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Studies on *Limulus* Amoebocyte Lysate

III. PURIFICATION OF AN ENDOTOXIN-BINDING PROTEIN FROM *LIMULUS* AMOEBOCYTE MEMBRANES*

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A protein that has been isolated from *Limulus polyphemus* amoebocyte membranes binds endotoxin. The protein was purified by two independent methods, organic solvent extraction and affinity chromatography, both followed by gel filtration. Immunologic studies confirm that the protein is a component of amoebocyte membranes. Although without enzymatic activity, the binding protein enhances *Limulus* lysate gelation. As a membrane-associated endotoxin binding "protein," it may be involved in *Limulus* lysate coagulation, which is initiated by minute amounts of Gram-negative bacterial endotoxin. The protein has an apparent molecular weight of 80,000.

Limulus amoebocyte lysate forms a gel when exposed to minute quantities of bacterial lipopolysaccharides (1). This unique property may allow increasing biomedical applications. For example, the lysate assay has been employed to detect contaminating pyrogens in pharmaceuticals and drugs intended for human use (2). Our laboratory has been investigating the enzymatic mechanism of the lysate gelation. We have purified a proclotting enzyme (3), a coagulogen (4), and, more recently, an active enzyme (5). Our study is directed toward understanding the chemical and physiological principles underlying the *Limulus* coagulation system.

Limulus amoebocytes are normally discoid and highly granulated. However, minute amounts of endotoxin cause cellular degranulation and lysis, followed by the formation of an extracellular gel (6). No enzymatic activity related to clotting occurs within the amoebocyte. All factors which eventually contribute to a clot must be expelled into the blood plasma when the cells degranulate and lyse. Activation of a zymogen by Ca^{2+} and endotoxin is an early stage in the clotting process (3), in spite of the fact that endotoxin cannot enter the cell intact. Consequently, *in vivo,* endotoxin must mediate the degranulation and lysis of amoebocytes before activation of the zymogen occurs. We propose the existence of an endotoxin-binding protein on the outer membrane of *Limulus* amoebocytes. The existence of such a protein has been shown in the human red blood cell (7).

This paper describes the purification of a protein from *Limulus* amoebocyte membranes which binds endotoxin. Although the protein shows no enzymatic activity, it enhances the coagulation process when added to *Limulus* lysate. As a membrane component, the endotoxin-binding protein could possibly act as an endotoxin receptor and mediate the characteristic degranulation and lysis of amoebocytes exposed to bacterial endotoxin.

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EXPERIMENTAL PROCEDURES

Materials

Endotoxin (*Escherichia coli* 026-B6 and *Klebsiella* 1B) was a gift from Dr. Donald Hochstein, Food and Drug Administration. Sephadex G-100, Sephacryl S-200, Sepharose CL-4B, and 6-aminohexyl (AH)-Sepharose 4B were purchased from Pharmacia. Pyrogen-free water was purchased from Cutter Laboratories. Pyrotest *Limulus* amoebocyte lysate was purchased from Difco Laboratories and Pyrotell *Limulus* amoebocyte lysate was purchased from Associates of Cape Cod, Inc., Woods Hole, MA. Chromogenic substrates were from Ortho Diagnostics. All reagents were of the purest grade commercially available.

Glassware was freed from pyrogens by heating at 180°C for at least 4 h.

Limuli, obtained from beaches on Long Island, NY, were bled by cardiac puncture as described by Jorgensen and Smith (8). The blood, collected in ice-cold containers, was centrifuged at 100 × *g* for 15 min at 4°C. The plasma portion was removed and the residual layer containing the amoebocytes was washed twice with sterile 3% saline, frozen, and stored at −70°C. Lipopolysaccharide (LPS) was isolated from *E. coli* (K1) by a variation of the method of Westphal *et al.* (9). The endotoxin was purified by extensive hot phenol extraction and was judged free of protein contamination by amino acid analysis. A general tritium label was applied by New England Nuclear. The labeled endotoxin had a specific activity of 3.58 mCi/mg.

