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THE ROLE OF ENDOTOXIN IN THE EXTRACELLULAR COAGULATION OF LIMULUS BLOOD¹

JACK LEVIN AND FREDERIK B. BANG

Marine Biological Laboratory, Woods Hole, Massachusetts, the Department of Medicine, The Johns Hopkins University School of Medicine and Hospital, and the Department of Pathobiology, School of Hygiene and Public Health, The Johns Hopkins University

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INTRODUCTION

The generalized Shwartzman reaction is described as the occurrence of renal cortical necrosis in rabbits following appropriately spaced injections of endotoxin (1). During the course of this reaction, there is a fall in the numbers of circulating platelets and granulocytes; and there may be an abrupt decrease in the level of circulating fibrinogen and shortening of the clotting time (2). Intravascular coagulation may be an important part of this phenomenon (2, 3), and the mechanism by which endotoxin affects the coagulation system appears to be mediated through the platelet (4, 5).

In 1956, it was shown (6) that a bacterial infection of *Limulus*, the horseshoe crab, caused fatal intravascular clotting. This extensive clotting, resembling in some respects the generalized Shwartzman reaction, was readily induced by a heat stable derivative of the bacterium, subsequently shown to be a *Vibrio* species (7).

Limulus blood contains only one type of cell called the amebocyte. When whole blood is withdrawn from the *Limulus*, a clot quickly forms. Thereafter, this clot shrinks spontaneously, and a liquid phase appears. Under appropriate conditions, this liquid material has the capability of gelling when it is exposed to bacterial endotoxin (6), and is defined here as *pre-gel*.

In the current work, an attempt has been made to study the mechanism by which endotoxin affects coagulation in the *Limulus*, and to elucidate the mechanism by which endotoxin exerts its effect in a biological system that may be less complex than that found in mammals. The results demonstrate that cellular material from the amebocyte is necessary for coagulation of *Limulus* plasma, and that plasma free of all cellular elements does not clot spontaneously nor gel after addition of endotoxin. Data relating to the conversion of pre-gel to gel by endotoxin are reported.

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MATERIALS AND METHODS

All of the Limuli were obtained from the Marine Biological Laboratory Supply Department, and were kept in running sea water. Their sizes ranged from 8–12 inches across the cephalothorax. Blood was obtained by cardiac puncture by inserting a 20 gauge needle at the joint between the thoracic and abdominal segments, after the area had been cleansed with 70 per cent ethyl alcohol. Sterile and pyrogen-free glassware and syringes, prepared by steam sterilization at 16 lbs. of pressure for 45 minutes, followed by two hours at 170° C. in a drying oven, were used throughout. All tests were carried out in silicone-free 13 x 100 mm. glass test tubes which were immediately capped after reaction mixtures were added to prevent contamination from air borne moisture containing endotoxin. All experiments were carried out at room temperature, which ranged from 20 to 25° C.

Pre-gel was obtained by vigorously agitating a tube of fresh blood and allowing this to stand at room temperature for 20–24 hours. At the end of that time, a clear supernate (the pre-gel), which was free of cellular debris on microscopic examination, was removed.

Cell-free plasma was obtained from animals which were precooled at 4° C. for 24 hours. Prior to coagulation, the shed blood was centrifuged immediately in a refrigerated centrifuge at 20,000 G for 15 minutes.

Except where indicated, the endotoxin used was that obtained from the originally isolated *Vibrio* species (5). It was prepared from a 48–72 hour culture grown in ZoBell's sea water broth. The organisms were removed by centrifugation, and lysed in sterile, pyrogen-free distilled water. The liquid containing the lysate was placed in a boiling water bath for 20 minutes, and recentrifuged to remove cellular debris. The supernatant obtained constituted the *Vibrio* endotoxin. The *E. coli* endotoxin used was lyophilized lipopolysaccharide of *Escherichia coli* (Difco Laboratories, Inc., Detroit).

