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THE DIVERGENT EFFECTS OF ENDOTOXIN FRACTIONS ON HUMAN PLASMA AND LEUKOCYTES*†

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Abstract—Bacterial endotoxin is a lipid–polysaccharide–protein complex with many biological effects. We have fractioned crude endotoxin by centrifugation, acid hydrolysis and column chromatography into preparations of various molecular weights containing primarily polysaccharide or polysaccharide with known amounts of lipid. These fractions were tested *in vitro* for stimulation of kininogen depletion in heparinized human plasma and for stimulation of lysozyme leak from isolated human polymorphonuclear granulocytes. All fractions regardless of molecular size were capable of stimulating kininogen depletion in plasma, with the fraction highest in polysaccharide being the most potent. In contrast, only the large molecular size fractions were capable of interacting with granulocytes to produce a lysozyme leak, and the polysaccharide fraction was completely ineffective. These data are consistent with the hypothesis that endotoxin has a polysaccharide antigen which reacts with antibody and complement in plasma to stimulate kinin-forming enzymes. However, chemically unaltered endotoxin of large molecular size is necessary for interaction with granulocytes and complement to stimulate phagocytic activity by the cells. Correlating these data with those previously obtained, we conclude that the endotoxin–granulocyte interaction is likely to be more important in producing toxicity and kinin formation *in vivo* than the endotoxin–plasma interaction.

ENDOTOXIN, as usually prepared, is a lipid–polysaccharide–protein complex of variable molecular weight and numerous biological effects.[1] The simplest chemical structure and minimal molecular size which still possess endotoxic activities have not been determined and it is probable that the many biological effects are mediated by different aspects of the endotoxin complex.[2]

Endotoxin induces kinin formation in the monkey.[3] Two possible mechanisms of kinin production are the interaction of endotoxin with either plasma or leukocytes. When the interaction occurs with plasma, kinin-forming enzymes are activated.[4] When the interaction is with leukocytes, kallikrein or a kallikrein activator is released as well as lysosomal and other enzymes capable of influencing the course of endotoxemia or gram-negative sepsis. The plasma–endotoxin interaction requires the presence of complement and antibody to endotoxin;[4] the granulocyte–endotoxin interaction does not require the presence of antibody but does require complement.[5] The differing

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requirements for these two interactions suggest that they may be mediated by different physical or chemical (or both) properties of crude endotoxin. Using endotoxin fractions, we have further investigated the interaction of endotoxin with plasma and leukocytes in order to clarify which aspects of the endotoxin complex are responsible for kinin formation.

METHODS

Preparation and characterization of endotoxin fractions

Cultural conditions. Escherichia coli, type B, were grown at 37° in 28 l. of "minimal" medium consisting of 0·056 M K_2HPO_4, 0·031 M KH_2PO_4, 0·0024 M sodium citrate, 0·011 M ammonium sulfate, 0·016 M dextrose, and $5{\cdot}8 \times 10^{-4}$ M magnesium sulfate.

