

DIRECT EVIDENCE FOR HAGEMAN FACTOR (FACTOR XII)
ACTIVATION BY BACTERIAL LIPOPOLYSACCHARIDES
(ENDOTOXINS)*. ‡

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Bacterial lipopolysaccharides (LPS)¹ or endotoxins elicit a variety of host responses, the combination of which result in the deposition of fibrin in capillaries and disseminated intravascular coagulation (DIC). These various host responses to endotoxins have recently been summarized (1) and include: (a) aggregation of platelets and release of platelet factor 3; (b) activation of Hageman factor; (c) inhibition of fibrinolysis; (d) aggregation of leukocytes and release of intracellular constituents; (e) stimulation of the α -adrenergic receptor sites in the kidney; and (f) impairment of reticuloendothelial system function. Whereas there exists substantial experimental evidence to support most of these effects of endotoxin, the evidence for the initiation of the intrinsic clotting system by the direct activation of Hageman factor by endotoxin has been limited and indirect.

Considerable indirect evidence has inferred that Hageman factor may be activated after the injection or release of endotoxin. Patients in shock from gram-negative bacteremia, but not from hemorrhagic shock, have been estimated to have decreased levels of both Hageman factor and prekallikrein (2). Increased levels of plasma kinins have, in addition, been demonstrated within 30 min of the intravenous injection of purified LPS into human volunteers (3). Indirect evidence in vitro for endotoxin-induced activation of Hageman factor has been demonstrated by the experiments of Rodriguez-Erdmann (4), who showed that LPS significantly altered the thromboplastin-generating times in plasma but had no effect on purified prothrombin.

Injections of endotoxin in rabbits have led to a diminution of circulating Hageman factor levels (5, 6). However, the reduction was likely secondary to other effects of the endotoxin since treatment with vitamin K analogues, or depletion of granulocytes prevented the fall of Hageman factor levels.

That the activation of Hageman factor in vivo may participate in DIC has been inferred from several studies. Injection of the Hageman factor activators sodium stearate or ellagic acid into rabbits induced DIC when accompanied by administration

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¹ *Abbreviations used in this paper:* BAEe, benzoyl arginine ethyl ester; DIC, disseminated intravascular coagulation; KDO, 2-keto-3-deoxyoctanoic acid; LPS, bacterial lipopolysaccharides; OMTI ovomucoid trypsin inhibitor.

of epsilon-aminocaproic acid (to inhibit fibrinolysis) and norepinephrine (to stimulate α -adrenergic receptor sites in the kidney) (7, 8). In another study, injection of activated Hageman factor into pregnant (fibrinolysin inhibited) rats, when accompanied by norepinephrine and inosithin (a substitute for platelet factor 3) produced the generalized Schwartzman reaction in six of seven animals (9). Further administration of endotoxin to fowl which are naturally deficient in Hageman factor, failed to elicit DIC, although fibrin was precipitated intravascularly upon administration of thromboplastin (10). Hereditary deficiency of factor VII of the extrinsic clotting system in dogs also failed to prevent a fall in fibrinogen levels after injection of endotoxin, suggesting participation of the intrinsic clotting system (11).

In contrast to the above experiments, data also indicate participation of the extrinsic clotting system in DIC. Rabbits, depleted of factor VIII of the extrinsic clotting system with heterologous antibody, demonstrated a fall in fibrinogen levels after injection of endotoxin that equalled the drop found in normal rabbits. In fact, one of the factor VIII-depleted rabbits developed a generalized Schwartzman reaction (12). The use of lysozyme to block contact activation of Hageman factor in vivo failed to block DIC of the generalized Schwartzman reaction in endotoxin-treated rabbits (5), although lysozyme, together with vitamin K analogues did inhibit DIC after the injection of liquid (1, 13).

If endotoxin (bacterial lipopolysaccharide, LPS) is capable of initiating clotting via the intrinsic clotting system it is essential to determine which component in the series is activated by the LPS. In this report, we demonstrate that purified LPS can activate directly purified precursor Hageman factor, the initial component of the intrinsic clotting system, that the interaction of LPS and precursor Hageman factor results in the formation of a complex between these two molecules, and that this complex possesses the capacity to activate prekallikrein.