Endotoxin Purification—Before use, endotoxin was dissolved with sonication in pyrogen-free water. Solutions then were placed on Sephadex G-100 and eluted with pyrogen-free 3% saline. Fractions causing gelation of *Limulus* lysate were pooled and used in other procedures. Studies with [^{3}H]endotoxin showed that two radioactive peaks were eluted, one near the void volume and one with the salt fraction. Only the material from the void volume peak caused gelation of *Limulus* lysate.

Preparation of Endotoxin Affinity Column—The procedure used was modified from that of Cambiaso *et al.* (10). AH-Sepharose 4B activated by glutaraldehyde was coupled to endotoxin. The resin was washed with 10 volumes of 0.1 M sodium phosphate buffer, pH 7.5, on a sintered-glass filter. Glutaraldehyde in the same buffer was added under stirring to the gel to give a final concentration of 2.5%. The reaction was allowed to proceed for 10 min at room temperature. Unreacted glutaraldehyde then was eliminated by washing with phosphate buffer (five times, 7 ml/ml of packed gel). Endotoxin (1 mg/ml of packed gel), mixed with a trace of radioactive endotoxin in the phosphate buffer, was added under stirring to the activated gel. The reaction was allowed to proceed at room temperature for 15 min and the sample was washed with phosphate buffer until no radioactivity appeared in the eluent (five times, 7 ml/ml of packed gel). The gel was further incubated with 0.2 M glycine buffer, pH 8.5, at 4°C for 24 h to block unreacted aldehyde groups. The radioactivity of all washings and gel were counted to determine the amount coupled. About 50% of the total radioactivity of the endotoxin was coupled to the gel.

Gel Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed essentially as described by Weber and Osborn (11) using 7.5% gels. Protein samples were denatured in 1% sodium dodecyl sulfate and 10 mM dithiothreitol at 100°C for 3 min. Protein standards from BDH Chemical Ltd., Poole, England, were prepared similarly. The molecular weight of the binding protein was

calculated on the basis of a linear relationship of the log molecular weight to the distance migrated in the sodium dodecyl sulfate gel.

Amino Acid Analysis—Amino acid compositional data were obtained by automated ion exchange chromatography on a Beckman 121 M analyzer according to the general procedures of Spackman *et al.* (12). Proteins were hydrolyzed in 4 M methanesulfonic acid as described by Simpson *et al.* (13) and Liu (14).

Trichloroacetic Acid Precipitation—Protein was incubated with [^{3}H]-endotoxin in a ratio of about 500:1 by weight at room temperature for 30 min. Trichloroacetic acid was added to yield a final concentration of 5%. The mixture was placed on ice for 30 min and then centrifuged. The precipitate was washed twice with 1% trichloroacetic acid. Only a trace of radioactivity appeared in the second wash. After dissolving the precipitate in 0.1 ml of 1 N NaOH, radioactivity was counted in a Packard liquid scintillation counter. A control containing endotoxin but no protein was run to ensure that no endotoxin was precipitated.

Methods

Purification of Endotoxin-binding protein—The endotoxin-binding protein was isolated using two methods. First, a butanol/water extraction procedure was designed after the method of Springer *et al.* (7) on the isolation of a human red blood cell LPS[1] receptor. Second, affinity chromatography on a column of endotoxin linked to a glutaraldehyde-activated aminohexyl-Sepharose 4B was employed.

Butanol/Water Extraction—The amoebocytes from 100 ml of fresh *Limulus* blood were washed twice with sterile 3% saline. The cells were lysed by agitating in 10 ml of water. The supernatant lysate was discarded and the remaining stroma were homogenized in a Waring Blendor at 4°C in 100 ml of water. After adding 100 ml of 1-butanol, the mixture was again blended. The butanol layer was removed following centrifugation at 10,000 × *g* and storage at 4°C for 24 h. The aqueous layer was brought slowly to 0.5 saturation in ammonium sulfate by dialyzing at 4°C against 50% ammonium sulfate. The small amount of fluffy white precipitate which formed in the dialysis bag was dissolved in a minimum of water and dialyzed at 4°C against 0.05 M Tris, 0.1 M NaCl (pH 7.3), 10% glycerol buffer. The sample was applied to a column of Sephacryl S-200 (2.6 × 78 cm) which had been equilibrated in the same buffer. The fractions were analyzed for protein content by measuring A_{280}. Alkaline hydrolysis and ninhydrin reaction (15) were also performed on aliquots of each fraction. The elution profile obtained is shown in Fig. 1. The fractions forming the first peak were pooled, dialyzed against water, and concentrated in S & S collodion bags. SDS-gel electrophoresis in 7.5% gels gave one band.