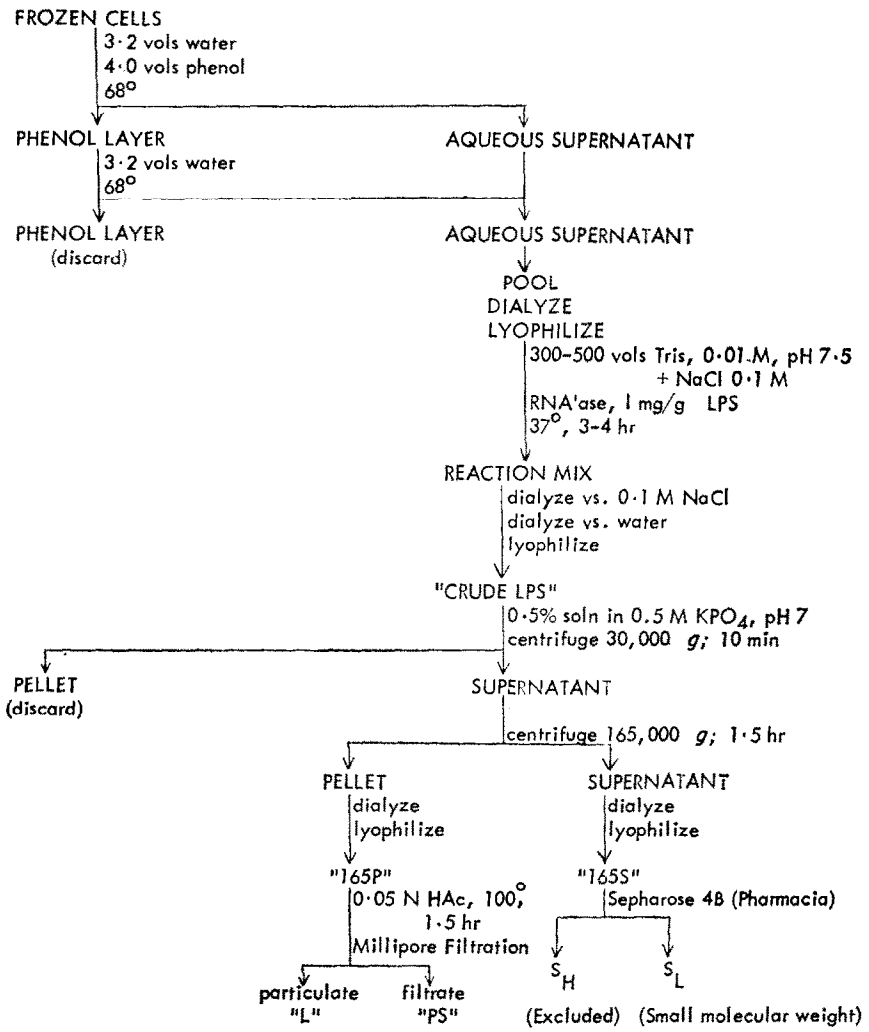

FIG. 1. Flow sheet for the preparation of endotoxin fractions. Abbreviations used in this figure are repeated in the text and tables. 0·5 M KPO_4, pH 7, is a 0·5 molar potassium phosphate buffer ($K_2HPO_4 + KH_2PO_4$) of pH 7.

They were harvested in the plateau phase of growth and resuspended in 16 l. of "minimal" medium enriched with 24 g yeast extract (Difco Laboratories), 104 g nutrient broth (Difco Laboratories), 30·4 g $MgSO_4$, 80 g dextrose, 112 g K_2HPO_4 and 48 g KH_2PO_4. The resuspended cells were incubated for 3 hr at 37°, harvested and frozen.

Preparation of lipopolysaccharide (LPS) fractions. The frozen cells were extracted by the hot phenol–water method of Westphal *et al.*[6] and further purified by dialysis, ribonuclease degradation, centrifugation, gel filtration and hydrolysis as shown in Fig. 1.

Analysis of LPS fractions (Table 1). The lyophilized fractions were dissolved or suspended in distilled water at concentrations ranging from 1 to 10 mg/ml. The intact LPS and subfractions were analyzed for heptose by Osborn's modification[7] of the procedure of Dische,[8] for rhamnose by the procedure of Dische and Shettles,[9] for total and free inorganic phosphate by the method of Chen *et al.*,[10] and for ribose by a modification of the orcinol method[11] using 0·04% $FeCl_3$ — 6 H_2O, which allows for estimation of ribose in the presence of other carbohydrates. For the determination of glucose, the fractions were hydrolyzed for 3 hr in 1 N sulfuric acid at 100°, neutralized and aliquots were assayed directly for glucose using glucose oxidase (Glucostat-Worthington). The galactose analyses were performed with galactose oxidase (Galactostat-Worthington) on similar hydrolysates that had been deionized by serial passage

TABLE 1. COMPOSITION OF LIPOPOLYSACCHARIDE (ENDOTOXIN) PREPARATIONS*

		μmoles/mg of lyophilized substance†								
Preparation	Fraction	Glc	Gal	Rham	GlyManHep	KDO	$HexNH_2$	FA	Bound P_i‡	Rib§
A	165P	0·48	0·51	0·40	0·18	0·21	0·53		0·42	0·11
B		0·52	0·45	0·41	0·20	0·19	0·62	0·50	0·39	0·11
C		0·42	0·47	0·45	0·23	0·23	0·54	0·58	0·38	0·09
D		0·47	0·55	0·48	0·22	0·23	0·54	0·54	0·41	0·10
A	165S	0·98	0·70	1·30	0·03	0·05	0·63	0·13	0·75	0·36
B		1·02	0·76	1·24	0·03	0·07	0·76	0·12	0·72	0·38
C		0·86	0·68	1·27	0·05	0·09	0·57	0·14	0·78	0·32
D		0·82	0·65	1·33	0·04	0·06	0·54	0·11	0·70	0·42
A	S_H	1·20			0·05	0·06	0·87	0·21	0·31	0·16
B		1·36			0·05	0·06	0·81	0·18	0·27	0·16
C		1·08			0·06	0·08	0·44	0·23	0·30	0·20
D		1·08			0·10	0·03	0·71	0·16	0·25	0·20
A	S_L	0·12			0·01	0·003	0·14	0·06	1·25	0·92
B		0·09			< 0·01	0·006	0·10	0·04	1·30	0·87
C		0·11			< 0·01	0·014	0·18	0·03	1·21	0·79
D		0·12			0·01	0·006	0·06	0·01	1·29	0·99
B	PS	0·45			0·24	0·21	0·28	0·01		
C		0·33			0·23	0·22	0·28	0·02		
D		0·38			0·26	0·23	0·25	0·01		