Materials and Methods

Plasma Proteins.—Both rabbit and human Hageman factor were purified in precursor form by methods previously published (14). The resulting Hageman factor failed to activate prekallikrein unless exposed to activators. All Hageman factor preparations were used at a concentration of 30 $\mu\text{g}/\text{ml}$, i.e., at approximately the concentration found in whole human plasma.² For some experiments, purified human precursor Hageman factor was labeled with ¹²⁵I by the procedure of McConahey and Dixon (15). The purity of these preparations has been previously described.² Purified rabbit prekallikrein was also prepared as previously described (14), and was used at a concentration of 12.5 $\mu\text{g}/\text{ml}$ (1,000 mU/ml).

Plasma.—Hageman factor-deficient plasma was generously donated by Mrs. Gunda Hiatt. Blood was collected into acid citrate anticoagulant (13.65 g citric acid, 25.0 g NaCitrate, 20.0 g dextrose, pH 4.7; 1 part to 6 parts blood) and the plasma was separated from the formed elements by centrifugation at 4,000 rpm for 30 min and stored at -70°C until just before use.

Bacteria.—*Escherichia coli* 0111:B4 was grown in minimal medium as previously described (16). The epimeraseless mutant of this strain (J-5) obtained from Dr. Loretta Leive, as well

² Revak, S. D., C. G. Cochrane, A. R. Johnston, and T. E. Hugli. 1974. Structural changes accompanying enzymatic activation of human Hageman factor. Manuscript submitted for publication.

as the heptose-deficient mutant from *Salmonella minnesota* (R595) obtained from Dr. John Ryan were grown with aeration in trypticase soy broth (Baltimore Biological Laboratories, Baltimore, Md.). Cells were harvested by centrifugation and washed twice with 0.85% sodium chloride.

Lipopolysaccharides.—LPS was extracted from 0111:B4 by the phenol-water procedure of Westphal and Jann (17) and purified as previously described (18, 19). This preparation of LPS was then further fractionated by column chromatography on Sepharose 4B, (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), as described elsewhere,³ into two fractions, LPS-I and LPS-II containing approximately 1% and 10% by weight of lipid A, respectively. The LPS fractions prepared by this method have been estimated to contain less than 1% protein and 5% nucleic acid.³ Both of these LPS fractions have been demonstrated to be lethal to mice,³ cause DIC in rabbits,⁴ and to be mitogenic for B lymphocytes (20). LPS from the mutant strains J-5 and R-595 were extracted and purified by the phenol-petroleum ether-chloroform procedure of Galanos et al. (21). These latter preparations were insoluble in water and were briefly sonicated to prepare a fine suspension immediately before use. Lipid A was prepared from purified preparations of 0111:B4-LPS by mild acid hydrolysis in 0.1 N HCl for 25–35 min at 100°C. The precipitated lipid A was then solubilized by the addition of triethylamine to 0.1% suspensions of lipid A, and the solubilized lipid A then dialyzed extensively against distilled water (22).

Chemical Assays.—The somatic O-antigen determinant of *E. coli* 0111:B4 contains a dideoxyhexose (colitose) which is specifically and conveniently measured by the thiobarbituric acid assay described by Cynkin and Ashwell (23), after mild acid hydrolysis of LPS. If the hydrolysis conditions are modified (0.01 N H₂SO₄ instead of 0.2 N H₂SO₄) and the periodate oxidation carried out for 60 min at 0°C instead of 20 min at 55°C, the reaction with colitose is effectively suppressed and this same assay may then be used to estimate relative levels of the eight carbon saccharide 2-keto-3-deoxyoctanoic acid (KDO) (the specific carbohydrate linkage between the lipid A portion of the LPS molecule and the heptose backbone of the polysaccharide region) after correction for residual reactive colitose.⁴ This assay is a highly sensitive and extremely specific assay for dideoxyhexoses which are found almost exclusively in LPS (23).

Determination of Hageman Factor Activation.—For all of the experiments described in the following paragraphs, activation is defined as the conversion of the enzymatically inactive precursor Hageman factor to a form in which it may interact with one or more of its natural substrates. All assays were performed in plastic tubes to avoid the spontaneous activation of Hageman factor by glass. Two methods have been used to estimate the ability of LPS to activate purified precursor Hageman factor. In the first, preparations of LPS plus Hageman factor, in a final vol of 100 μ l in Tris-buffered saline (0.01 M Tris, 0.14 M NaCl, pH 7.4) after incubation for 20 min at 37°C, were added to purified preparations of rabbit prekallikrein (1.25 μ g in 100 μ l) (24) and incubated for a further 20-min period. Conversion of prekallikrein to the active enzyme was assayed after the addition of 3.0 ml of 1 mM benzoyl arginine ethyl ester (BAEe). Enzymatic activity was determined by cleavage of the ethyl ester as estimated by a change in absorbance at 253 nm. LPS alone was shown to have no effect on either prekallikrein, BAEe, or a mixture of the two. In the second method, the ability of LPS-activated Hageman factor to reduce the clotting time of XII-deficient plasma was determined (14).