Affinity Chromatography—Frozen amoebocytes from 150 ml of blood were thawed and extracted at 4°C with 25 ml of 1.0 M $CaCl_2$ containing 0.2% Triton X-100. The extract was dialyzed against 3% saline and then gently re-extracted with chloroform overnight at 4°C to remove lipid and endotoxin. The aqueous layer then was passed through the endotoxin affinity column (0.9 × 17 cm). The column was washed with buffer containing 0.02 M Tris, 0.35 M NaCl, and 0.01 M $CaCl_2$ at pH 7.7 until A_{280} of the effluent was less than 0.03. Protein bound to the column then was eluted with 150 ml of a buffer containing 0.02 M Tris, 0.05 M NaCl, and 0.025 M EDTA at pH 7.7 and finally with 300 ml of a buffer containing 0.02 M Tris, 0.15 M NaCl, and 0.25 M EDTA at pH 7.7. The eluent was monitored for the presence of binding protein by using antibody prepared as described below. Fractions containing binding protein were concentrated using collodion bags and rechromatographed on a Sepharose CL-4B column (0.9 × 48 cm) in the same EDTA buffer. The protein in peak II (see Fig. 3) showed a single band on SDS-gel electrophoresis.

Physicochemical Studies—In order to relate chemical properties of the LPS receptor to the physiological events in coagulation, an *in vitro* system must mimic natural conditions. The following two studies were designed to demonstrate the effects of the LPS-binding protein on the amoebocyte lysate coagulation system.

Turbidity Test—The observation of a linear relationship between endotoxin concentration and clot formation as measured by turbidity at 360 nm was employed (16). Endotoxin solutions (120 μl) of known concentration were added to 500-μl quantities of Pyrotell *Limulus* lysate reconstituted with pyrogen-free water in sterile polypropylene capped tubes (12 × 75 mm). The endotoxin, Food and Drug Administration (FDA) *Klebsiella* 1B, was sonicated to ensure homogeneous dispersal. The mixture was incubated for 60 min at 37°C and turbidity was recorded at 360 nm. Turbidity of the blank, endotoxin-free lysate, was subtracted from experimental values. A linear relationship between endotoxin amount and turbidity was confirmed for final endotoxin concentrations of 0.01 to 0.05 ng/ml. Next, 60-μl aliquots of 1 ng/ml of protein solution were added to 500-μl volumes of lysate and 60-μl volumes of endotoxin solution. Two tubes were used for each amount of endotoxin. In the control tube, 60 μl of pyrogen-free water replaced the receptor. Protein solutions were freed from endotoxin by gently extracting with chloroform.

Chromogenic Substrate Tests—Tai *et al.* (4) reported that clot formation in *Limulus* is caused by clotting enzyme action on the coagulogen involving the cleavage of a single peptide bond (Arg-Lys). This limited proteolysis indicates that the endotoxin-activated clotting enzyme may be monitored by introducing a synthetic chromogenic substrate. Benzoyl-Ile-Glu-(γ-OCH_3)-Gly-Arg-*p*-nitroanilide (S-2222) has been shown to be a sensitive substrate for clotting enzyme in *Limulus* (17). In the experiments described below, the synthetic substrate was employed to monitor the effects of introducing the binding protein into the *Limulus* lysate system.

Preliminary studies were performed to determine the maximum concentration of product that can accumulate before the product concentration *versus* time and velocity *versus* enzyme concentration response becomes nonlinear. A buffer, 0.01 M Tris-HCl, (pH 8.0), 0.01 M $CaCl_2$, containing 0.1 mM S-2222 synthetic substrate, was prepared using pyrogen-free water. FDA *Klebsiella* 1B endotoxin was sonicated as before.