* The following abbreviations are used: lipopolysaccharide, LPS; polysaccharide, PS; glucose, Glc; galactose, Gal; rhamnose, Rham; glyceromannoheptose, GlyManHep; 2-keto-3-deoxyoctulosonic acid, KDO; hexosamine, HexN; fatty acid, FA; ribose, Rib; inorganic phosphate, P_i.

† Note similarity of chemical composition among different batches of lipopolysaccharide.

‡ Bound P_i = total P_i − free P_i.

§ Ribose values represent maximal values because some increase in absorbance is obtained from nonribose carbohydrate.

through small Dowex-50-H^+ and Dowex-1-CO_3 columns to remove galactosamine. 2-Keto-3-deoxyoctulosonic acid (KDO) was determined by the method of Warren,[12] using *N*-acetylneuraminic acid as a standard after 20 min of hydrolysis in 0·02 N acetic acid at 100°. Fractions were hydrolyzed in 4 N HCl at 100° for 5 hr, after which they were analyzed for hexosamine by the method of Boas[13] modified by extraction of the acetylated aminosugars into isoamyl alcohol before addition of Ehrlich's reagent (made with 0·4 ml *p*-dimethylaminobenzaldehyde, 1·5 ml concentrated HCl, and 13·5 ml isoamyl alcohol). The fatty acids were quantified by the method of Duncombe[14] after hydrolysis.

The ribose in these fractions is thought to represent oligonucleotides that are bound to LPS by nonspecific forces that were exceedingly difficult to break. The quantitative values for ribose content in these fractions are undoubtedly overestimations, since even with the reduced amounts of $FeCl_3$ in the orcinal reaction, the other carbohydrates add a small but significant amount of nonspecific color.

The composition of the 165P and the 165S fractions differs as indicated in Table 1. In addition to these differences, it is notable that the lipid moiety of 165S is not easily cleaved from the polysaccharide (PS) as it is in 165P, possibly as a consequence of the low KDO content in the former fraction, since it is presumed that KDO is the sugar linking the lipid portion to the PS.[13]

Leukocytes

The methods for isolation and incubation of viable leukocytes (70–90 per cent neutrophils) have been described.[5,15] The cells were suspended in Eagle's minimal essential medium containing 33 per cent heparinized (2 units/ml) autologous plasma and preincubated in a Dubnoff metabolic shaking incubator in 95% O_2–5% CO_2 at a concentration of 2×10^7 cells/ml for 60 min. Saline or endotoxin fraction was then added at a final concentration of 5 μg of the fraction/ml. After 60 min, the suspending medium was assayed for the difference in lysozyme activity in micrograms per milliliter in the endotoxin-treated and saline-treated leukocyte suspensions.[5,16]

Plasma

Fresh, heparinized (2–5 units/ml) human plasma was separated into 2-ml aliquots. Four-tenths ml of 0·9 per cent saline was added to one aliquot and 0·4 mg of the different endotoxin fractions in a concentration of 1 mg/ml of 0·9 per cent saline was added to the other aliquots. At 0, 5 and 10 min after addition of saline or fraction, samples were removed for kininogen determination.[17] The kininogen depletion in the system *in vitro* can be used as an estimate of the *maximum amount* of kinin formed.[4] Polypropylene or siliconized glass was used throughout for handling of the plasma.

RESULTS

The results of the endotoxin fraction–plasma interaction are shown in Table 2. Kininogen is expressed as a per cent of the zero time value, which averaged 4524 $\pm$ 100 (S.E.) ng trypsin-releasable bradykinin/ml of plasma. Significant kininogen depletion as compared with saline controls occurred with crude LPS, the 165,000 *g* pellet (165P) and the PS portion obtained from the pellet by acid hydrolysis. The kininogen depletion produced by the supernatant fractions was quite variable and not statistically different from controls.

