ON THE ABSORPTION OF BACTERIAL ENDOTOXIN FROM THE GASTRO-INTESTINAL TRACT OF THE NORMAL AND SHOCKED ANIMAL*

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There is considerable evidence that the circulating toxin in the blood of the animal in severe and prolonged hemorrhagic shock is an endotoxin derived from the intra-intestinal bacteria, and that this endotoxin is responsible for the failure of the circulation in late shock to respond to blood volume therapy (1). This thesis is of general biologic significance. It demands unequivocal proof that bacterial endotoxin is absorbed from the intestine in sufficient quantity and potency to account for the state of irreversibility to transfusion. This report presents evidence that this is the case.

Methods

The bacterium, *Escherichia coli* $0111B_4$, employed in this study is a stable, antigenically well defined organism, characterized by a single oligosaccharide end-group antigen. It is occasionally pathogenic for man, but is generally not found in the intestinal flora of the rabbit. The organism was cultured in a nutrient broth (Difco) rendered phosphate-poor by precipitation of phosphate ion as a magnesium salt. The broth was adjusted to pH 7.4 after which 16 to 25 mc. of carrier-free $Na_3HP^{32}O_4$ were added per liter of broth. A heavy inoculum of a 6 hour broth culture of the stock strain was then introduced into each of three 2 liter flasks containing 1 liter of the sterile radioactive medium, and incubated for 16 to 22 hours on a shaker platform at 37°C. The labelled organisms were harvested by centrifugation at 4°C., yielding an average of 3 to 3.5 gm. of wet bacteria per liter. The supernatant radioactive medium was sterilized, adjusted to pH 7.4, charged with glucose, and re-inoculated with a fresh heavy 6 hour broth culture of *E. coli* $0111B_4$. This process could be repeated six to eight times before the yield of bacteria declined significantly. The labeled organisms were pooled, suspended in 0.15 M saline, and stored in the cold until a sufficient amount had been accumulated for a complete experiment, at which time the pooled bacteria were washed in chilled saline, and dialyzed overnight against running cold tap water.

Adult albino rabbits (average weight 2.3 kg.), reared from birth on an antibiotic-enriched diet, were employed. One day prior to each experiment the intestinal flora was examined by culture of rectal swabs. With the exception of one small group noted below they were all "coliform-free"; *i.e.* the plate cultures yielded only an occasional colony of coliform organisms.

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In each experiment eight to ten coliform-free rabbits, fasted for 16 hours, were given 2 to 3 gm. of dialyzed labeled *E. coli* $0111B_4$, suspended in 20 ml. of saline, by stomach tube. Three hours after gavage alternate rabbits were subjected to hemorrhagic shock by bleeding into an elevated heparinized reservoir from a cannula in the femoral artery. Mean arterial pressure was maintained at 50 mm. Hg. Alternate control animals were treated in precisely the same manner, except that they were not bled into the reservoir. All animals were held in the supine position throughout the experiment.[1]

Five to six hours after the onset of hemorrhage the shocked animals were rapidly transfused with all their shed blood. Fifteen minutes later all animals were exsanguinated into clean sterile Erlenmeyer flasks. Samples of liver, spleen, kidney, and heart of both normal and shocked animals were also taken. The tissues and blood were submitted to a procedure, described below, for the recovery of the lipopolysaccharide fraction, which was then assayed for its content of antigenically specific bacterial polysaccharide. The method of Westphal *et al* (2) was employed for the isolation of bacterial lipopolysaccharide as follows: 90 per cent phenol (U.S.P. aqueous) was added to an equal volume of whole blood or 20 to 25 per cent homogenized tissue suspension in physiologic saline. The mixture was heated, with constant stirring, to 68° C. on a water bath for 30 minutes. After cooling, the mixture was centrifuged for 30 minutes at 3000 R.P.M. and the aqueous upper phase was carefully removed. The residue was discarded. A small amount of sodium acetate was added to the water extract, followed by five volumes of absolute ethyl alcohol. The resultant precipitate was separated by centrifugation for 10 minutes at 3000 R.P.M., taken up in 2 ml. of physiologic saline, and dialyzed against a large volume of saline overnight. These dialyzed extracts were then assayed for their content of antigenically specific polysaccharide of *E. coli* $0111B_4$ by the hemagglutination-inhibition technic of Crumpton *et al.* (3), as described below.