In one experiment, 0.60 ml of the substrate solution was preincubated at 37°C with 50 μl of 1 ng/ml of endotoxin. Ten microliters of reconstituted Pyrotell lysate and pyrogen-free water were added to give a final reaction volume of 1.0 ml. After 30 min of 37°C incubation, the reaction was stopped by adding 100 μl of glacial acetic acid and the color was read at 405 nm. Further experimentation consisted of independently varying amounts of lysate and incubation time for a given amount of endotoxin. Several endotoxin concentrations were employed. It is important to note that lysate activity varies from vial to vial so that data obtained from different lysate reconstitutions cannot be confidently compared. Attempts were made to run a maximum number of experiments with each vial of reconstituted lysate so that data could be better evaluated. Optimal conditions for investigating the effect of receptor on the coagulation were chosen to be 10 μl of lysate and 1 ng/ml of endotoxin incubated for 30 min. The concentrations of substrate were also chosen so that changes in enzyme substrate affinity induced by the protein might be detected. The above procedure was repeated within the chosen range of substrate concentrations for a given amount of binding protein. The protein was added in the form of a 1 ng/ml of solution prepared in pyrogen-free water, then extracted with chloroform at 4°C with gentle stirring. The chloroform extraction was necessary to remove any LPS already bound to the protein.

Experiments were repeated with another commercial lysate preparation. Difco Pyrotest lysate, provided in small tubes, was reconstituted with 0.4 ml of 0.1 mM S-2222 substrate in the same buffer as above. Substrate, endotoxin, and binding protein concentrations were kept constant while incubation time was varied.

Proteolytic Activity Assay—Proteolytic activity of the purified endotoxin binding protein was examined by the method of Kunitz using casein as substrate (18). One hundred microliters of a solution containing 10–15 μg of test protein was added to 1 ml of 1% casein in 0.1 M phosphate buffer, pH 7.6, and incubated at 40°C for 60 min. Measurement of the proteolytic activity was made by addition of 3 ml of 5% trichloroacetic acid and, after centrifugation, the absorbance of the supernatant solution was measured at 280 nm. Appropriate controls were run by using trypsin and pronase as proteolytic enzymes.

Preparation of Anti-Endotoxin-binding Protein Antibody—A rabbit was injected in the footpads and neck with 0.5 ml of antigen prepared by emulsifying 0.25 ml of endotoxin-binding protein (100 μg) obtained as described in the legend Fig. 1 with 0.25 ml of Freund's incomplete adjuvant. Six weeks later, the rabbit was reinjected in the footpads with 20 μg of protein without adjuvant. Blood was withdrawn 10 days later and serum was prepared.

RESULTS

Purification and Characterization of Binding Proteins—Two independent methods were used to isolate a protein from *Limulus* amoebocyte membranes. First, 1-butanol was used to solubilize amoebocyte membranes. This procedure has been used in isolating an LPS receptor from human red blood cells

[1] The abbreviations used are: LPS, lipopolysaccharide; SDS, sodium dodecyl sulfate.

(7) and in solubilizing many membranes (19, 20). Proteins pass into the aqueous phase and lipids into the butanol phase. The membrane protein then was purified by ammonium sulfate precipitation and gel filtration. Fig. 1 shows the elution profile of the membrane proteins, precipitated by 50% ammonium sulfate solution, on Sephacryl S-200. The peak eluting near the void volume gave one band on SDS-gel electrophoresis (see Fig. 2). The molecular weight of the protein based on the distance migrated in the gel is 80,000. Typical yields of the protein were 30 to 40 μg from 100 ml of fresh *Limulus* blood as judged by amino acid analysis. Several preparations were combined to study the biological activity and properties of the protein.

