IN VIVO DETOXIFICATION OF ENDOTOXIN BY THE RETICULO-ENDOTHELIAL SYSTEM*

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Macrophages harvested from the peritoneal cavity of the rabbit and suspended in plasma or serum at body temperature inactivate bacterial endotoxin (1). This observation does not reflect the kinetics of the *in vivo* process, or permit a definitive study of the effects of various types of injury to the reticulo-endothelial system (RES) upon this function. *In vivo* experiments were devised to disclose such additional information in order to illuminate some of the problems in host resistance to Gram-negative bacteria.

Materials and Methods

Mongrel dogs weighing between 17 and 28 kg. were given morphine subcutaneously, and anesthetized 1 hour later with nembutal intravenously. Sodium heparinate (1.5 mg./kg.) was given intravenously at the same time. One femoral artery and vein were cannulated for purposes of blood pressure measurement and transfusion. The spleen was then delivered through an upper midline abdominal incision. A plastic Y-tube was placed in the main splenic vein, and a fine polyethylene tube in a marginal artery at the superior pole (Fig. 1). Flow through the main artery was not interrupted. All tissue in the splenic pedicle except the cannulated vessels and splenic artery was then divided. After the spleen had been restored to its normal site, the venous cannula was brought out of the abdominal incision, which was then closed for the duration of the experiment. In several control experiments the left kidney, instead of the spleen, was prepared in a similar fashion. After exposing the kidney through a muscle-splitting incision, a fine polyethylene tube was inserted into the renal artery without interrupting flow, and a plastic Y-tube was inserted into the renal vein. The abdominal incision was closed around these tubes for the duration of the experiment. Aseptic technic was used in performing all the surgical procedures described.

On completion of the preparation 1.5 to 2.0 mg. of endotoxin[1] in 2 ml. of saline was slowly injected into the cannulated marginal artery. Collection of all the effluent blood was simul-

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[1] Extracted by the technic of Boivin and Mesrobeanu from a strain of P^{32}-labeled *E. coli* $0111B_4$ prepared by growth in a phosphate-poor nutrient broth (Difco) containing 16 to 25 mc. of $Na_2HP^{32}O_4$ per liter. The rabbit M.L.D./100 was 3.0 to 4.0 mg./kg.

taneously initiated and continued for 10 minutes. An equivalent amount of fresh normal dog blood was concurrently infused *via* the femoral vein. The flow during each minute, which was some 40 ml. in all dogs, was collected in a separate flask. After the 10 minute period, normal flow of blood from the spleen was restored. Sixty minutes and 2 hours later small splenic ve-