Sucrose Gradient Ultracentrifugation.—Preparations of LPS, Hageman factor, or a mixture of the two were layered onto 5–20% linear sucrose gradients and centrifuged in the Spinco

³ Morrison, D. C. and L. Leive. 1974. Two fractions of lipopolysaccharide from *Escherichia coli* 0111:B4 as prepared by two extraction procedures. Manuscript submitted for publication.

⁴ D. C. Morrison. Unpublished observations.

Model L preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 15 h at 30,000 rpm using an SW 50 rotor. Fractions of approximately 0.35 ml were then collected from the bottom of the tube and were assayed by addition of 0.05-ml aliquots of the fractions to 0.1 ml prekallikrein as noted above, or by treating a similar volume aliquot with 0.1 μg trypsin (Worthington Biochemical Corp., Freehold, N. J.) for 20 min at 37°C to activate Hageman factor, inhibiting the trypsin with 25 μg ovomucoid trypsin inhibitor (OMTI, Worthington Biochemical Corp.) and then assaying the fraction for its ability to activate prekallikrein. To measure LPS in the gradient fractions, aliquots were dialyzed extensively vs. distilled water before assaying levels of colitose, which reflects the concentration of LPS (sucrose in high concentration interferes with this assay).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.—Aliquots of ^{125}I -labeled human Hageman factor were electrophoresed and assayed for radioactivity as described elsewhere (25).

RESULTS

The Direct Activation of Hageman Factor by LPS.—To determine the relative ability of LPS to activate Hageman factor, increasing concentrations of LPS-I and LPS-II were incubated with a constant amount of purified rabbit Hageman factor and the resulting ability of activated Hageman factor to convert prekallikrein to its active form then measured. The results of this experiment, shown in Fig. 1, demonstrate that although both preparations of LPS can activate Hageman factor, LPS-II is more than 10-fold more efficient than LPS-I. It has been demonstrated³ that LPS-II contains significantly higher amounts of lipid A on a weight basis than does LPS-I.

Demonstration of Lipid A as the Active Principle of the LPS Molecule.—The

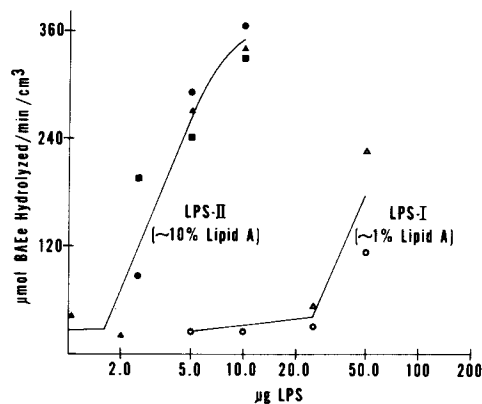


FIG. 1. The activation of Hageman factor by LPS. Increasing amounts of purified LPS from *E. coli* 0111:B4 containing approximately 10% lipid A (LPS-II) or 1% lipid A (LPS-I) were added to 0.3 μg of purified rabbit Hageman factor in a final vol of 100 μl . After incubation for 20 min at 37°C, 1.25 μg of purified prekallikrein in 100 μl of Tris-buffered saline was added and the mixture incubated an additional 20 min. Then 3.0 ml of 1 mM BAEe was added and the change in absorbance measured as a function of time of incubation at 37°. The circles and triangles represent individual experiments.

fact that LPS-II is biochemically more potent than LPS-I suggests that the lipid A region of the LPS molecule may be responsible for Hageman factor activation (see Fig. 2). To examine this question further, the lipid A portion of the LPS-II molecule was chemically removed by mild acid hydrolysis. The rate of disappearance of the ability of the treated LPS to activate rabbit Hageman factor was then compared with the rate of removal of lipid A as measured by the appearance of free KDO. Hydrolysis was carried out in 0.01 N sulfuric acid for various times at 100°C. To assay for free KDO, samples were then chilled rapidly and assayed by the thiobarbituric acid assay as described in the Materials and