The changes noted after the addition of endotoxin to pre-gel were: first the appearance of viscous material on the sides of the test tube, shortly followed by macroscopically visible flocculation; and then by a marked increase in viscosity. If the test tube was allowed to remain undisturbed a solid gel formed. However, if the sample was agitated during gelation, a viscous and ropey material was noted and a solid gel did not form, presumably because of disruption of the gel as it was forming.

RESULTS

The ability of the cell free plasma to clot or gel was tested. Cell free plasma did not clot spontaneously for periods up to one week when incubated at room temperature. Furthermore, the addition of 0.1 ml. of

Vibrio endotoxin or of pre-gel to 1 ml. of cell free plasma did not produce a gel or clot when incubated at room temperature for 24 hours. Addition of the washed amebocytes to the cell free plasma from which they had been removed resulted in the formation of a clot within 10 minutes. The liquid phase that was removed from these clots 24 hours later gelled upon addition of endotoxin, indicating the presence of pre-gel.

To determine whether agitation of the blood and subsequent disruption of the cells would affect production of the pre-gel, the following experiments were performed. In 10 experiments, blood from different animals was divided into equal portions and placed in non-siliconized glass tubes. One tube of each pair was allowed to remain undisturbed and the other was vigorously agitated for two minutes, and then was left undisturbed. All of the sample clotted within five minutes. Samples of the liquid phase were removed at intervals for periods up to 24 hours, and tested for the presence of pre-gel by the addition of 0.1 ml. of undiluted *Vibrio* endotoxin to 1.0 ml. of the samples. Pre-gel was demonstrable in approximately one-half the samples of the liquid material removed within a half hour and was present in all of the samples removed after seven hours from the blood that had been agitated. In contrast, the liquid material removed within three hours from samples of blood that had not been agitated only rarely gelled. Thereafter, pre-gel was present in increasing numbers of the samples removed from blood that had been undisturbed, until at 10 hours, all samples of the liquid material contained pre-gel (Fig. 1).

The time of onset of flocculation following the addition of endotoxin to pre-gel was affected by the method of handling the whole blood, and the duration of exposure of the liquid phase to amebocytes before separation as shown in Figure 2. The onset of flocculation was more rapid in the liquid phases obtained at periods up to four hours from blood that had

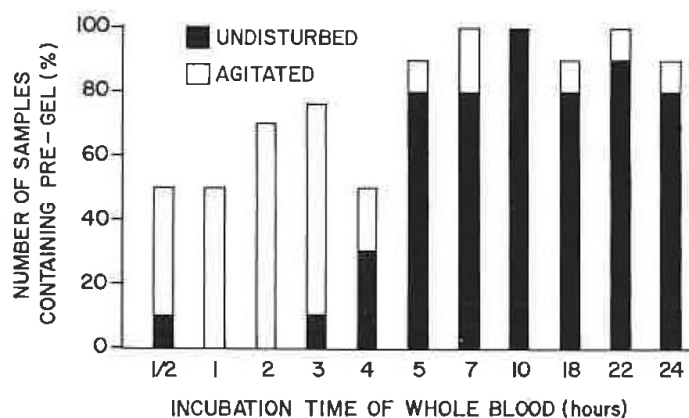


FIG. 1. Effect of incubation time and agitation of blood on appearance of pre-gel (10 animals tested).

been shaken. Thereafter, the onset of flocculation was similar in both groups, and reached a minimum time of 12–15 minutes.

To determine whether all Limuli possessed the capability of producing pre-gel, the following experiments were carried out. The liquid phase, removed from blood that had been agitated and incubated for 24 hours at room temperature, was prepared from each of 28 Limuli. All of the pre-gel samples formed a gel after the addition of endotoxin. In addition, 10 of these animals (five female and five male) were tested at weekly intervals for seven weeks to test the consistency of the reaction with endotoxin. Blood from all of these animals repeatedly produced pre-gel that formed a gel after the addition of endotoxin. Pre-gel prepared in this manner and diluted to 15–25 per cent by volume also became viscid upon addition of endotoxin. Once pre-gel had been gelled by the addition of endotoxin, it did not re-gel if the gel was disrupted and endotoxin was again added.