The lipopolysaccharide from an aliquot of the pooled P^{32}-labeled bacteria employed in each experiment was also prepared by this technic. This fraction was dialyzed against distilled water and then lyophilized. A carefully weighed amount of the lyophilized bacterial lipopolysaccharide was incubated with 0.02 N NaOH for 2 hours at 37°C., and neutralized with 0.02 N HCl. Fresh sheep erythrocytes suspended in Alsever's solution were washed three times in physiologic saline, and diluted to a 2 per cent V/V suspension in a fourth saline wash. Seven ml. of this suspension was then incubated with 25 μg. of alkali-treated bacterial lipopolysaccharide for 1 hour at 37°C. After incubation, the sensitized cells were washed three times with a 1:100 dilution of normal rabbit serum in saline, and the washed cells suspended in 14 ml. of 1:100 diluted rabbit serum to give a 1 per cent suspension of sensitized (coated) erythrocytes.

Two hemagglutinating doses of specific rabbit antiserum to *E. coli* $0111B_4$[2] were mixed with successive twofold dilutions of the extracts to be tested, and the mixture incubated at 37°C. for 1 hour. At the end of this time 0.2 ml. of sensitized sheep cells were added to 0.2 ml. of the extract/antiserum mixture, and the incubation continued 1 more hour. The incubated mixtures were then cooled to 4°C., and the hemagglutination titer read after standing in the cold for 18 hours. Serial twofold dilutions of the known weight of bacterial lipopolysaccharide were titrated in the same manner, and the smallest amount giving complete inhibition determined. The polysaccharide antigen content of tissue and blood extracts was calculated as the

[1] In six control experiments employing three rabbits with a normal, *i.e.* coliform-bearing flora, and three with a "coliform-free" flora, the introduction of the *E. coli* $0111B_4$ was omitted (See Table I).

[2] One hemagglutinating dose of antiserum is defined as the smallest amount of antiserum which produces visible agglutination of sensitized sheep erythrocytes. Specific E. Coli $0111B_4$ rabbit antiserum was prepared by immunization of rabbits with a heat-killed suspension of 5×10^8 bacteria/ml. according to the method of the Gardener *et al.*(4).

product of the highest serial dilution of the unknown antigen and the minimum amount of weighed bacterial lipopolysaccharide producing complete inhibition.

0.1 ml. aliquots of the fractions isolated from the blood and tissues in each experiment were pipetted onto 2.5 cm. circles of Whatman No. 1 filter paper cemented onto glass slides, and the P^{32} content estimated with a thin end-window Geiger-Müller tube. Comparison of this value with the P^{32} content of the lipopolysaccharide in the endotoxin isolated from the bacteria fed to the same animal provided an independent measure of the endotoxin content of these fractions.[3]

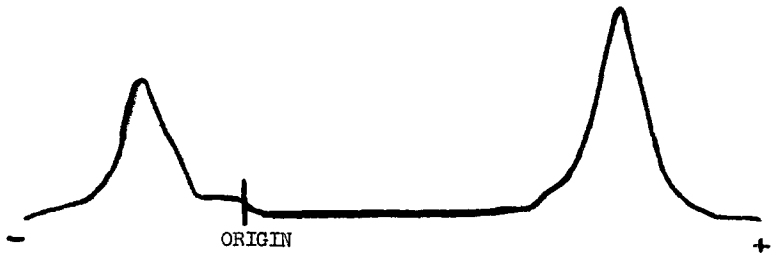

FIG. 1. Radioelectrophotogram of P^{32} labeled lipopolysaccharide of *E. coli* $0111B_4$ isolated by the phenol/water extraction method of Westphal *et al.* (3). The sample is an aliquot of that employed in Experiment 4 determination of P_{32} activity of the antigen. The cathodal migrating peak (left) is lipopolysaccharide containing 40 per cent of total sample radioactivity, while the anodal migrating peak (right) is nucleic acid containing 45 per cent of total sample of radioactivity. The remaining 5 per cent of P^{32} radioactivity is contained within the small peak trailing the lipopolysaccharide.