TABLE 2. EFFECT OF VARIOUS ENDOTOXIN FRACTIONS ON PLASMA KININOGEN

Fraction	N	Kininogen concn* (5 min)	(10 min)
Saline	13	99 ± 2	96 ± 2
Crude LPS	6	85 ± 4†	75 ± 5†
165P	9	92 ± 4	86 ± 3†
PS	13	78 ± 5‡	63 ± 6‡
S_L	9	83 ± 10	76 ± 9
S_H	6	90 ± 8	83 ± 4

* The kininogen values are expressed as a percentage of the zero time values (± S.E.).
† Significantly different from saline control ($P < 0.05$).
‡ Significantly different from saline control ($P < 0.01$).

The results of the leukocyte–endotoxin fraction interaction were very consistent (Table 3). Only the pellet and the crude endotoxin produced any appreciable release of lysozyme into the suspending medium. In each of the three experiments, the release of lysozyme produced by the pellet was greater than that produced by crude endotoxin.

TABLE 3. EFFECT OF VARIOUS ENDOTOXIN FRACTIONS ON HUMAN GRANULOCYTES

Fraction	N	Increment in lysozyme activity* (μg/ml ± S.E.)
Crude LPS	5	30 ± 5†
165P	3	53 ± 17†
PS	3	3 ± 2
S_L	3	9 ± 5
S_H	3	7 ± 3

* Increment in lysozyme activity in the suspending medium after incubation of granulocytes with endotoxin fractions for 60 min.
† Significantly different from controls ($P < 0.01$).

DISCUSSION

Endotoxin is a complex material derived from the cell walls of gram-negative bacteria; it has lipid, polysaccharide and protein moieties and a molecular weight of 1–2 million.[1] The numerous biological effects of endotoxin include shock, leukopenia, leakage of lysosomal enzymes from tissues, histamine release, kinin formation, fever, interferon production, intravascular coagulation and antibody production.[18–20] The exact physical structure and chemical composition required to produce the biological effects have eluded many investigators. Although still controversial,[21] the protein moiety has been removed without loss of biological activity.[22–25] The role of the lipid portion is not resolved. Westphal and Lüderitz[25] and Kasai[26] have shown that the lipid portion does possess toxic properties. However, Ribi *et al.*[23,24] have prepared a

potent, large molecular weight polysaccharide endotoxin free of protein and with very little lipid, arguing against an absolute requirement for lipid. The polysaccharide moiety is generally believed to carry the antigenic determinants of endotoxin[20,27] and the complexity of the polysaccharide is correlated with toxicity in mice.[28] *In vitro*, we had kininogen depletion in plasma with all endotoxin fractions, each of which contains polysaccharide. The most potent, however, was the pure PS formed by acid hydrolysis of the pellet. Previous studies have shown that the polysaccharide of endotoxin, when degraded to units of 20,000–30,000 molecular weight by acid hydrolysis, behaves like a hapten, possesses little endotoxic activity *in vivo*, and will not stimulate antibody production, but will react with preformed antibody.[27,29] The necessary requirements for the formation of kinins by crude endotoxin in cell-free plasma are complement and antibody to endotoxin.[4] Likewise, the polysaccharide hapten, as well as all other fractions containing polysaccharide, presumably reacts with antibody and complement in plasma to activate kinin-forming enzymes. Although we have not yet tested our polysaccharide portion *in vivo*, the literature available on low molecular weight polysaccharide indicates that endotoxic activity in terms of producing shock is unlikely,[27,29] and suggests that kinin formation in cell-free plasma may not be very important in the cardiovascular events produced by endotoxin *in vivo*. Similar conclusions can be reached by studying species variation in response to endotoxin.[30] Kinin production occurs *in vitro* when endotoxin is added to rabbit plasma, even though kinins play no obvious role in the cardiovascular events produced by an endotoxin infusion in rabbits.[30]

Molecular size or shape may be as important as chemical composition in confirming toxicity of the endotoxin molecule.[31–33] In the present study, the only endotoxin fractions which interacted with leukocytes *in vitro* were the crude endotoxin and the 165,000 *g* pellet, which contained unaltered endotoxin molecules and aggregates of large molecular weight. The supernatant fractions (molecular weights of about 1 million) did not react with the leukocytes. The interaction of white cells with endotoxin has been shown to be similar to phagocytosis; it requires complement and is characterized by a leak of intracellular enzymes, increased $^{14}CO_2$ production from glucose-1-^{14}C, and increased incorporation of ^{3}H-uridine into RNA.[5] The finding that lysozyme leakage from leukocytes occurred only with the fractions containing large molecules and aggregates strengthens the theory that the interaction of endotoxin with leukocytes is in fact phagocytosis of endotoxin by granulocytes.[5] The amount of enzyme leak has been suggested by Weissmann and Thomas[34] to be greater than one would expect with simple phagocytosis, however.

