IN VITRO DETOXIFICATION OF BACTERIAL ENDOTOXIN BY MACROPHAGES*

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The reticulo-endothelial system (RES) is known to be the primary repository of endotoxins injected into the circulation (1, 2). It is assumed to be the site of their detoxification as well. A variety of hypotheses based on this assumption have been formulated (4). If we are to gain more understanding of the process of resistance to endotoxins, it would be well to validate this assumption by direct evidence. This report provides data on the *in vitro* capacity of macrophages to detoxify endotoxin. The results of a parallel *in vivo* study will follow.

Materials and Method

1. Macrophages were harvested in iced gelatin–Locke's solution, by a technic described elsewhere (5), from the peritoneal cavities of normal rabbits 48 hours after intraperitoneal instillation of beef-infusion broth. The cells were centrifuged at 4°C., washed in iced gelatin–Locke's solution, and finally resuspended in Tyrode's solution to an average concentration of 10^8/ml. Granulocytes were harvested in the same way except that these cells were collected 6 hours instead of 48 hours after injection of the broth.

2. Serum was obtained from clotted normal rabbit's blood and plasma from blood collected in ACD solution. Immune serum was similarly obtained from rabbits which had been injected intramuscularly three times a week for 6 weeks with 1 ml. of a heat-killed culture of a strain of *E. coli* $0111B_4$. This serum had a 1:5000 agglutinating titer against this organism.

3. Two different endotoxins were employed. One was prepared by the Boivin technic from a culture of the same strain of *E. coli* $0111B_4$ labeled with P^{32}. The MLD/100 of a stock solution of the endotoxin in adult rabbits pretreated with thorotrast 3 hours previously was 0.3 ml./kg. The other endotoxin, a Difco preparation (lot 129:B8), had an MLD/100 of 10^{-3} mg./kg. in such thorotrast-pretreated rabbits. Toxicity of the reaction mixtures to be described below was also determined in such rabbits. Test rabbits that did not die were killed after 48 hours. Detoxification of the endotoxin was considered not to have occurred if postmortem examination revealed the presence of intramural intestinal hemorrhage or the generalized Shwartzman reaction, whether the rabbit died or survived the injection. Survival together with the absence of any reaction was taken as evidence of detoxification.

In some experiments toxicity of the reaction mixtures was determined by the 10 day old chick embryo test, as well as by the rabbit test. Six chick embryos were used in each test. At

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least 4 of the 6 were regularly killed by exposure to 5×10^{-6} mg. of the Difco endotoxin. Detoxification was considered not to have occurred if the reaction mixture killed at least four of the six embryos. Survival of at least four embryos was taken to indicate substantial detoxification.

Procedure

In the first series of experiments four tubes (A) were set up, each containing 1 ml. macrophages (10^8/ml.), 1 ml. of ACD plasma or serum, and 1 MLD/100 (*i.e.* 0.3 ml./kg.) of the P^{32}-labeled endotoxin in 0.6 ml. saline. Two similar tubes (B), omitting the macrophages, and two others (C) omitting the plasma or serum, were also set up.[1] All tubes were brought to the same volume with Tyrode's solution, and incubated at 37°C. for 1 or more hours, as indicated below. During the entire period of incubation the tubes were agitated frequently by hand, or in a shaking machine.

Thereafter the tubes were treated as follows: The contents of the A tubes were ground in a mortar and pestle to rupture the cell membranes. The content of one of these was then dialyzed overnight in running tap water at 4°C., while that of the other was held overnight at this temperature without dialysis. The P^{32} activity in both tubes was then determined, the difference yielding the measure of release of P^{32} from the endotoxin. The contents of each of these two tubes were then tested for toxicity by injection intravenously into thorotrast-pretreated rabbits.