RESULTS

The experimental data are summarized in Tables I and II. They demonstrate

1. That there was no detectable quantity of the type-specific bacterial polysaccharide in the blood of 22 of 23 normal rabbits, whereas a substantial quan-

[3] The total P^{32} activity of the endotoxin minus the P^{32} content of its non-lipopolysaccharide components, primarily the bacterial nucleic acids, gives the value of the P_{32} in the lipopolysaccharide used in this comparison. These values were obtained as follows: The bacterial endotoxin preparation and the extracts of blood and tissues were subjected to paper electrophoresis in barbital buffer at pH 8.4 dried and scanned for localization of P^{32} with an automatic recording gas-flow Geiger-Müller tube. Under these conditions of analysis P^{32}-labeled polysaccharide or lipopolysaccharide is virtually immobile or migrates slightly toward the cathode, whereas nucleic acid (verified by elution and ultraviolet-spectrophotometric examination) migrates rapidly toward the anode (Fig. 1). Only one peak, corresponding to lipopolysaccharide, is encountered in the antigen-containing extracts prepared from the tissues, whereas both lipopolysaccharide and nucleic acid peaks are encountered in the bacterial preparation. The area under each peak was measured by planimetry, and the specific activity of the lipopolysaccharide component of the bacterial endotoxin corrected by multiplying P^{32} activity/mg. lyophilized endotoxin by that fraction of the total P^{32} which was found in the lipopolysaccharide peak.

tity was present in the blood of 20 of 23 rabbits subjected to irreversible hemorrhagic shock.

TABLE I

*E. coli 0111B$_4$ Polysaccharide Recovered from Normal and Shock Blood**

Experiment No.	Normal rabbit whole blood	Shocked rabbit whole blood
	μg. per cent	*μg. per cent*
1	0	160
	0	16
	0	6
	0	10
2	0	0
	0	100
	0	0
	0	80
	0	40
3	0	4 (4.5)‡
	0	16 (20)
	0	24 (27)
	0	44 (63)
	0	24 (27)
4	0	31 (60)
	0	63 (60)
	0	55 (67)
	0	75 (90)
	0	38 (30)
5	0	15 (18)
	0	8 (8)
	0	8 (7)
	0	8 (10)

* Three "coliform-free" rabbits and three rabbits with a normal, *i.e.* coliform-bearing, flora which did not receive *E. coli* 0111B$_4$ and which were subjected to severe hemorrhagic shock, were studied for the presence of circulating 0111B$_4$ or cross-reacting antigens. All six were uniformly free of these substances.

‡ Figures in parentheses represent results of P^{32} analysis of *E. coli* endotoxin content of serum extracts, corrected for nucleic acid as described in footnote 3.

2. That a substantial amount of this polysaccharide was present in the liver of both the normal and the shocked rabbit.

3. That the concentration of this polysaccharide in the liver and spleen of the shocked animal, and in the liver of the normal animal, was significantly

higher than in the blood; whereas the concentration in the kidney was approximately the same as in the blood.

4. That the blood of rabbits in severe and prolonged hemorrhagic shock, which had not received the *E. coli* 0111B$_4$, contained none of this polysaccharide or of any other cross-reacting polysaccharide.