A second isolation procedure involved solubilizing *Limulus* membranes with Triton X-100. This detergent has proved useful in isolating a cholinergic receptor (21) and in solubilizing an insulin receptor (22) from plasma membranes. After Triton X-100 treatment, affinity chromatography on a column of endotoxin linked to glutaraldehyde-activated AH-Sepharose 4B followed by gel filtration was used to purify a membrane protein. The elution profile on Sepharose CL-4B of material retained on the affinity column during washes with buffer then eluted with EDTA is shown in Fig. 3. Peak II gave a single band on SDS-gel electrophoresis which migrated the same distance as the protein purified by the butanol/water extraction procedure. Based on amino acid analysis, protein yields were typically 0.5 to 0.6 mg from 100 ml of *Limulus* blood.

The proteins isolated by the two methods discussed above are similar, as judged by SDS-gel electrophoresis and amino acid composition. Table I presents amino acid composition data. The molar ratios of the amino acids in the proteins isolated by each procedure are similar. No amino sugars were detected in the protein.

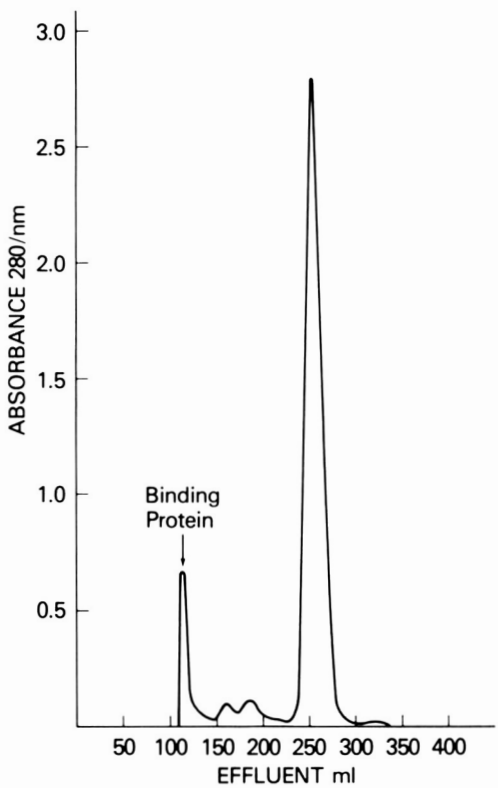

FIG. 1. **Gel filtration of 50% ammonium sulfate-induced precipitation of butanol/water extract on Sephacryl S-200.** The column (2.6 × 78 cm) was equilibrated in 0.05 M Tris, 0.1 M NaCl (pH 7.3), 10% glycerol buffer.

FIG. 2. **Sodium dodecyl sulfate-gel electrophoresis on acrylamide gels (7.5%).** *A*, purified binding protein from *Peak I* in Fig. 1; *B*, purified binding protein from *Peak II* in Fig. 3.

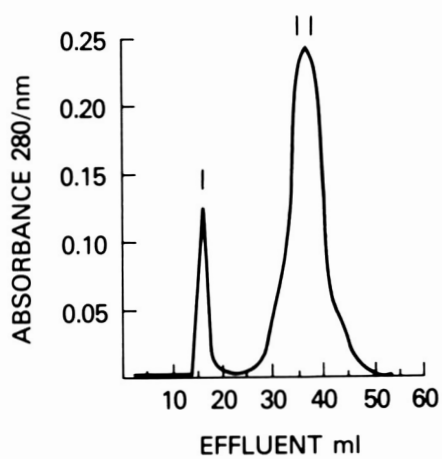

FIG. 3. **Gel filtration of binding protein on Sepharose CL-4B (0.9 × 48 cm) following affinity chromatography of Triton X-100 extract as described under "Experimental Procedures".** The column was equilibrated and eluted with 0.25 M EDTA in 0.02 M Tris, 0.15 M NaCl (pH 7.7) buffer.

Protein-Endotoxin Binding—One isolation method described above relies on binding of the protein to endotoxin covalently linked to Sepahrose 4B. In the presence of Ca^{2+} at pH 7.7, the protein is retained on the column. The protein-endotoxin binding is probably mediated by Ca^{2+} ions. Protein bound to the endotoxin affinity column can be eluted with EDTA.