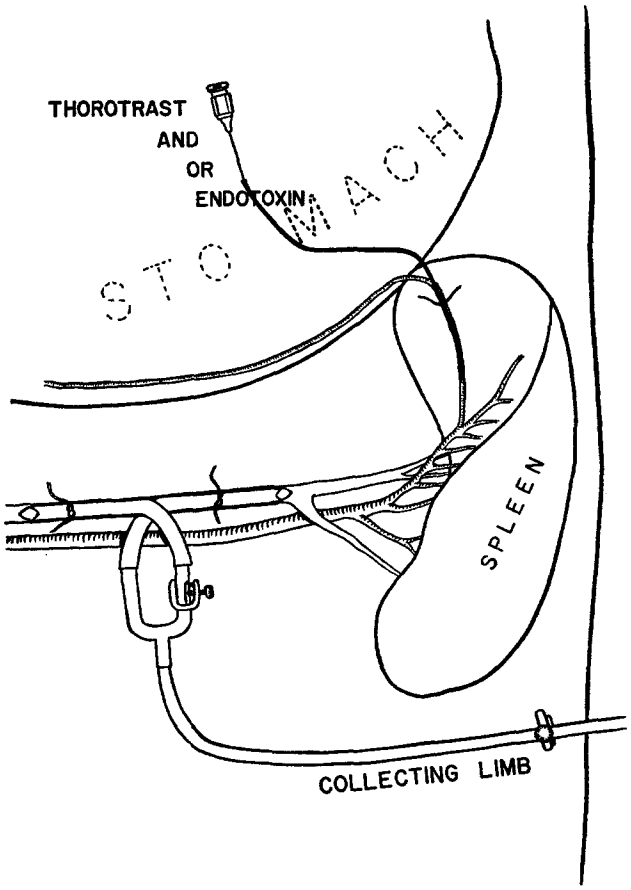

FIG. 1. The spleen perfusion system. The endotoxin or thorotrast is injected into a marginal artery of the spleen without interrupting the main arterial flow. The splenic vein is cannulated by a Y-tube. When the collecting limb is clamped, *i.e.* no blood is being collected, the clamp on the other limb of the Y-tube is open. The Y-tube system is primed with heparinized saline.

nous blood samples were taken. Then the spleen was excised and homogenized in an autoclaved Waring blendor. The kidney experiments were performed in a similar manner.

Each sample of blood, and each spleen or kidney was tested for radioactivity. Most of the samples of blood and all spleens and kidneys were tested for toxicity. The radioactivity measurements were made in a deep well quartz β-ray scintillation counter on 1.0 ml. aliquots of whole blood or of homogenate placed in cellulose dialysis bags. The measurements were repeated after dialysis for 24 hours in running tap water at 4°C. The probable error in

counting technic did not exceed 4 per cent. Toxicity was determined by the chick embryo test of Smith and Thomas (2). Multiple hemorrhages and death of at least four of the six embryos injected with 0.1 ml. of the same sample were taken as evidence of the presence of endotoxin. Survival of at least four of the six embryos was taken as evidence of the absence of endotoxin.

Four types of experiment were performed. In type I the procedure as already described was carried out. This experiment gave us a measure of the spleen's capacity to ingest and destroy a given amount of endotoxin. In type II the procedure was the same, except that the endotoxin was not injected until 3 hours after 10 ml. of thorotrast[2] was injected slowly into the marginal artery of the spleen during a 3 minute period. The data of this experiment allowed a comparison of uptake and detoxifying capacity of the intact RES in the spleen with that of the same

TABLE I

Per cent of P^{32} Retained by Spleen or Kidney 10 Minutes and 2 Hours after Infusion of Radioactive Endotoxin

Exp. No.	Normal spleen		Thorotrast spleen		Shock spleen		Kidney	
	10 min.	2 hrs.	10 min.	2 hrs.	10 min.	2 hrs.	10 min.	2 hrs.
1	79	50	20	20	0	0	3	0
2	68	38	22	22	0	0	0	0
3	78	34	30	18	12	12	0	0
4	92	35	27	27	23	23	—	—
5	88	35	—	—	42*	4*	—	—
6	69	22	—	—	—	—	—	—
7	78	70	—	—	—	—	—	—
Mean....	78.8	40.5	24.7	21.7	8.3	8.3	1	0

* This experiment has not been included in the mean for this group because the spleen in this animal behaved more like a normal spleen with respect to uptake, release of P^{32}, and detoxification. This is in accord with our experience that prolonged hemorrhagic shock does not produce significant injury in about one of every five dogs.

RES "blocked" by thorotrast. In type III the procedure was also the same as in type I except that the endotoxin was not injected until 2 hours after the dogs had been transfused with all their shed blood for the treatment of hemorrhagic shock (30 mm. Hg) of 2 hours duration. The data of this experiment allowed a comparison of the uptake and detoxifying capacity of an intact RES with that of the same RES damaged by exposure to severe hemorrhagic shock for 2 hours. In type IV the type I experiment was performed, employing the kidney instead of the spleen, in order to determine the ability of an organ without a significant number of RE cells to take up and detoxify endotoxin.