TABLE II

*E. coli 0111B$_4$ Polysaccharide Recovered from Normal and Shock Tissue**

Experiment No.	Normal rabbits				Shocked rabbits			
	Liver	Spleen	Kidney	Heart	Liver	Spleen	Kidney	Heart
3	50				40			
	40				110			
					360			
					200			
4	100	0	25		140	250	25	
	180	0	0		120	250	50	
	50	0	0		200	250	25	
	100	0	25		100	250	75	
	120	0	0		25	0	50	
5	0	0	0	0	15	0	9	0
	0	0	0	0	12	0	0	0
	0	0	0	0	12	0	0	0
	0	0	0	0	12	0	0	0

* Aliquots of tissue were homogenized and extracts for determination of *E. coli* antigen content according to the following schedule: 10 gm. aliquots were taken of liver and kidney, 3 gm. of heart (usually the entire organ), 0.5 to 1.0 gm. of spleen (entire organ). Within the limits imposed by the sample size and the sensitivity of the hemagglutination-inhibition test, the lower level of detectability of *E. coli* 0111B$_4$ antigen in the tissues 10 μg./100 gm. of liver and kidney, 25 to 33 μg./100 gm. of heart muscle, and 150 to 250 μg/100 gm. of spleen. A recorded value of "0" in the table indicates that the tissue was sampled and tested, but contained less than the lower limit of detectability in that organ. Concentration Recorded in μg./100 gm.

DISCUSSION

The foregoing data provide unequivocal evidence that the circulating toxin is the antigenically intact endotoxin derived from the strain of *E. coli* introduced into the gastro-intestinal tract a few hours prior to the onset of shock. Although the hemagglutination-inhibition reaction identifies only the polysaccharide component of the lipopolysaccharide, previously reported data (1) provide ample evidence that the substance isolated from shock blood, characterized here

antigenically, exhibits all the known biological properties of the intact lipopolysaccharide. That the polysaccharide identified in these experiments was derived only from the particular strain of Gram-negative bacteria introduced into the gut is evident from the fact that the amount of lipopolysaccharide, as estimated from its P^{32} content, is in good agreement with the amount measured by the hemagglutination-inhibition assay. This finding precludes the likelihood of admixture with unlabeled polysaccharides derived from normal tissues, from breakdown products of anoxic tissues, or from multiplication of labeled organisms within the tissues.

Isolation of the specific antigen from the liver of both normal and shocked animals is evidence that endotoxin is absorbed, probably continuously, in the normal as well as in the shocked animal. Although the tissues of the normal rabbit contain some bacteria, which multiply during the shock state (5), the number of organisms is far too small to account for a significant fraction of the endotoxin recovered from the blood and tissues. For example, some 8 to 10 hours after administration of *E. coli* $0111B_4$ to rabbits, and induction of shock for 5 to 6 hours, approximately 200 to 300 μg. of the type-specific polysaccharide was recoverable from the blood and liver. This amount represents the total lipopolysaccharide content of approximately 1.2×10^9 organisms, or an average concentration of 600,000 bacteria per gm. throughout the entire animal. Since the number of bacteria recovered from the blood and tissues in such shocked animals is rarely more than a fraction of 1 per cent of this number, it may be presumed that endotoxin entered the circulation as such by passive diffusion or active transport mechanisms. Since only 1 per cent of the amount of endotoxin contained within the administered oral dose of 3 gm. of intact *E. coli* $0111B_4$ was recovered from the blood and tissues, passive diffusion would appear to be an adequate mechanism to account for the observed data, without invoking active transport by pinocytosis, phagocytosis, or other possible mechanisms.

Sanford and Noyes (6) question the validity of the hypothesis that endotoxin derived from the intra-intestinal flora is involved in the phenomenon of irreversible shock because they were unable to detect Cr^{51} in the blood or tissues following the introduction of Cr^{51}-labeled endotoxin into the gastro-intestinal tract. These authors recognized the fact that altered diffusibility of chromium-labeled endotoxin might account for their negative results. Nevertheless, they assumed that their experimental conclusions were valid. It is now known, however, that Cr^{51} attaches firmly to the lipid A moiety of endotoxin, rendering it much less emulsifiable and diffusible,—indeed precipitating the lipid as an insoluble chromium complex (7).