To determine the distribution of the total bound P^{32} activity between the cells and the plasma, and the presence or absence of toxicity in either, the remaining two A tubes were treated as follows: The cells from both tubes were recovered immediately after incubation, washed twice in Tyrode's solution and recentrifuged. All washings were added to the supernatant fluid. There were now two tubes containing washed cells, and two tubes containing supernatant fluid. The cells were ground with mortar and pestle to rupture the cell's walls. One tube of ground cells and one tube of supernatant fluid were dialyzed overnight in running tap water at 4°C.; the other two tubes were stored at 4°C. for the same period, after which the P^{32} content and the presence or absence of toxicity in each of the four tubes was determined.

The B tubes were treated as follows: Immediately after the incubation period, one of these tubes was dialyzed overnight in running tap water at 4°C., while the other was held for the same period at 4°C. The P^{32} activity and the presence or absence of toxicity in the contents of each tube were then determined.

In the second series of experiments the incubation period was extended to 3 hours. Serum was used instead of plasma, and the Difco endotoxin was employed. The amount added to the mixture was one MLD/100 (*i.e.* 10^{-3} mg./kg. for the rabbit to be tested). In this series of experiments the tests for toxicity were done not only in the rabbit, but also in the chick embryo. A few preliminary experiments with immune serum were also performed.

In the third series of experiments the second series was repeated, but with granulocytes in place of macrophages.

Results.—Table I shows that normal plasma alone released an average of 19 per cent of the bound P^{32} from the labeled endotoxin, without effecting detoxification.[1] On the other hand, the macrophages in plasma released an average of 51 per cent of the bound P^{32}, and achieved a simultaneous detoxification[2] of the

[1] Experiments with C tubes were discontinued after several series of such experiments showed no release of P_{32} and no detoxification.

[2] As defined under Materials and Methods.

endotoxin in 3 of 4 experiments. Since at the end of the reaction period most of the radioactivity was found in the supernatant fluid, and the cells were not toxic by the rabbit test, it appears that the ingested endotoxin does not accumulate intact within the cells, and is rapidly broken down. The degree to which the plasma detoxified the toxin without the aid of cells could not be assessed by the rabbit assay method, because this method does not reveal gradations in breakdown. But the extent of release of bound P_{32} may be a rough index of detoxifica-

TABLE I

*Detoxification of P_{32}-Labeled Endotoxin by Macrophages in Plasma after Incubation for 1 Hour**

Experiment	Plasma + toxin		Macrophages + plasma + toxin		Supernatant fluid		Cells	
1. Undialyzed	18171 } 12	+	17853 } 42	+	15393 } 41	+	3001	–
Dialyzed	16225 }	+	10393 }	–	8830 }	–	2107	–
2. Undialyzed	10846 } 35	+	10894 } 54	+	9334 } 57	+	183	–
Dialyzed	7104 }	+	5002 }	–	3968 }	–	542	–
3. Undialyzed	9247 } 4	+	9520 } 50	+	8414 } 62	+	1009	–
Dialyzed	8832 }	+	4874 }	+	3207 }	–	1016	–
4. Undialyzed	7223 } 24	+	6493 } 58	+	5832 } 54	–	573	–
Dialyzed	5579 }	+	2780 }	–	2672 }	–	185	–

* The plus and minus signs signify the presence or absence of toxicity in the reaction mixture before and after incubation. They also signify toxicity in the supernatant fluid and in the cells separately before and after dialysis. Toxicity in these experiments was assayed by the rabbit test.

The bracketed figures show the counts per minute before and after dialysis to remove released P_{32}. The figure outside the bracket shows the percentage of decline in counts per minute of the labeled endotoxin after 1 hour of incubation. This percentage is omitted for the cells because the total radioactivity content of the cells is relatively insignificant.

tion, for detoxification by the rabbit test was observed when half the initially bound P^{32} was released. This accords with the observation of Tal and Goebel that when acid hydrolysis of the somatic antigen of *Shigella paradysenteriae* released 40 per cent of the total phosphorus, the toxicity was destroyed (5).