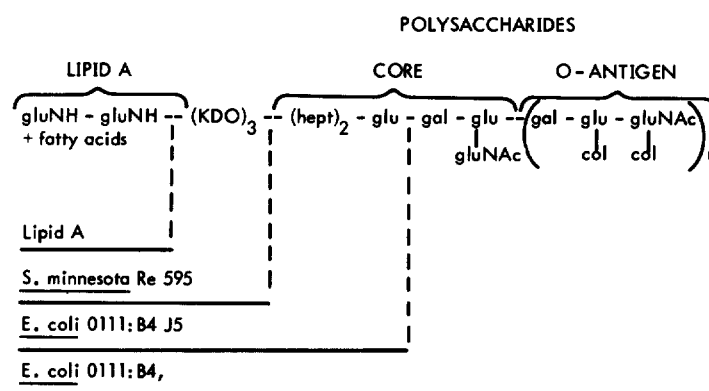


FIG. 2. The schematic structure of LPS. The basic structure of the LPS of *E. coli* 0111:B4 consists of a repeating polysaccharide portion and a lipid portion (lipid A). The polysaccharide composition of the O-antigen is varied within a given species of bacteria, the core polysaccharide is common within a species of bacteria but varies between bacterial species and the lipid A and KDO is common to all bacterial species. The chemical structure of the preparations of LPS obtained from the complete and mutant strains of bacteria used in these experiments is indicated (*E. coli* 0111:B4, LPS-I, $n \sim 10$, LPS-II, $n \sim 2$).

Methods. To determine Hageman factor activation, samples were neutralized with NaOH, the lipid A extracted with chloroform, and the partially hydrolyzed remaining LPS assayed for ability to activate Hageman factor at several concentrations of LPS. The results of this experiment, shown in Fig. 3 *a*, demonstrate that very short periods of hydrolysis are sufficient to increase the amount of LPS required for Hageman factor activation. By measuring the quantity of LPS, after acid hydrolysis, required to activate approximately 50% of the Hageman factor, an approximate correlation is apparent between the loss of Hageman factor-activating capacity and the appearance of relative amount of free KDO (Fig. 3 *b*).

In order to determine more accurately the role of lipid A and other portions of the LPS molecule in the activation of Hageman factor, preparations of LPS from mutant bacteria, as well as purified lipid A were prepared (see Fig. 2). The LPS from the J-5 strain of *E. coli* does not contain O-antigenic polysaccharide

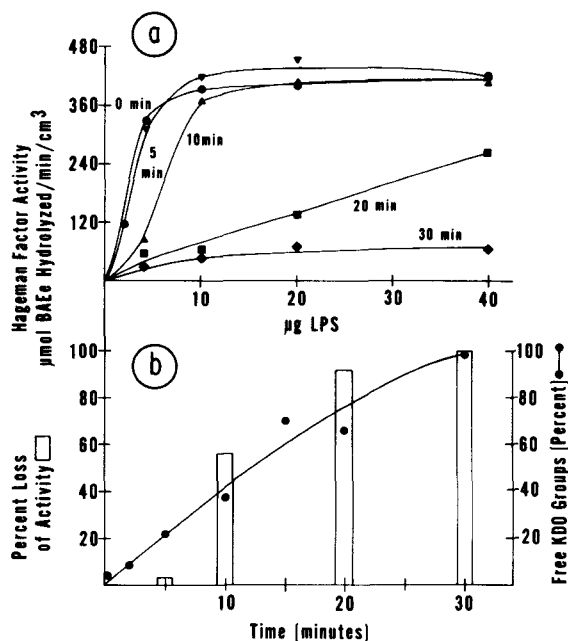


FIG. 3. The removal of lipid A by mild acid hydrolysis and its effect on Hageman factor activation. Samples of LPS-II were hydrolyzed in 0.01 N H_2SO_4 at $100^\circ C$ for various times. The generation of liberated lipid A was estimated by the appearance of free KDO. After extraction of the lipid A into $CHCl_3$, the remaining polysaccharide was assayed for its ability to activate Hageman factor as described in the legend to Fig. 1. (a) The activation of rabbit Hageman factor by increasing amounts of partially hydrolyzed LPS-II. (b) The appearance of free KDO and the approximate amount of partially hydrolyzed LPS-II required to give 50% of maximal Hageman factor activity are plotted as a function of time of hydrolysis of LPS-II.

and contains a high concentration of lipid A. The R-595 mutant contains only the KDO trisaccharide and lipid A. Both of these LPS preparations activated Hageman factor with respect to its action on prekallikrein. While they were less active than LPS-II, accurate comparisons are difficult owing to the fact that LPS-II is soluble in water, while the other two are obtained as insoluble aggregates. Further evidence for the action of lipid A on Hageman factor was obtained with purified lipid A which was found to be a highly effective activator of Hageman factor (see Fig. 4).