These results suggest that disruption of amebocytes resulted in either the liberation of pre-gel from the amebocytes, the release of a factor necessary for the production of pre-gel from a substance normally present in the plasma, or the release of a pro-coagulant that was activated by endotoxin.

The relationship of endotoxin concentration to the appearance of gel was next studied. Serial dilutions of *Vibrio* endotoxin, in 0.1 ml. of sterile, pyrogen-free distilled water were added to a series of tubes containing 1 ml. of pre-gel. The tubes containing these mixtures were gently tilted every three minutes for 15 minutes, every five minutes for 30 minutes, and then every 15 minutes until a marked increase in viscosity occurred. The time of onset of increased viscosity in pre-gel was delayed as the endotoxin concentration was decreased, but marked viscosity occurred in all

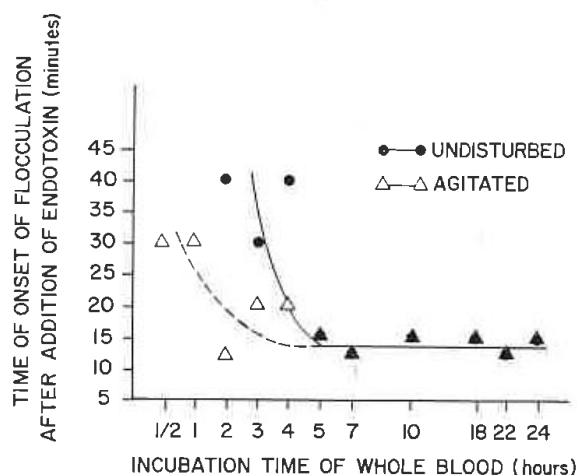


FIG. 2. Effect of incubation time and agitation of blood on time of onset of flocculation (10 animals tested).

tubes up to dilutions of endotoxin to 10^{-6} (Table I). Solid gelation did not occur in these tubes presumably because of the mechanical disruption of the gel as the tubes were tilted. However, paired tubes, which were not tilted, did achieve solid gelation at dilutions of endotoxin up to 10^{-6} .

Similar results were obtained using a second preparation of *Vibrio* endotoxin and *E. coli* endotoxin. Marked increase in viscosity was observed following the addition of as little as 0.0005 gamma of *E. coli* endotoxin to 1 ml. of pre-gel. An extract of a species of *Gaffkya* Tetragenesis, a Gram positive coccus, pathogenic for the lobster, and muscle extracts prepared from the *Limulus* did not produce gelation when added to pre-gel.

The presence of a substance, during the reaction between pre-gel and endotoxin, capable of producing additional gelation was sought. One-tenth ml. of undiluted endotoxin was added to one ml. of pre-gel and at varying intervals 0.1 ml. portions of the reacting mixture were removed and added to 1 ml. samples of pre-gel. The reaction mixture, incubated for a period up to 20 minutes, still contained a substance that when transferred, produced gelation of pre-gel. As expected, however, addition of this substance to cell-free plasma did not cause clotting or gelation of the plasma. The reaction mixture gelled at 20 minutes. Samples taken from the reaction mixture after it had gelled were incapable of causing gelation of pre-gel. The transferable material that caused gelation was most likely endotoxin. However, material taken after gelation had occurred was incapable of causing secondary gelation, implying that the endotoxin had been inactivated, or utilized.

Studies were performed in an attempt to detect the presence of a sub-

TABLE I

Effect of Endotoxin Concentration on the Time of Onset of Increased Viscosity

Endotoxin Concentration	Minutes after Addition of Endotoxin						
	10	12	15	20	30	40	60
Undil.	0	F	V	V			
2×10^{-1}	0	0	F	V	V		
1×10^{-1}	0	0	0	F	V	V	
1×10^{-2}	0	0	0	+	V	V	
1×10^{-4}	0	0	0	0	+	+	V
1×10^{-6}	0	0	0	0	+	V	V
1×10^{-8}	0	0	0	0	+	+	F
Control	0	0	0	0	0	0	0

0 = no change.

