flasks immersed in ice baths. The glassware and wire mesh were changed between the two collection periods. In certain experiments, urine was precipitated in 5 per cent trichloracetic acid and the soluble portion concentrated 4 or 5 times by pervaporation.

Toxicity.—Toxicity measurements were obtained after tail vein inoculation of the various test samples into white mice of common stock, weighing 20 to 25 gm. Lethality was determined during 24 hours in mice which had been adrenalectomized 2 or 3 days previously. Toxicity was also measured by abortion in 15- to 18-day pregnant mice, the fetuses being examined 24 hours after injection (4).

Ring Tests and Agar Diffusion.—High titer–specific antiserum, prepared in the horse, was kindly supplied by Dr. A. M. Staub. Ring tests and the double diffusion experiments in agar were performed according to the usual techniques. After development of the precipitating lines, the agar diffusion plates were washed 3 to 4 days with frequent changes of physiologic saline. Filter paper was placed in contact with the agar surfaces and the plates were put into an oven at 37°C until the agar had dried (15). Autoradiographs were prepared by direct contact of the dried agar plates with radiosensitive film. Lastly, the plates were stained with azocarmine to better visualize the lines of precipitation.

RESULTS

Endotoxin Preparations. Toxicity.—By intravenous route, the LD_{100} of preparation A is 300 μg in normal mice (6), whereas in carefully adrenalectomized mice the LD_{100} was reduced to 0.05 μg, or approximately 6000 times less. The effects of centrifugation on the distribution of toxicity are shown in Table I. On a weight basis, preparations A, B_s, and B_p were of equal toxicity, preparation C_p was slightly more toxic, but preparation C_s proved to be about 100 times less toxic than the other preparations. Centrifugation at 40,000 RPM for 8 hours resulted in the separation of a pellet comprising 80 per cent of the weight, 93 per cent of the radioactivity, and more than 99 per cent of the toxicity of the non-centrifuged preparation. The supernatant contained approximately 20 per cent of the starting weight, 7 per cent of the radioactivity, and less than 1 per cent of the toxicity.

Agar Diffusion Patterns.—Under optimum conditions of diffusion, the antiserum utilized in these experiments demonstrated the presence of at least four distinct lines of precipitation with preparation A. Two of these lines represent fast diffusing fractions which precipitate near the antiserum reservoir whereas the others represent more slowly diffusing fractions of higher molecular weight. After drying the agar plates, autoradiographs were prepared with varying periods of exposure, but not longer than 2 weeks, according to the specific activity of the chromium label. These autoradiographs revealed clearly that the specifically precipitated endotoxin bore the chromium label although only on the slow diffusing fractions (well A, Fig. 1).

TABLE I

Lethality of Endotoxin Preparations in Adrenalectomized Mice

Preparation	Centrifugation	Fraction	Dose injected, μg						
			0.02	0.05	0.10	0.20	0.50	1.0	3.0
A	None	Total	1/6*	7/8	13/14	13/15	17/17	17/17	—
B_s	10,000 RPM, 1 hr.	Supernatant	—	6/8	8/8	10/10	10/10	10/10	—
B_p	10,000 " , 1 "	Pellet	—	5/8	8/8	9/10	10/10	10/10	—
C_s	40,000 RPM, 8 hrs.	Supernatant	—	0/6	—	0/7	0/6	1/6	5/6
C_p	40,000 " , 8 "	Pellet	6/6	—	5/5	6/6	7/7	6/6	—

* Dead/total of mice injected.

In order to obtain a fully toxic and well labeled starting product, preparation A was subjected to high-speed centrifugation. The agar diffusion patterns of preparation A after centrifugation at 10,000 RPM for 1 hour showed the presence of all four lines of precipitation in both the supernatant (B_s) and the pellet (B_p). However, after 8 hours at 40,000 RPM, the heavy intensively labeled fractions were separated adequately from the lighter, non-labeled fractions of the supernatant. The upper right well in Fig. 1 contains the supernatant (C_s) and the lower well, the resuspended pellet (C_p). The line of identity formed between C_s and C_p indicates that a small amount of the fast diffusing material was still present in the pellet. An autoradiograph of this plate revealed that in C_p, the label is readily detected on the slow diffusing lines of precipitation (Fig. 2). The autoradiograph also showed that in the supernatant, the slow diffusing fraction which was weakly precipitated in the supernatant was also weakly radioactive. Considering that the actual amount of radioactivity put into this well was approximately 50 times greater than the minimum detectable in preparation C_p, it can be judged that most of the label in the C_s preparation is attached to a product which is not precipitable by the antiserum.