Protein-endotoxin binding was also shown by precipitation

of a complex with trichloroacetic acid (see Table II). Endotoxin alone was not precipitated. However, a significant amount of radioactivity was incorporated into the trichloroacetic acid precipitate after incubation of [^{3}H]endotoxin with purified binding protein. If the protein was incubated with excess unlabeled endotoxin before addition of [^{3}H]endotoxin and precipitation, then radioactivity incorporated into the trichloroacetic acid precipitate decreased.

Enhancement of Limulus Lysate Activity—The endotoxin-binding protein purified by the methods described above possessed neither proteolytic activity toward casein nor amidase activity using benzoyl-Ile-Glu-(γ-OCH_3)-Gly-Arg-*p*-nitroanilide (S-2222) as a substrate (17). However, when the protein was added to *Limulus* lysate preparations, clotting activity was consistently enhanced. Two methods were used to detect this enhancement and are described under "Experimental Procedures." Turbidity tests and chromogenic substrate tests using different commercial lysate preparations showed that clotting activity as related to turbidity or color formation increased from 15 to 30%. A summary of a typical set of results is shown in Table III.

Experiments were also performed using the active clotting enzyme recently purified in our laboratory (5). Results indicate that the binding protein does not enhance clotting enzyme activity as measured by S-2222 hydrolysis.

Immunology—The results of a double diffusion study using antibody developed against purified endotoxin binding protein are shown in Fig. 4.

TABLE I

Amino acid composition of endotoxin-binding protein from Limulus amoebocyte membranes

Amino acid	Protein purified by affinity chromatography	Protein purified by butanol/water procedure
	molar % total residues	
Tyr	3.9	3.8
Phe	5.1	4.8
Try	1.0	0.8
Lys	5.8	5.5
His	5.7	6.2
Arg	4.8	5.0
Asp	12.2	12.7
Thr	5.1	4.9
Ser	6.0	6.1
Glu	11.2	12.0
Pro	5.0	4.4
Gly	6.9	7.7
Ala	5.6	5.2
Val	5.1	5.9
Met	2.2	1.9
Ile	4.7	4.4
Leu	8.6	8.8

TABLE II

Detection of [^{3}H]endotoxin binding to protein by means of trichloroacetic acid precipitation

In each experiment, 40 μg of protein was used and constant amount of [^{3}H]endotoxin as shown was used.

Experiment	Total [^{3}H]-endotoxin used	Radioactivity retained in trichloroacetic acid precipitation			Radioactivity incorporated into trichloroacetic acid precipitation	
		[^{3}H]-Endotoxin alone	[^{3}H]-Endotoxin + protein	Excess cold endotoxin + protein then [^{3}H]-endotoxin	[^{3}H]-Endotoxin + protein	Excess cold endotoxin + protein, then [^{3}H]-endotoxin
	cpm	*cpm*			*%*	
1	14750	132	5310	1995	35	12.6
2	28376	260	8782	3867	30	12.4

TABLE III

Effect of binding protein on lysate activity

Procedure	Endotoxin concentration	ΔA/min (average rate)		Activity increase
		Lysate	Lysate + binding protein	
	ng/ml			*%*
Turbidity test	0.01	0.0125	0.016	28
	0.02	0.0175	0.023	31
	0.03	0.0235	0.030	29
	0.04	0.029	0.034	17
	0.05	0.034	0.040	18
Chromogenic substrate test	1.0	0.084[a]	0.103[a]	23

[a] Values for ΔA/min are given as initial rates.