Table II lists the results of the second series of experiments. They show that by the rabbit test serum[3] failed to detoxify the endotoxin in 16 of 17 experiments, but by the chick embryo test serum achieved some detoxification, for the percentage of surviving embryos was increased from 26 per cent (23 of 90) on exposure to toxin alone, to 51 per cent (46 of 90) on exposure to the serum plus toxin.

[3] Our data show no difference between the detoxifying potency of serum and plasma.

TABLE II

*Detoxification of Endotoxin By Macrophages in Serum after Incubation for 3 Hours**

Experiment No.	Toxin		Toxin + serum		Toxin + serum + macrophages		Toxin + immune serum‡		Toxin + immune serum + macrophages‡	
1.	6/6	+	3/6	+	1/6	−	3/6	−	1/6	−
2.	5/6	+	4/6	+	1/6	−	0/6	−	0/6	−
3.	5/6	+	4/6	+	1/6	−	4/6	+	1/6	−
4.	4/6	+	4/6	+	0/6	+	2/6	0	1/6	0
5.	4/6	+	3/6	+	0/6	−	3/6	−	1/6	−
6.	4/6	+	1/6	+	0/6	−	3/6	+	3/6	−
7.	0	+	0	+	0	−	3/6	0	2/6	0
8.	4/6	+	1/6	−	0/6	−	3/6	+	1/6	+
9.	5/6	+	3/6	+	1/6	+	2/6	+	2/6	+
10.	5/6	0	6/6	+	6/6	+	0	−	0	−
11.	5/6	+	3/6	+	1/6	−	0	+	0	−
12.	2/6	−	0/6	+	0/6	−	3/6	+	3/6	−
13.	4/6	+	3/6	+	1/6	−	2/6	+	2/6	+
14.	5/6	+	3/6	0	2/6	0	0	+	0	−
15.	4/6	−	3/6	+	1/6	−	0	+	0	−
16.	5/6	+	3/6	+	2/6	+	0	0	0	0
17.	0	+	0	+	0	−	0	0	0	0
18.	0	+	0	+	0	−	0	0	0	0

* Each sample was assayed by the rabbit test and the chick embryo test. The plus sign signifies no detoxification and the minus sign signifies detoxification by the rabbit test. The numerator in the fractions indicates the number of chick embryos killed out of a total of six (denominator) tested with the same sample. Zero signifies no observation.

‡ The amounts of toxin in the reaction mixtures with immune serum were 2, 3, or 4 times the usual dose.

TABLE III

*Detoxification of Endotoxin by Leukocytes in Serum after Incubation for 3 Hours**

Experiment No.	Toxin		Toxin + serum		Toxin + serum + leukocytes	
1.	6/6	+	4/6	+	4/6	+
2.	5/6	+	5/6	+	1/6	+
3.	5/6	+	3/6	+	3/6	−
4.	6/6	+	3/6	+	3/6	+
5.	4/6	+	2/6	+	2/6	−
6.	4/6	+	3/6	+	3/6	−
7.	4/6	+	3/6	+	2/6	+
8.	4/6	+	3/6	+	1/6	−
			dead 48 hrs.			

* Each sample was assayed by the rabbit test and the chick embryo test. The plus sign signifies no detoxification and the minus sign signifies detoxification by the rabbit test. The numerator in the fractions indicates the number of chick embryos killed out of a total of six (denominator) tested with the same sample.

The superiority of the macrophage in serum over serum alone is evident in the survival rate of 81.2 per cent (73 of 90) of chick embryos, and the survival of 13 of 17 test rabbits without pathological evidence of toxicity.