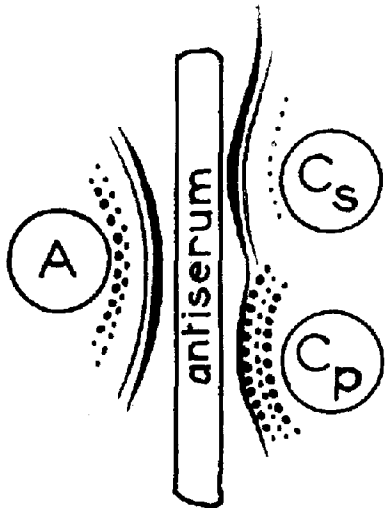

FIG. 1. Precipitation patterns of chromate-labeled endotoxin in agar double diffusion. Well (*A*), 200 μg of preparation A, non-centrifuged; (C_s) supernatant, and (C_p) pellet, from 200 μg of preparation A after centrifugation at 40,000 RPM for 8 hours. The interrupted line represent the precipitated fractions which are autoradiograph-positive.

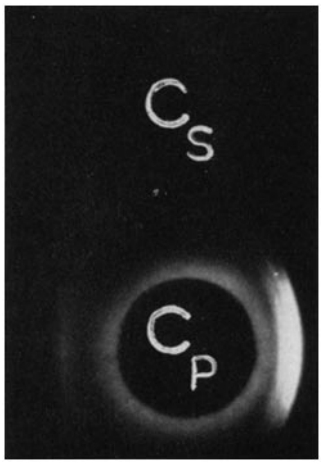

FIG. 2. Autoradiograph of the precipitation pattern of preparation A after ultracentrifugation; (C_s) supernatant, and (C_p) pellet.

In the experiments which follow, only preparations A and C_p were employed. The lower limits of sensitivity for saline suspensions of the two preparations were the following; LD_{100} between 0.02 and 0.05 μg in adrenalectomized mice, minimum ring test at 0.2 μg, autoradiograph-positive at 0.3 to 0.5 μg after precipitation in agar.

Recovery and Identification of Endotoxin in Blood and Urine.—Normal mice received 300 μg of preparation A or C_p by intraperitoneal route (approximate LD_{50}, reference 6) and were bled 6 hours later. Plasma and urine samples were examined for radioactivity, toxicity, and immunologic reaction. The radioactivity of whole blood was found in the plasma and not on the erythrocytes. Of the total label injected, an average of 12 per cent was found in the plasma, 9 per cent in urine collected during the first 6 hours, and 6 per cent in urine collected between 6 and 24 hours. About 10 per cent of the label in plasma and 80 per cent of that present in urine was dialyzable.

TABLE II

Toxicity of Plasma and Urine Obtained from Mice Receiving an LD_{50} Dose of Endotoxin

Sample	Radioactivity	Ring test (per ml)*	Lethality (volume injected in ml)				Abortion (volume injected in ml)			
			0.05	0.10	0.25	0.50	0.05	0.10	0.25	0.50
	μg/ml									
6 Hr. plasma (diluted)	20‡	200	15/16§	16/19	13/13	—	3/9§	3/13	25/28	
Urine (0–6 hrs.)	20‡	200	—	1/15	0/10	3/5‖	—	—	1/13	3/13‖
Endotoxin in saline	20	100	10/11	23/26	36/41	6/6	11/18	11/11	20/22	14/14

* Reciprocal of maximum dilution giving a positive ring test.

‡ This value is based on a radioactive count equivalent to 20 μg of the stock endotoxin.

§ Adrenalectomized mice, No. dead/total: Pregnant mice, No. aborted/total.

‖ Normal urine was equally toxic for adrenalectomized (4/7) and pregnant (3/7) mice at this volume.