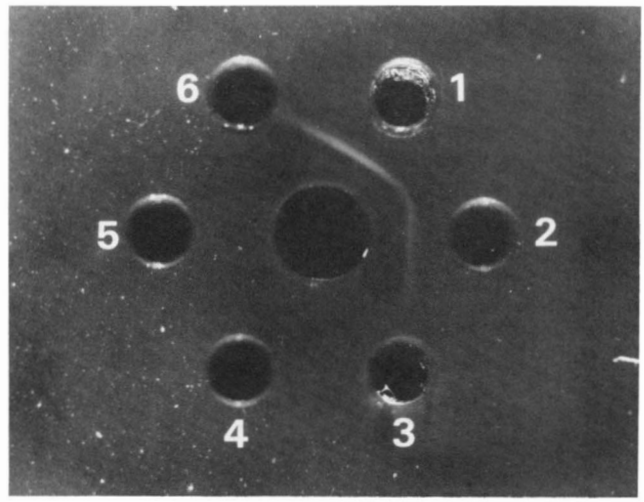

FIG. 4. **Double diffusion of endotoxin-binding protein.** *Center well*, antibody against purified binding protein obtained as described in the legend to Fig. 1; *Well 1*, purified binding protein obtained by organic extraction (Fig. 1); *Well 2*, purified binding protein obtained by affinity chromatography (Fig. 3). *Well 3*, amoebocyte lysate containing soluble intracellular components (see Ref. 3); *Well 4*, *Limulus* serum; *Well 5*, saline.

DISCUSSION

A protein from *Limulus* amoebocytes has been isolated by two independent methods. The protein is probably a plasma membrane protein for the following reasons: 1) it can be extracted by treatment with butanol/water from the water-insoluble residue left after lysis of amoebocytes; 2) it can be extracted with Triton X-100 and Ca^{2+} from previously frozen cells without lysis; and 3) antibody raised against purified binding protein failed to react with serum or with amoebocyte lysate containing only soluble intracellular components.

The protein has been shown to bind with endotoxin from Gram-negative bacteria. This binding is the basis for the purification procedure described above using an affinity column of endotoxin linked to glutaraldehyde-activated AH-Sepharose 4B. The binding is apparently dependent on Ca^{2+} ions and can be disrupted with EDTA. Also, an endotoxin-protein complex was precipitated with trichloroacetic acid. Binding studies were facilitated by the use of [^{3}H]endotoxin. However, the state of aggregation of LPS in solutions of various compositions complicates conventional binding studies. LPS is believed to be arranged in a bilayer which may

break down into M_r = 300,000 to 1,000,000 micelles upon removal of divalent cations. The micelles can be further broken down into M_r = 10,000 to 20,000 in the presence of detergent or bile salts (23). Even after sonication and gel filtration, uncertainty about the state of LPS aggregation limits quantitative interpretation of the results.

The binding protein displayed no amidase activity characteristic of the active clotting enzyme in lysate nor does it exhibit any detectable proteinase activity when assayed with casein as substrate. However, several experimental approaches revealed that binding protein incubated with endotoxin enhanced the clotting activity of the lysate. Furthermore, upon heating the binding protein for 3 min at 100°C, pH 7.0, in 0.1 M Tris buffer, the endotoxin binding protein lost its ability to enhance the clotting activity of the lysate. The activity of the purified clotting enzyme (5) was not enhanced by the binding protein. Thus, the enhancement observed in lysate depends on the integrity of the protein structure and is most likely due to a stimulation of the conversion of proclotting enzyme zymogen to the active form. A similar situation occurs in the mammalian system. Prothrombin to thrombin conversion is stimulated by phospholipids (24). Although only a 15 to 30% enhancement in lysate activity was caused by the endotoxin binding protein, its physiological role should not be underestimated. *In vitro* assay methods cannot adequately represent physiological conditions. The interaction of endotoxin and proteins in association with amoebocyte membranes may, in fact, play a central role in the regulation of the *Limulus* clotting system. *In vivo* studies in which the endotoxin-binding protein is in its native environment are currently underway.

The mechanism of clotting in *Limulus* involves degranulation of blood cells, starting a chain of enzymatic events. The degranulation is initiated by minute quantities of endotoxin. Apparently, the endotoxin must interact with the amoebocyte membrane. The membrane protein which binds with endotoxin described in this article may be a "receptor" for endotoxin. A possible physiological advantage of such a receptor system may be to localize and immobilize minute quantities of endotoxin in *Limulus* blood without the occurrence of massive intravascular coagulation. Receptor site-bound endotoxin may stimulate the release of clotting zymogen which has an affinity for this endotoxin. The zymogen is converted to an active enzyme and the invading endotoxin is quickly engulfed by a clot.

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