RESULTS

Tables I and II and Figs. 2 and 3 contain the essential data, which may be summarized as follows:—

I. Normal Spleen.—In seven experiments with the normal spleen 79 per cent

[2] A sterile 25 per cent colloidal suspension of thorium dioxide in dextrins—"testagar."

of the P^{32} injected as labeled endotoxin was still present in the spleen at the end of the first 10 minutes. Most (17 per cent) of the remainder was found in the splenic effluent during the first 2 minutes after the injection (Fig. 2). Samples of splenic vein blood taken after 1 hour and 2 hours also contained P^{32}. Some 40 per cent of the injected radioactivity was recovered in the spleen 2 hours after injection (Fig. 3).

Of the radioactivity in the splenic effluent collected during the first 10 minutes, 26 per cent was dialyzable. A fraction of every blood sample taken during

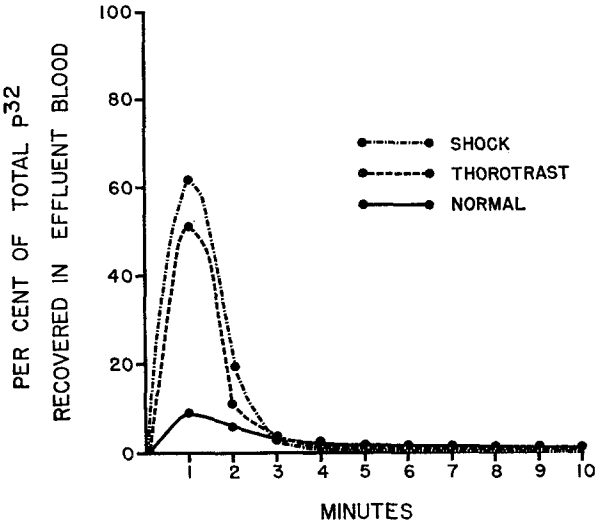

FIG. 2. Per cent of P^{32} recovered in splenic vein blood of normal dogs, dogs with thorotrast-primed spleens, and dogs recovering from 2 hours of hemorrhagic shock after the injection of P^{32}-labeled endotoxin. Each point on each curve is the mean of several experiments.

this period and also at 1 hour and 2 hours was dialyzable. But the splenic homogenate was essentially non-dialyzable (less than 4 per cent).

All tests for toxicity of blood specimens collected during the first 10 minutes and subsequently, and of the splenic homogenates, were negative without exception.

II. Thorotrast-Pretreated Spleen.—Of the P^{32} injected as labeled endotoxin into all thorotrast-treated spleens, only 25 per cent was still present in the spleen at the end of 10 minutes. Over 90 per cent of the remainder was found in the splenic effluent during the first 2 minutes after the injection. Little or no release of P^{32} occurred after the first 10 minutes, for virtually all (22 per cent) the radioactivity present in the spleen after 10 minutes was still present after 2 hours and samples of the splenic blood taken at 1 hour and 2 hours showed no radioactivity. Of the P^{32} in the splenic effluent only 9 per cent was dialyz-

able. None of the P^{32} in the splenic homogenate was dialyzable. The bloods collected during the 1st minute, and the splenic homogenates were consistently positive for endotoxin by the chick embryo test.

III. Spleen after 2 Hours of Hemorrhagic Shock.—In four experiments on dogs recovering from 2 hours of hemorrhagic shock only 8 per cent of the P^{32} injected as labeled endotoxin was present in the spleen at the end of the first 10 minutes.[3] Almost all the remainder was recovered in the splenic effluent within the first 2 minutes (Fig. 2). After 5 minutes there was no further release