Initiation of the Intrinsic Clotting System by Hageman Factor Treated with LPS.—As the above experiments indicate that LPS (and in particular lipid A) can activate Hageman factor with respect to its ability to convert prekallikrein, it was also of interest to examine the ability of LPS to activate Hageman factor to initiate clotting. For these experiments, purified human Hageman factor was incubated with various preparations of LPS and the mixture tested for clot promotion in Hageman factor-deficient plasma. The results of these experiments, shown in Fig. 5, demonstrate that both LPS preparations tested had the ca-

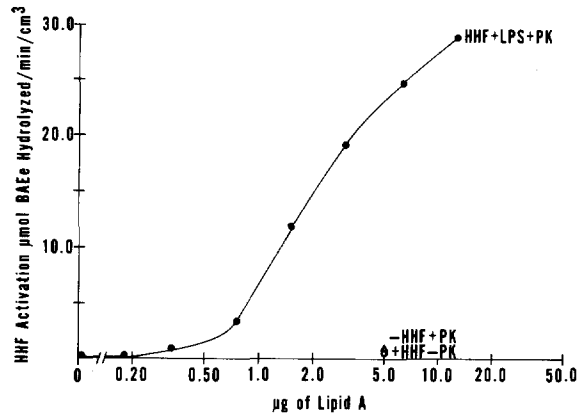


FIG. 4. The activation of Hageman factor by isolated lipid A. Increasing amounts of lipid A, isolated from *E. coli* 0111:B4 LPS-II, were incubated with purified human Hageman factor and assayed for activity as described in the legend to Fig. 1.

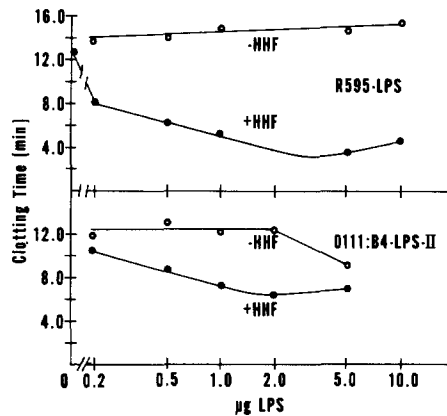


FIG. 5. The influence of LPS-activated Hageman factor on the clotting of factor XII-deficient plasma. Approximately 0.3 μg of purified human Hageman factor were incubated with increasing concentrations of either *S. minnesota* R-595-LPS or 0111:B4-LPS-II for 10 min at 37°C in a final vol of 100 μl. Then 50 μl of XII-deficient plasma were added and incubation continued for an additional 5 min. Finally 50 μl of a 1:50 dilution of cephalin containing 10 mM calcium were added and the time for clot formation determined. Each time point shown is the average of triplicate clot time determinations.

capacity to decrease clotting times in XII-deficient plasma by activating purified precursor Hageman factor, and that responses were concentration dependent. Further, with only one exception (5 μg of LPS-II) LPS alone did not affect the clotting times of Hageman factor-deficient plasma, although the reason(s) for this are not known. The absence of a demonstrable decrease in clotting time of Hageman factor incubated in the absence of LPS demonstrates that the Hageman factor was essentially in precursor form.

Demonstration of Binding of Hageman Factor to LPS.—Purified human Hageman factor (supplemented with trace ^{125}I -labeled human Hageman factor) was incubated with approximately $100\ \mu\text{g}$ of LPS-II at 37°C for 20 min. This mixture was then sedimented in a 5–20% linear sucrose gradient as described in the Materials and Methods. Both LPS-II and ^{125}I -labeled Hageman factor incubated alone were also centrifuged as controls. The results of this experiment are shown in Fig. 6. Hageman factor, incubated in the absence of LPS-II, sedimented near the top of the gradient, (Fig. 6 a), at its characteristic 4.5S rate. The sedimented Hageman factor contains no spontaneous activity, but could be activated by incubation with trypsin as shown. LPS-II and Hageman factor