Preliminary experiments with immune serum prepared by repeated injections of the specific antigen demonstrate a very considerable endotoxin-detoxifying potency of such serum in the absence of macrophages (Table II). Such sera protected 57 per cent of 66 chick embryos exposed to 2, 3, or 4 lethal doses without the aid of macrophages. With macrophages in the reaction mixture 74 per cent of the embryos survived the same exposure. The superiority of macrophages in immune serum over immune serum alone is also indicated by the results of the rabbit test, which yielded a survival rate for the macrophages in serum of 79 per cent (10 of 13) as compared to 31 per cent (4 of 13) for immune serum alone.

Table III lists the data on the detoxifying potential of granulocytes. The mortality of chick embryos caused by toxin alone (77 per cent) was considerably reduced by serum (54 per cent) and still more by granulocytes in serum (39 per cent). But by the rabbit test the granulocytes in serum reduced the mortality rate only 50 per cent.

DISCUSSION

The foregoing data demonstrate the very considerable anti-endotoxic power of serum or plasma. At the same time they demonstrate the clearly superior detoxifying activity of the cellular component in the destruction of bacterial endotoxins.[4] 10^8 macrophages possess greater detoxifying power than a thorotrast-pretreated rabbit. Since the normal rabbit possesses a very large multiple of 10^8 macrophages, the failure of a thorotrast-pretreated rabbit to survive a dose which 10^8 intact cells can destroy reveals the thoroughness with which thorotrast impairs the detoxifying function of the RE system.[5]

However, the relevance of these *in vitro* data to the fate of endotoxin *in vivo* is uncertain, for the way in which the endotoxin is presented to the cell, the ratio of the concentration of cells to endotoxin, the extent of injury inflicted by the endotoxin on the cell, which may affect its detoxifying potential (8), the hemodynamic factors affecting the time of interaction between cell and endotoxin,—these are but a few of the factors in the animal which cannot be reproduced *in vitro*. Hence, only the roughest approximation can be made from the functional behavior of the cell in an experimental model which is defective in so many directions, not the least of which is that the test cell is one which has already responded to the stimulus of an irritant injected into the peritoneal cavity.

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[4] Comparison of the data in Table III with that of Tables I and II show that the superiority of granulocytes in serum to serum alone is less impressive than that of macrophages in serum to serum alone.

[5] It should however be stated that a lethal dose of endotoxin *in vivo* is not solely the amount administered. An important contributing factor is the endotoxin absorbed from the gut (7).

The behavior of the granulocyte *in vitro* is even more artificial. The data showing an antiendotoxic property of granulocytes *in vitro* conflicts with the fact that circulating granulocytes, which are plentiful in the thorotrast-pretreated rabbit, do not protect against the death from extremely small doses of endotoxin in such rabbits. And endotoxin injected intravenously not only causes a flight of the granulocytes to the liver, spleen, and lung, in which they appear to behave abnormally, but suppresses the movement of fresh granulocytes into the circulation from the bone marrow.

Even more contradictory is the *in vitro* behavior of immune serum, as compared to its action in the living animal. The preliminary *in vitro* data here reported show an impressive detoxifying power, which is clearly at variance with the well established observation that such immune serum will not protect the animal from which it was taken against endotoxin used to produce the immune serum.

Thus it is necessary to secure *in vivo* data, which are presented in the paper to follow.

SUMMARY AND CONCLUSIONS

In the *in vitro* model described normal serum or plasma alone does not detoxify endotoxin, as determined by the response of the thorotrast-primed rabbit to the injection of the reaction mixture after 1 hour of incubation at 37°C. Partial detoxification, however, is achieved by serum or plasma after 3 hours of incubation of the same reaction mixture, as determined by the chick embryo test.

Macrophages in serum or plasma do detoxify endotoxin, as determined by both tests. Granulocytes, which are also able to detoxify endotoxin in the presence of serum, are less potent than macrophages.

Immune serum alone is much more potent than normal serum alone. Macrophages in immune serum are more potent than immune serum alone.

The relevance of the anti-endotoxic properties of these *in vitro* systems to the processes in the living animal are in doubt, and must await the results of comparable observations in the living animal.

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