The variability in degree of endotoxemia among animals within a given category (*e.g.* 3 of 23 shocked rabbits exhibited no recoverable circulating endotoxin) is consistent with the facts (*a*), that approximately 20 per cent of animals bearing an apparently normal intestinal flora survive severe and prolonged

hemorrhagic shock (8); and (*b*) that the blood of about the same per cent of severely shocked animals is toxin-free by biologic assay (9, 10). Such quantitative variations are expected, and indeed, are inevitable in view of the many variables governing the size of the pool of endotoxin within the gut, its rate of diffusion across the intestinal membrane, and differences in the detoxifying potential of the reticulo-endothelial system. Since all of the rabbits in this series were initially "coliform-free," and since all received the same amount of *E. coli* $0111B_4$ by gavage at the same time, the varying amounts of type-specific lipopolysaccharide recovered from their blood and tissues probably reflect (a) differences in rate of caudal movement; (*b*) differences in distribution of administered endotoxin on available absorbing surfaces; (*c*) possible differences in the physical state of endotoxin on its release from the bacteria within the gastrointestinal tract; (*d*) differences in the state of the intestinal membrane of individual animals; and (*e*) differences in the endotoxic content of different preparations of the same bacteria. Inspection of the data of Experiment 5 suggests that the amount of endotoxin available for absorption was smaller than usual even though the weight of bacteria administered was equal to that employed in other experiments.[4] The wide disparity in the amount of circulating endotoxin which results from the operation of these and other factors account, in our opinion, for the differences in mortality rate, in survival time, and in the severity of endotoxic damage encountered in shocked animals at postmortem examination.

Whatever the relative importance of these several factors in determining the quantitative differences in endotoxin content of blood from one group of shocked animals to another, there can be no doubt that the consistent differences between the amount of circulating endotoxin in the normal and shocked animal within the same group signifies an inability of the shocked animal to clear the blood of absorbed endotoxin. The possibility that this difference is due to greater absorption from the gut in the shocked animal is unlikely for two reasons: (*a*) the amount of P^{32} recovered from the blood and tissues (Table III) and the amount of antigenically identifiable polysaccharide recovered from the liver (Table II) was about the same in the normal and shocked animal. (*b*) The amount recovered does not include what was absorbed and excreted or destroyed prior to exsanguination, so that the measured difference in the amount of endotoxin cleared and detoxified underrates the performance, of the normal animal, which has been detoxifying and excreting at a better rate than the shocked animal for at least 6 hours.

[4] This surmise was confirmed by the subnormal yield of endotoxin obtained on processing the pool of *E. coli* employed for this experiment. The low endotoxin content of these organisms was probably due to the use of an enriched medium, Difco tryptic digest beef heart broth, in an attempt to increase the over-all yield of bacteria. Bacterial yield was better, but endotoxin yield was reduced to 32 per cent of the usual yield obtained from *E. coli* $0111B_4$ grown in nutrient broth.

The extent of the injury to RE function in the shocked animal can be gauged by the following considerations: The maximum concentration of antigen that could go undetected in the hemagglutination-inhibition test is 2.5 μg./100 ml. of blood. If we assume that the blood from the normal animal contained this much *undetected antigen*, the ratio between the measured concentration of endotoxin in normal liver (40 to 180 μg./100 gm.) and this concentration in the blood ranges between 16/1 and 72/1 in 7 experiments with normal rabbits. This ratio ranges from 0.6/1 to 10/1 in nine experiments with shocked animals. Since the clearance load was no greater for the shocked liver than for the normal liver,

TABLE III

*Recovery of P^{32} in Organs of Normal and Shocked Rabbits after Oral Administration of Labeled E. coli $0111B_4$**

	Normal recipient	Shocked recipient
	Per cent administered dose	Per cent administered dose
Liver	2.1	1.3
Lung	0.23	0.12
Spleen	0.01	0.01
Kidney	0.61	0.58
Muscle	2.35	1.30
Total	5.29	3.31

* Each of three rabbits in each group received 20 ml. of a dialyzed suspension of *E. coli* $0111B_4$-P^{32} 8 hours prior to sacrifice by exsanguination. The shocked group were bled into the reservoir 2 hours after gavage and retransfused 30 minutes before sacrifice. Recorded value is arithmetic mean for each group.