Preliminary evidence that endotoxin was present in the radioactive samples of plasma and urine was obtained by means of positive ring tests employing the specific antiserum. Occasionally, undiluted normal urine gave a weak precipitation in ring test tubes but at a dilution of 1:2 or greater such non-specific precipitation was not seen. Urine specimens from animals receiving endotoxin were of equal ring test titer before and after dialysis.

Both urine and plasma were assayed for toxicity in adrenalectomized and pregnant mice (Table II). Test plasma was toxic at the lowest volume utilized (equivalent to 1 μg of endotoxin by radioactivity and 10 ring tests) whereas urine was not toxic at the maximum injectable volume, which represented 5 μg of endotoxin by radioactivity and 50 ring tests. Normal mouse plasma was tolerated without observable effect at 0.5 ml in both adrenalectomized and pregnant mice. Normal urine proved to be toxic for these animals at a volume of 0.5 ml; however, a volume of 0.25 ml or less was well tolerated. Neither dialysis nor deproteinization of normal or test urine resulted in a diminution of this inherent toxicity.

The control animals received endotoxin in saline at a minimum dose of 1 μg by weight and radioactivity, which represented about 20 LD_{100} doses, and 5 ring tests. The results in the abortion assay indicated that 6-hour plasma contained less toxicity than would be expected on the basis of radioactivity. This difference was not seen in the lethality data since the latter is a more sensitive assay and the end-point was not determined in these experiments.

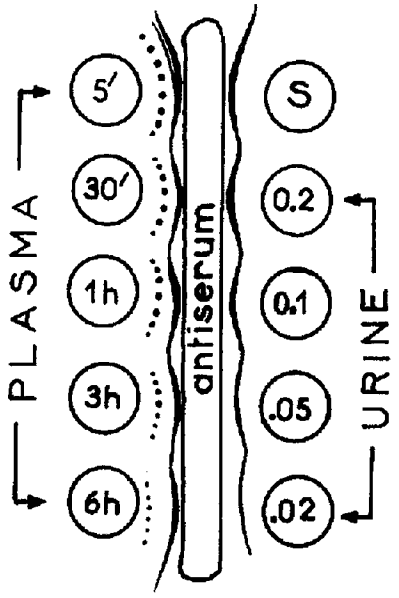

FIG. 3. Agar precipitation patterns of the endotoxin present in plasma and urine samples obtained after injection of chromate-labeled preparation C_p. The plasma samples were taken from groups of mice which had received 50 μg; 0.2 ml of plasma was added to each well. The pooled urine, collected between 0 and 6 hours after the administration of 300 μg, was dialyzed and concentrated five times before being added to the wells at the volumes indicated (milliliters). Well (*S*) received approximately 10 μg of preparation C_s. The interrupted line of precipitation indicated in the plasma wells is autoradiograph-positive.

In experiments in which normal mice received a sublethal dose (50 μg of preparation C_p) by intravenous route, the endotoxin was readily demonstrated in plasma and urine samples taken up to several hours after injection. Plasma collected 6 hours later contained the equivalent of 4.5 μg of endotoxin by radioactivity, was ring test–positive at a dilution of 1/90, and killed adrenalectomized mice at a dilution of 1/45. Urine samples collected between 0 and 6 hours or 6 and 12 hours were also positive in ring tests and were radioactive, although more than 90 per cent of this radioactivity was dialyzable.

Identification of Endotoxin in Plasma and Urine Specimens by Specific Pre-

cipition in Agar.—Plasma taken from mice 6 hours after intraperitoneal administration of an LD_{50} dose of preparation A reacted specifically with the antiserum in agar diffusion experiments. These precipitation patterns revealed one