+

F = macroscopically visible flocculation.

V = marked increase in viscosity.

stance in the gel, which would enhance the gelation of pre-gel by endotoxin. Pre-gel was mixed either with gel which had been mechanically disrupted, or with distilled, pyrogen-free water. To 1 ml. of the mixtures, 0.1 ml. of undiluted endotoxin was added, and the time of onset of increased viscosity was measured. There was no acceleration of the time of onset of increased viscosity following the addition of endotoxin to the pre-gel (Table II).

The effects of various anticoagulants on gelation of pre-gel by endotoxin were studied. High concentrations of heparin (final conc. 1000 U per ml.) and sodium citrate (final conc. 3 per cent) were unable to prevent this reaction. EDTA in a final concentration of 0.6 per cent was inhibitory. The inhibition by EDTA could not be reversed by the addition of either calcium or magnesium ions, in concentrations ranging from M/5 to M/800. The concentration in sea water of Ca^{++} is 0/009 M and of Mg^{++} is 0/02 M. The addition of calcium and magnesium ions in the proportions found in normal sea water also did not reverse the effect of EDTA. Hemocyanin, a copper containing pigment, is present in *Limulus* blood, and may be centrifuged from pre-gel without loss of the ability to form gel (6). Therefore, cupric and cuprous ions were added in an effort to reverse the inhibitory effect of EDTA on gelation. The results were not interpretable because of the precipitation of a copper-protein complex.

The pyrogenicity of the *Vibrio* endotoxin, used in these experiments, was compared with that of *E. coli* endotoxin. These substances produced similar febrile responses when administered intravenously to rabbits (Fig. 3). Lyophilization of the liquid *Vibrio* endotoxin revealed it to contain 9.9 mg. of protein per ml.

DISCUSSION

Coagulation of shed *Limulus* blood and the formation of pre-gel have been shown to require the presence of amebocytes. The current work has

TABLE II
Effect of Addition of Disrupted Gel to Pre-Gel on the Gelation Produced by Endotoxin

Final Concentration of Pre-Gel	Time of Onset of Increased Viscosity of Pre-Gel after Addition of Endotoxin (minutes)	
	Pre-Gel Diluted with	
	Disrupted Gel	Distilled H ₂ O
100%	10	10
75%	10	10
50%	10	10
25%	20*	20*

* At this low concentration of pre-gel, the time of onset of increased viscosity was delayed.