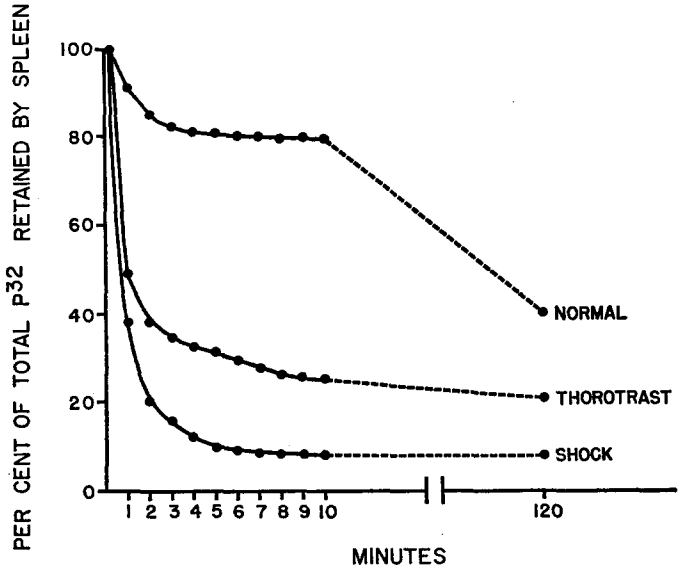

FIG. 3. Per cent of P^{32} retained by the spleen of normal dogs, dogs with thorotrast-primed spleens, and dogs recovering from 2 hours of hemorrhagic shock after the injection of P^{32}-labeled endotoxin. Each point on each curve is the mean of several experiments.

of P^{32}. In two of the four experiments the amount calculated as retained by the spleen after the first 10 minutes was recovered in full 2 hours later in the splenic homogenate. In the remaining two experiments none was expected and none was found.

The dialyzable P^{32} in the effluent blood was 8 per cent. None of the P^{32} in the splenic homogenate was dialyzable. The effluent during the first minute and the splenic homogenate after 2 hours were consistently positive for endotoxin by the chick embryo test.

[3] A fifth experiment was excluded from this mean because the spleen behaved like the normal spleen in that there was (*a*) a large retention of the injected toxin, (*b*) a high percentage of dialyzability of the effluent blood which was *non*-toxic on bioassay, and (*c*) a considerable and continuous release of P^{32} until the end of the experiment.

Normal Kidney.—When the three normal kidneys were perfused with P^{32}-labeled endotoxin, 97 to 100 per cent of the radioactivity was recovered in the venous outflow during the first 2 minutes after injection. *None* of the radioactivity in this blood was dialyzable. All effluent blood collected during the first 2 minutes and all the kidney homogenates were positive for endotoxin by the chick embryo test.

DISCUSSION

It is noteworthy that dialyzable P^{32} was present in the blood leaving the normal spleen within the 1st minute after injection as well as subsequently, so

TABLE II

Per cent of Dialyzable P^{32} in Splenic or Renal Venous Blood Collected for 10 Minutes after Injection of P^{32}-Labeled Endotoxin

Exp. No.	Normal spleen	Thorotrast spleen	2 hr. shock spleen	Normal kidney
1	44	18	0	0
2	18	16	11	0
3	—	0	13	0
4	38	4	9	—
5	7	—	40*	—
6	25	—	—	—
7	24	—	—	—
Mean.....	26	9.5	8.2	0

* This experiment has not been included in the mean for this group because the spleen in this animal behaved like a normal spleen with respect to uptake, release of P^{32} and detoxification. This is in accord with our experience that prolonged hemorrhagic shock does not produce significant injury in about one of every five dogs.

that within the first 10 minutes 26 per cent of the P^{32} which had left the spleen was dialyzable (Table II).

In a preceding paper (1) *in vitro* data were presented indicating that when 50 per cent of the P^{32} bound to endotoxin was released by macrophages in plasma, significant detoxification had occurred. Such a quantitative correlation is difficult to determine in the *in vivo* experiment, because much of the released P^{32} is promptly incorporated by the tissues at the site of release from the endotoxin. This is evident from the observation that some 96 per cent of the P^{32} in the splenic homogenate was non-dialyzable at a time when the spleen was free of toxin.