The interaction of granulocytes and endotoxin may have significance *in vivo* in terms of kinin formation and cardiovascular response to endotoxin. Human granulocytes contain a kallikrein or kallikrein activator which is active at physiological pH.[16,35] The release of this kallikrein by endotoxin could account in part for the kinin formation seen *in vivo* after endotoxin. If this is true, the 165,000 *g* pellet should result in cardiovascular changes and kinin formation when tested *in vivo*.

On the basis of our studies and the literature, we propose the following hypothesis (Fig. 2). Endotoxin infused *in vivo* interacts with complement and antibody to the polysaccharide antigens to activate plasma kinin-forming enzymes. During a slow infusion, this is relatively unimportant but may account for some of the acute hypersensitivity-type reactions seen after rapidly injected endotoxin,[36–38] and may modify

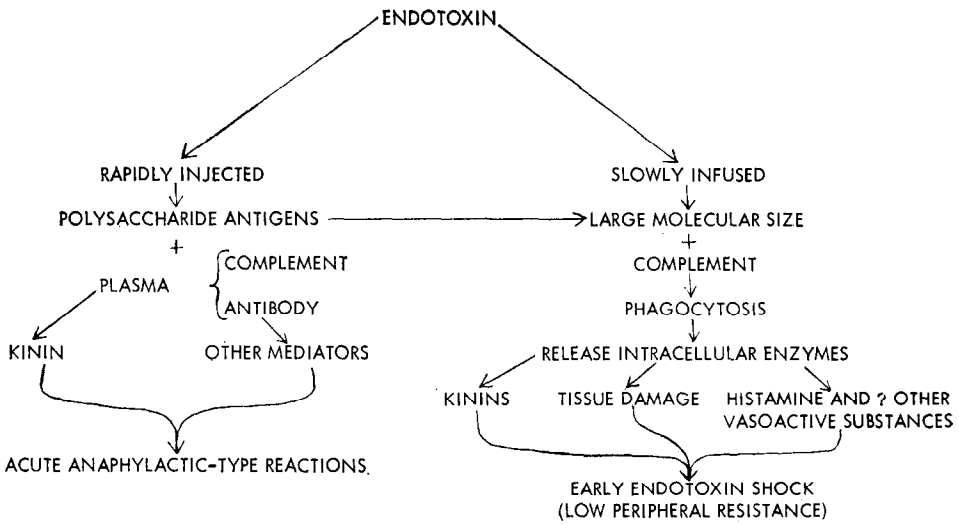

FIG. 2. Schematic representation of a hypothesis on the mechanism of endotoxin-induced changes *in vivo*.

subsequent events in some species.[38,39] However, endotoxin is rapidly cleared from the plasma in the presence of complement by phagocytic cells which release their intracellular enzymes.[3,5,34,40,41] These enzymes are capable of forming kinins, damaging tissues directly, and releasing other vasoactive mediators such as histamine.[42] The early phase of endotoxin shock is the result, which in monkey and man is characterized by low peripheral resistance. Whether continued tissue damage or other events mediated by endotoxin are responsible for the late phases of shock is unknown. Experiments are in progress to evaluate the endotoxin factors *in vivo* and test this hypothesis. We suspect that, after a slow endotoxin infusion into monkeys, the only endotoxic effects will be seen with the pellet (165P) and the crude endotoxin (LPS) and not with the other fractions. The conclusion is in contrast to the speculation we discussed in our original communications on kinins and endotoxin shock.[3] In the original paper we stated that, since the peak rise in measurable lysosomal enzymes occurred somewhat after the early rise in blood kinin levels, then the kinin rise probably was due to plasma–endotoxin interaction. Our data from this and a previous paper[30] now lead us to the conclusion that the endotoxin–cellular interaction is the most important in kinin formation. The leukocytes may be able to mediate kinin formation with a minimal disturbance in the metabolism[16] prior to measurable release of lysosomal enzymes associated with the phagocytosis of endotoxin. The postulate of Weissmann and Thomas[34] that lysosomal enzyme release contributes to the tissue damage and toxic effects of endotoxin is consistent with this concept and explains some of the late phases of endotoxin shock.

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