the reduced capacity of the shocked liver is obvious. This could in part result from reduced contact between the blood and the hepatic reticulo-endothelial cells secondary to changes in intrahepatic distribution of blood flow. However, the difference between normal and shocked animals applies not only to the capacity for clearance, but also to detoxification. With respect to the latter function, the antigenically intact bacterial polysaccharide recovered from the normal liver was tested and found to be biologically inactive (*i.e.* free of pyrogenicity, toxicity, Shwartzman-inducing activity, etc.), whereas the same fraction isolated from the blood and liver of the shocked animal retained the characteristic biologic properties of a potent endotoxin. Hence the RE system of the shocked animal is notably less able to detoxify lipopolysaccharide.

There is little doubt that the amount of endotoxin demonstrated in the blood and tissues of the shocked animals is enough to account for the development of irreversibility and death. This judgment is based on the following data: The amount of endotoxin present in the blood and tissues of the animals in this

study is well in excess of the amount of endotoxin which can convert a "reversibly shocked" animal to an "irreversibly shocked" animal (11), or the amount of toxin present in the volume of blood from an irreversibly shocked animal which will kill another in reversible shock (9). Recent, as yet unpublished, observations show that coliform-free rabbits, which regularly survive exposure to 6 hours of hemorrhagic shock, do not recover if fed 2 gm. of *E. coli* $0111B_4$ or an equivalent weight of endotoxin, prior to the induction of shock. For some months, during which a diarrhea was widespread among rabbits in this vicinity, we encountered rabbits with an apparently normal coliform flora which were resistant to hemorrhagic shock, and which likewise became irreversible when fed *E. coli* or endotoxin like the coliform-free rabbits (12). Such observations are among various lines of evidence which support the thesis that the intestinal pool of Gram-negative organisms is of variable size, and contributes materially according to its size to the injury sustained by the animal from parenterally administered endotoxin (13), and in the elicitation of the generalized Shwartzman reaction (14).

The implications of the evidence for continuous, but not necessarily constant, absorption of endotoxin from the gastro-intestinal tract in the normal animal are of major biologic significance. Thus, just as repeated injections of sublethal doses of endotoxin modify the response of animals to stress (*e.g.* Gram-negative infections, drum shock, hemorrhagic shock (1)), so the endotoxin being constantly absorbed from the intestinal tract can prime or condition the responsiveness of the reticulo-endothelial system to such stress. Considering the variables involved in the exposure and in the response to such endotoxins, one should expect the observed large variations in resistance from one animal to another.

SUMMARY AND CONCLUSIONS

Coliform-free rabbits fed P^{32} labeled *E. coli* $0111B_4$ prior to the induction of experimental hemorrhagic shock were shown to have a substantial amount of the type-specific $0111B_4$ antigen in the circulating blood, liver, and spleen, whereas normal rabbits fed the same amount of these bacteria and held under identical conditions, but not exposed to shock, have the antigen within the liver, and occasionally in the kidney, but not in the blood.

That the antigen recovered from the blood and tissues was derived from this specific strain of bacteria was demonstrated by the use of the hemagglutination inhibition reaction, by the absence of cross-reacting antigens in appropriate control animals, and by agreement in the amount of antigen as estimated by two different technics.

Transport of bacterial endotoxin across the intestinal membrane appears to be achieved primarily by passive diffusion.

The accumulation of biologically active endotoxin in the blood and tissues of the shocked animal appears to be due to a reduction in the detoxifying potential

of the reticulo-endothelial system, and not to a greater than normal absorption of endotoxin from the intestine.

The absence of toxicity in the specific antigen extracted from normal liver demonstrates that the degradation of endotoxic potency can be achieved without altering the chemical integrity of the polysaccharide moiety of the molecule.

The implications of the hypothesis that there is a continuous but fluctuating absorption of bacterial endotoxin from the intestine are briefly discussed, and the contribution of free circulating bacterial endotoxin of intestinal origin to the fate of the shocked animal is noted.

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