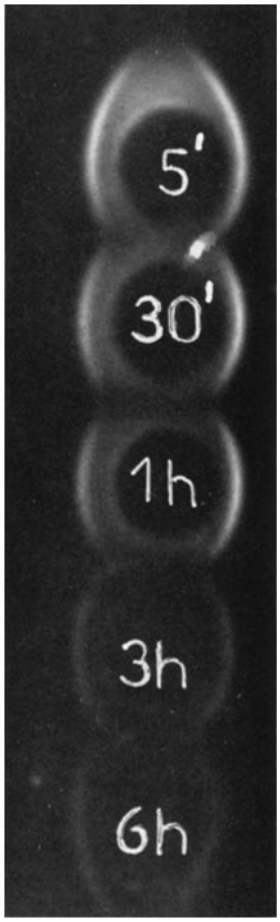

FIG. 4 Autoradiograph of the labeled endotoxin present in plasma samples obtained at various intervals after injection of 50 μg of preparation C_p. The film was prepared from a precipitation pattern similar to that represented diagrammatically in Fig. 3, left.

slow and two fast diffusing fractions and the subsequent autoradiographs showed that the label was associated only with the slow diffusing endotoxin. The same pattern of agar precipitation was seen as well with preparation C_p which, before injection, was composed almost entirely of slow diffusing fractions. Specific precipitation in agar was also evident in the plasma collected from mice which had received a sublethal dose of preparation C_p (Fig. 3). Autoradiography again showed that the label was bound only to the slow diffusing fraction which was demonstrated in all the plasma samples taken between 5 minutes and 6 hours (Fig. 4).

Endotoxin was readily identified in urine samples taken from mice receiving either the LD_{50} or the sublethal dose of preparation C_p. The urines were dialyzed usually to reduce the non-specific clouding of the agar in the immediate vicinity of the wells. The precipitation patterns with the urine samples demonstrated the existence of only a fast diffusing fraction in both the 0 to 6 hour and the 6 to 12 or 24 hour collections. Urine specimens from mice which had received an LD_{50} dose of C_p were precipitated with trichloroacetic acid and the clear supernatant was concentrated 4 or 5 times by pervaporation. Endotoxin was not lost during this procedure as witnessed by a 4- to 5-fold increase in ring tests per ml of urine. With such concentrated urine, the agar precipitation was greatly strengthened but in no case was a slow diffusing fraction of endotoxin brought into evidence (Fig. 3). The line of precipitation formed in urine samples exhibited antigenic identity with the fastest diffusing fraction of preparation C_s. Autoradiographs of these plates before or after urine concentration demonstrated the absence of radioactivity despite the fact that from 5 to 20 per cent of the label present in urine was not dialyzable. Control specimens of normal urine, concentrated or not, were uniformly negative in agar precipitation experiments.

DISCUSSION

The incubation of endotoxin with hexavalent Cr^{51} resulted in the specific labeling of the product as demonstrated by autoradiography of agar diffusion precipitation plates. From these precipitation patterns, it was seen that only the slow diffusing fractions carried the radioactivity. These heavier, intensively labeled fractions were adequately separated from the lighter, non-labeled fractions by high-speed centrifugation. Such treatment was useful moreover, in that a minimum of 99 per cent of the initial toxicity was sedimented. Thus, a fairly homogeneous preparation of endotoxin was obtained which was quite suitable for *in vivo* experiments.

The agar precipitation patterns of plasma samples taken from mice receiving the endotoxin demonstrated that there occurred with time, both a progressive decrease in a slow diffusing, labeled fraction and the continued presence of fast diffusing, unlabeled endotoxin. Continuous *in vivo* degradation and unlabeling of the large molecules of endotoxin afford a plausible explanation of this observation. The finding in urine of significant amounts of fast diffusing fragments of unlabeled and non-toxic endotoxin suggests that only these degraded molecules can pass the kidney. Whether these same products are eliminated under less stressful conditions or whether their presence in urine is the result of kidney insult is to be investigated.

A correlation between the presence in agar diffusion experiments of a slow diffusing fraction and the toxicity of an endotoxin has been reported (13). This relationship is supported by the *in vivo* experiments described here in

which plasma was shown to possess both slow diffusing endotoxin and toxicity, whereas urine, which did not contain slow diffusing endotoxin, was not toxic. Furthermore, the results of ultracentrifugation with preparation A also confirmed this correlation between large molecular size and toxicity, in that the pellet contained almost 100 per cent of the toxicity of the original preparation.