The evidence that the normal spleen had detoxified the endotoxin was survival of at least four of six chick embryos after exposure to an 0.1 ml. aliquot of the splenic homogenate in all seven experiments. This would not be evidence of detoxification if the amount of intact endotoxin retained by the spleen after

2 hours were so small as to set the fraction present in 0.1 ml. of homogenate below the sensitivity of the test. That this was not so is shown by the finding of toxicity, *i.e.* death of at least four of six embryos, in all similar tests of the thorotrast-treated spleens, the endotoxin content of which, as judged by the P^{32} retention data,[4] was well below that of the normal spleen.

It will be noted that two of the shock spleens which had retained no endotoxin were toxic by the chick embryo test. This signifies the presence of intact unlabeled endotoxin. Two sources for unlabeled endotoxin would be (*a*) endotoxin absorbed from the intestine, which might be present in the spleen at the end of the 2 hour shock period because of defective detoxifying power (3); and (*b*) endotoxin from bacteria which incorporated unlabeled rather than labeled P^{32} from the "phosphate-poor" broth in which they were grown. Endotoxin from the intestine in these two spleens can be excluded, because the corresponding livers, which proved to be free of toxin, would also have been toxic if the intestine were the source of the endotoxin. The second of these two sources of unlabeled endotoxin can account not only for the toxicity of these two spleens, but also for the toxicity of the three kidneys, and to some indeterminate extent for the toxicity of the other injured spleens. These considerations do not invalidate the conclusion that the capacity of the injured spleen to detoxify endotoxin is far below that of the normal spleen, for if the normal spleen had retained unlabeled as well as labeled endotoxin, the assay showing detoxification by the normal spleen would signify breakdown of more endotoxin than was thought to be present from the P^{32} data.

In all experiments an aliquot of liver was tested for P^{32} activity. P^{32} was found in three of seven normal spleens, and in no others except the liver of Experiment five of the shock spleen series in which the spleen released P^{32} into the portal system as well as the normal spleens. The absence of P^{32} from the remaining livers in the experiments with injured spleens was to be expected, since no P^{32} and presumably no toxin were released by these spleens after the first 5 to 10 minutes. All the livers that were tested for toxicity were negative.

Whatever the uncertainty with respect to these processes, it is apparent that the release of P^{32} cannot be attributed to the action of plasma components acting alone, because) in three kidney infusion experiments all the bound P^{32} injected was recovered in the renal vein blood, and none of it was dialyzable. This blood was toxic, whereas the blood leaving the normal spleen, with 26 per cent of its P^{32} dialyzable, was not toxic. The absence of dialyzable P^{32} in the effluent blood, which is indirect evidence of breakdown, also indicates that the plasma acting alone does not detoxify the endotoxin.

Evidence that detoxification by the spleen is due to RE cells, and not to

[4] A judgment based on P^{32} retention data would be valid if all the P^{32} were still tied to endotoxin. It is even more valid if some of the P^{32} is not tied to the endotoxin, for the normal spleen was releasing P^{32} throughout the period during which the injured spleen was not releasing P^{32} that was not bound to endotoxin.

other cells in the spleen, is derived from the experiments with thorotrast. Table I shows that the spleen infused with thorotrast 3 hours before injecting the endotoxin was not able to retain more than 25 per cent of the injected dose. Suppression of some two-thirds of the normal spleen's capacity for uptake of endotoxin by thorotrast might be accounted for (*a*) by blockade of most of the RE cells by an outer shell of cells swollen with thorotrast occluding the sinusoids (4), or (*b*) by RE cell injury, or both.

The spleen in shock, like the spleen blocked with thorotrast, cannot retain more than a very small per cent of the injected endotoxin. Since the RE system of the shocked spleen is not physically blocked (4), as it is in the thorotrast spleen, the defective performance of this system in the shocked spleen must be attributed to vasoconstriction, which restricts the clearance function of the cells, or to direct cell injury, or both.