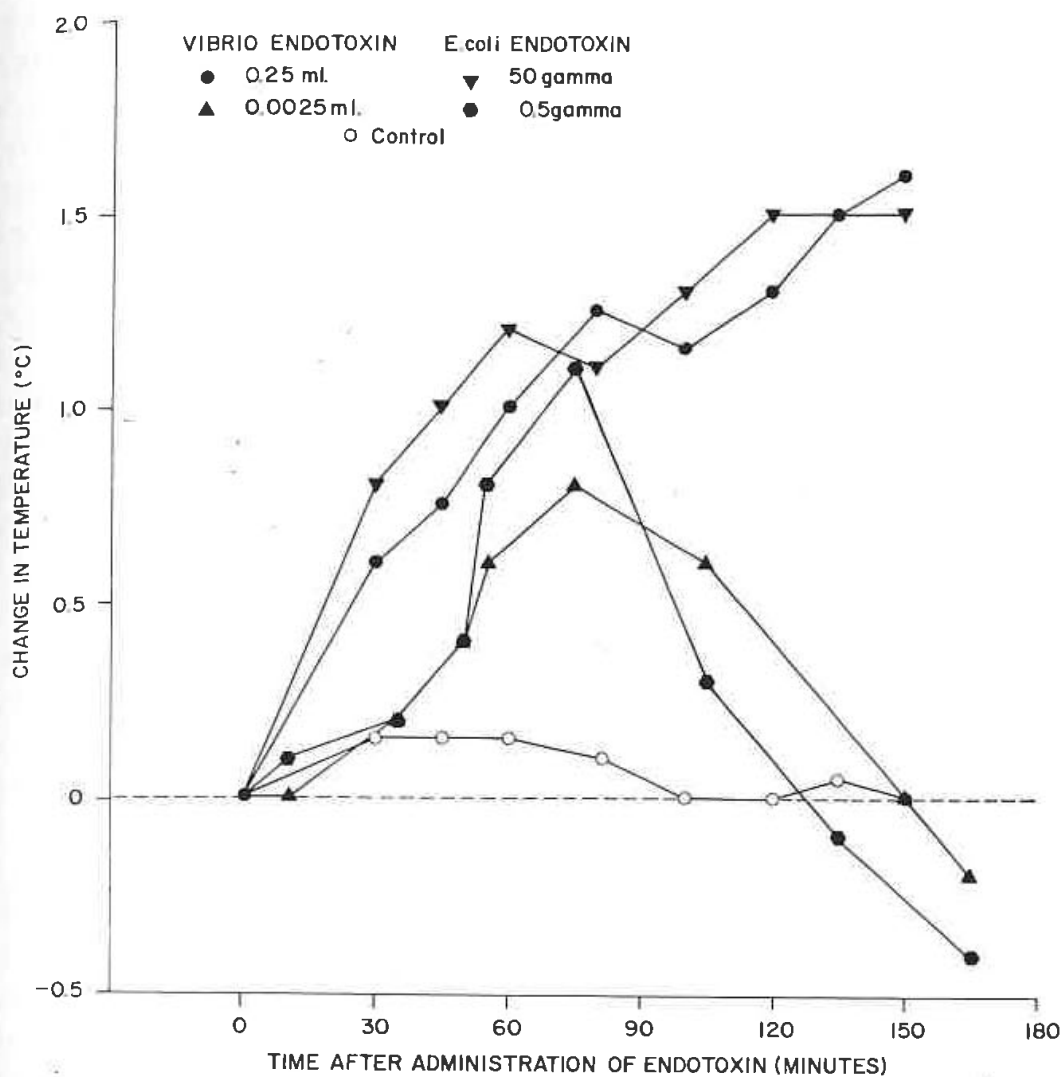


FIG. 3. Comparison of febrile response of rabbits to the intravenous administration of vibrio and *E. coli* endotoxin.

demonstrated that the production of pre-gel is enhanced by amebocyte disruption. Pre-gel may be present in the amebocytes and be released during clotting, or a factor may be liberated from the amebocyte during clotting which either reacts with a substance already present in plasma to form pre-gel, or constitutes a pro-coagulant which is activated by endotoxin.

Studies of comparative coagulation (8, 9) have revealed that as evolution has occurred, the coagulation system has become more complex. In invertebrates, plasma factors play a less prominent role in coagulation than they do in mammalian systems. The finding that *Limulus* plasma, which is free of all cellular material is incoagulable, is in keeping with previous observations (10, 11). The addition to cell free plasma of the amebocytes previously removed restored the property of coagulability. It is appropriate to consider the *Limulus* amebocyte as comparable to the mammalian platelet.

Observations made in conjunction with these studies (to be reported elsewhere) show striking morphological similarities between mammalian platelets and the amebocyte as they undergo changes associated with the coagulation process.