The choice of a radioactive marker is of crucial importance for *in vivo* studies on uptake and subsequent distribution of endotoxin. Radioactive labels such as $Cr^{51}Cl_3$ and P^{32} were not employed here but the results of others (7, 9, 11, 12, 14) have shown that these labels, presumably with endotoxin attached, are removed more rapidly from plasma than is endotoxin tagged with $Na_2Cr^{51}O_4$ (reference 3 together with the present report). No evidence is available to show that the $Cr^{51}Cl_3$ label is fixed onto the endotoxin either before or after its administration. That the difference between the *in vivo* distribution of endotoxin-bound $Cr^{51}Cl_3$ and free $Cr^{51}Cl_3$ indicates that the label remains associated with the endotoxin in the former case, is not necessarily a valid assumption (3, 7, 9). For example, if *in vivo* liberated chromium is not free but instead attached to a small dialyzable molecule (10), its subsequent distribution may be changed appreciably from that of the free label alone. In preliminary experiments, we have found that the dialyzable radioactivity present in urine and plasma samples taken from endotoxin treated mice cannot be fixed onto erythrocytes.

It is reasonable to expect that P^{32}, being an internal label, is well fixed in the endotoxin molecules. However, it is possible that an appreciable part of the phosphate marker is readily unlabeled in circulating blood and that this liberated radioactivity is more quickly cleared than the remaining but less strongly labeled endotoxin. In view of the findings of Rowley and colleagues (11, 12), that serum is capable of rapidly dephosphorylating a P^{32}-tagged endotoxin, this possibility becomes more tenable.

The quantitative or qualitative distribution *in vivo* of a tagged endotoxin in various tissues and fluids cannot be presumed merely on the basis of radioactivity counts. The urine samples examined in this study provide a good example of the difficulties inherent in such an assumption. The large amounts of radioactivity found in urine were no longer associated with the endotoxin but none the less the unlabeled endotoxin was present in the urine and identified immunologically. Thus, without other evidence, it would be reasonable to conclude that the presence or absence of radioactivity in a given specimen may or may not be related to the presence or absence of endotoxin. However, in the present investigation definitive evidence was obtained that radioactivity was associated with the endotoxin previously administered. This was accomplished by means of autoradiographs of the specifically precipitated endotoxin found in plasma samples. The presence of toxicity as well, was readily demonstrated by virtue of the highly sensitive assay in adrenalectomized mice.

The present results do not support the general view that endotoxin adminis-

tered intravenously, is rapidly removed from the circulation. The endotoxin used in this study was shown to persist in plasma, and in a toxic state, for several hours after its administration. Plasma taken 6 hours after injection of only 50 μg was lethal for adrenalectomized mice after a 45-fold dilution and contained in 0.2 ml, sufficient labeled endotoxin as to be detectable by autoradiograph after precipitation in agar. From these findings, one can be reasonably certain that identifiable endotoxin will be shown to persist in plasma for periods of time considerably longer than 6 hours.

The sodium chromate label is recommended as an *in vivo* marker to demonstrate the presence of endotoxin in plasma if precautions are taken to remove the dialyzable radioactivity and to reduce, by ultracentrifugation, the non-endotoxic labeled portion. Whether this marker will prove useful to study the uptake and distribution of endotoxin in other tissues of the body is now under investigation.

SUMMARY

The incubation of endotoxin with $Na_2Cr^{51}O_4$ yielded a product which was well labeled. That the label was fixed on the endotoxin itself was shown by autoradiography on specific lines of precipitation formed in agar. Ultracentrifugation at 40,000 RPM sedimented 80 per cent of the total weight of the starting Boivin preparation. Agar diffusion patterns with subsequent autoradiographs demonstrated that the chromium tag was associated only with the heavy fractions of the pellet. The supernatant contained precipitable, but unlabeled endotoxin. Toxicity measurements showed that more than 99 per cent of the total toxicity resided in the pellet fractions.

The chromate-tagged endotoxin was specifically identified in plasma samples taken up to 6 hours after intravenous administration of LD_{50} or sublethal doses. The endotoxin was not totally detoxified *in vivo* since plasma collected 6 hours after the injection of even the sublethal dose was toxic when assayed in adrenalectomized mice. The endotoxin was specifically identified in urine specimens but it was no longer toxic or radioactive. Agar diffusion experiments indicated that only degraded material was present.

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