The functional inferiority of the shocked and thorotrast-treated spleens is, at least in part, due to cell injury. This follows from the fact that none of the endotoxin retained after the first 10 minutes by these spleens loses P^{32} or toxicity within the next 2 hours. The same data demonstrate that uptake does not assure subsequent detoxification, just as phagocytosis of certain species or strains of bacteria is not always followed by their destruction.

The ratio of free to bound P^{32} in the venous blood from the two types of injured spleens within the first 3 minutes after injection of the endotoxin is about one-third of that in the venous blood of the normal spleen (*i.e.* 8.2 per cent and 9 per cent respectively *vs.* 26 per cent). But in terms of the endotoxin content of these bloods, the over-all capacity of the injured spleen to release P^{32} is at the outset equal in potency to that of the normal spleen. However, the fact that the injured spleens cannot release more P^{32} after the first 10 minutes, while the normal spleen continues to do so until the end of the experiment, is evidence that both injured spleens suffer further damage, presumably by the endotoxin which is not immediately taken up and destroyed.

The avoidance of damage to the normal spleen may be due to the capacity of the normal RE system to take up and destroy endotoxin very rapidly, as indicated by the observation that 17 per cent[5] of the endotoxin injected was released into the effluent blood in the first 2 minutes, that one-fourth of the P^{32} in this blood was already dialyzable, and that the released endotoxin was detoxified.[6]

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[5] In terms of P^{32} data.

[6] In a series of four preliminary experiments to determine directly the speed of detoxification by the normal spleen, the labeled endotoxin was injected as in the preceding experiments. After 3 minutes the third of the spleen nearest the injected artery was amputated between hemostatic bands. After 5 minutes the distal third was amputated, and after 10 minutes the middle third was removed. In two additional experiments the spleen was removed *in toto*

Previous studies have demonstrated the continuous absorption of endotoxin from the intestine (5). From this and the data herewith presented it follows that damage inflicted on the RES will result in a greatly reduced capacity to take up and destroy endotoxin, and hence must lead to endotoxemia with a resulting irreversible collapse of the peripheral circulation and death.

SUMMARY AND CONCLUSIONS

Evidence is presented that

The normal RES extracts endotoxin from the circulation and inactivates it very rapidly.

When the RES has been damaged, whether by a blockading agent, such as thorotrast, or by a reversible degree of hemorrhagic shock, it cannot extract more than a small per cent of the amount of the endotoxin the normal system can extract.

Of that fraction of endotoxin which is extracted, very little is detoxified.

An organ like the kidney, which does not contain a significant amount of RE tissue, does not extract more than an insignificant percentage of the injected amount, and therefore does not detoxify endotoxin.

Since dephosphorylation is a process concurrent with detoxification, and does not occur in an organ which does not extract or inactivate a significant amount of injected endotoxin, it is probable that this process is an indirect index of detoxification.

Within the time limits of these experiments, plasma alone does not inactivate endotoxin.

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after 3 minutes. All specimens were homogenized and the homogenate tested for toxicity as described. In three of the first four experiments the P^{32} content of the spleen was measured. There was retention of 69, 72, and 87 per cent respectively of the injected dose. Of the fourteen specimens tested for toxicity eleven were non-toxic; *i.e.*, not more than two of six chick embryos exposed to the same sample were killed. Of the remaining three which were toxic, two from the distal pole assayed borderline in one and toxic in the other. The third was a homogenate of one of the two whole spleens removed after 3 minutes.

It is thus evident that the detoxification of endotoxin by the normal RE cell is an extremely rapid process, and probably occurs immediately after uptake. In view of the fact that the injected pole uniformily showed about twice the radioactivity of the remainder of the spleen, it is possible that more, if not all, specimens would have assayed negative had the endotoxin entered the spleen *via* the main artery for uniform distribution.

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