The data presented show that the rate of gelation of pre-gel is directly related to the concentration of endotoxin employed. Hemocyanin, a pigment present in *Limulus* blood, is not required for the gelation of pre-gel (6). These results are consistent with the concept that an enzymatic system is responsible for the conversion of pre-gel to gel by endotoxin. The conversion of pre-gel to gel by endotoxin may be similar to the conversion of fibrinogen to fibrin, by thrombin, in mammalian systems; but the nature of the protein in the *Limulus* remains unknown. The occurrence of gelation of pre-gel is a very sensitive indicator of endotoxin activity, since minute amounts of endotoxin, prepared either from a *Vibrio* species or from *E. coli* produced this reaction. The data suggest that gelation, in some unknown way, inactivates endotoxin or interferes with its ability to produce further gelation. Because of its ability to absorb thrombin, fibrin has been described as a potent anti-thrombin (12). There is perhaps a parallelism between the anti-thrombin activity of fibrin and the inactivation of endotoxin by gel.

Heparin and sodium citrate did not prevent the gelation of pre-gel by endotoxin, but EDTA was inhibitory. Similarly, heparin and sodium citrate have been shown not to block the aggregation of human platelets by endotoxin, *in vitro*, but EDTA is inhibitory (5). The replacement of cations did not reverse the inhibition by EDTA of the reaction between pre-gel and endotoxin.

Pre-gel, from all *Limuli* tested, possessed the capability, *in vitro*, of producing a gel when exposed to *Vibrio* endotoxin. It has been suggested (13, 14) that immunological mechanisms play a role in some of the biologic activities of endotoxin, and that previous exposure of animals to endotoxin alters their response. Young rabbits (13) and young *Limuli* (6) are resistant to the lethal effects of endotoxin; both are environmentally exposed to Gram negative organisms.

Administration of endotoxin causes leucopenia and thrombocytopenia in the rabbit, and amebocytopenia in the *Limulus*. Endotoxin may, under conditions which produce the Shwartzman phenomenon, decrease the level of circulating fibrinogen in the rabbit; and is capable of making *Limulus* blood incoagulable. In both animals, intravascular coagulation and death result. *In vitro*, material from either platelets or amebocytes must be present for the demonstration of endotoxin induced alterations in coagulation.

It is interesting to speculate that the breakdown and disruption of

amebocytes by bacterial endotoxin, with resultant immobilization of bacteria, which may be demonstrated both *in vivo* (6), and *in vitro* (7), represents an important mechanism by which these animals protect themselves against the Gram negative organisms to which they are constantly exposed. Presumably, immobilization of the bacteria by the gel or clot caused by the interaction of bacterial endotoxin and the amebocyte constituents protects an animal against infection. Since minute concentrations of endotoxin are capable of producing gelation, this is a very sensitive defense mechanism. Perhaps this very primitive antibacterial mechanism is still represented in the mammalian system by the alterations of the coagulation system caused by endotoxin. There is measurable antibacterial activity in cell cultures of the *Limulus* amebocyte (7), and antibacterial activity has been described in mammalian platelets (5, 15, 16).

Limulus fossils have been found in Devonian rock, with an estimated age of 390×10^6 years; and Trilobites, which appear similar to embryonic Limuli, have been found among Cambrian fossil material with an estimated age of 500×10^6 years (17). The persistence of an apparently similar mechanism in the response of mammalian and *Limulus* coagulation systems to endotoxin suggests that this is one of the most ancient mechanisms involved in blood coagulation.

SUMMARY

The coagulation of *Limulus* pre-gel by the endotoxin of a *Vibrio* species has been studied. *Limulus* plasma, containing no cellular material, did not gel when endotoxin was added. There was a direct relationship between the endotoxin concentration and the time of onset of viscosity, but the final degree of gelation was not affected. EDTA inhibited this reaction. Endotoxin was inactivated following gelation of pre-gel. The reaction between endotoxin and pre-gel to form gel appears to be similar to the conversion of fibrinogen to fibrin, by thrombin, although the nature of the clottable protein in *Limulus* is unknown. The kinetics of the reaction suggest that an enzyme system is responsible for the conversion of pre-gel to gel. The characteristics of the reaction of *Limulus* to endotoxin are in many ways similar to those of the rabbit under conditions which produce the Schwartzman phenomenon.

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