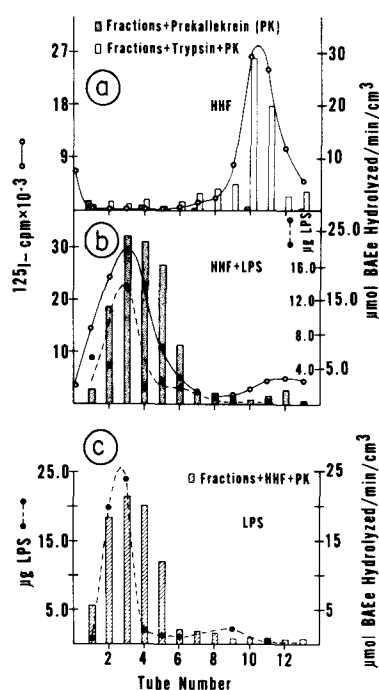


FIG. 6. The demonstration of Hageman factor binding to LPS. Samples containing $100\ \mu\text{g}$ of LPS-II, approximately $1\ \mu\text{g}$ of ^{131}I -labeled human Hageman factor or a mixture of the two were incubated in a final vol of $200\ \mu\text{l}$ for 20 min at 37°C . These samples were then layered onto 5–20% linear sucrose gradients and centrifuged for 15 h at $90,000 \times g$. Aliquots, collected from the bottom of the tube, were assayed, where appropriate, for the presence of ^{131}I , spontaneous Hageman factor activity, Hageman factor activatable by trypsin, ability to activate purified human Hageman factor, or the LPS-specific sugar colitose. (a) The sedimentation profile of Hageman factor. Gradient aliquots were assayed for ^{131}I (solid curve), spontaneous Hageman factor activity, or Hageman factor activity after treatment with trypsin. (b) The sedimentation profile of Hageman factor. Gradient aliquots were assayed for ^{131}I (solid curve), spontaneous Hageman factor activity, or LPS (dashed curve). (c) The sedimentation profile of LPS. Gradient fractions were assayed for LPS (dashed curve) and for their capacity to activate $0.3\ \mu\text{g}$ of purified human Hageman factor.

preincubated together at 37°C (Fig. 6 *b*) sedimented together as a complex in the region of the LPS-II peak,⁵ and the prekallikrein-activating capacity was associated with the LPS-Hageman factor complex. This experiment demonstrated that Hageman factor does bind to, and is, in turn, activated by the LPS molecule. The LPS-II preparation, incubated without Hageman factor (Fig. 6 *c*), sedimented near the bottom of the gradient. Only those fractions which contained LPS (as determined by colitose) had the capacity to activate Hageman factor added to the gradient fractions, confirming that it is the LPS-II, and not a contaminant in the LPS-II preparation, which was responsible for the activation of Hageman factor.

When equivalent preparations of LPS-II were incubated with radiolabeled Hageman factor, and then subjected to SDS polyacrylamide gel electrophoresis, no cleavage of the Hageman factor molecule could be demonstrated (see Fig. 7 *a*). Both LPS activated Hageman factor and control Hageman factor migrated as superimposable radiolabel peaks at a position of approximately 80,000 mol wt. The binding of Hageman factor fragments to LPS can be excluded by the observation³ that LPS-II is dissociated in SDS to approximately 90,000. If all of the Hageman factor fragments remained bound to the LPS it would have an aggregate mol wt of 170,000 and would not migrate into the gel. It may thus be concluded that LPS activation of Hageman factor does not involve peptide bond cleavage. This contrasts to the fluid phase activation observed with trypsin, kallikrein, or plasmin (25) in which Hageman factor is cleaved into lower molecular weight fragments during the activation (Fig. 7 *b*).

Inhibition of Hageman Factor Activation by LPS.—A consistent reversal of the decrease in clotting times has been observed when high concentrations of LPS have been used to activate Hageman factor (see Fig. 5). To examine whether a parallel observation could be obtained in the prekallikrein activating system, preparations of purified human Hageman factor were incubated with high concentrations of LPS (relative to that required to activate Hageman factor). When these preparations were then tested for prekallikrein conversion, a pronounced inhibition was also observed (see Fig. 8 *a*).

This inhibition of activity could occur as a result of either an interaction of LPS with Hageman factor or with prekallikrein. In order to assess the relative contribution of each of these factors to the LPS-induced inhibition, the concentration of both Hageman factor and prekallikrein in the reaction mixture were varied independently. The results of these experiments are shown in Fig. 8 *a* and 8 *b*. In Fig. 8 *a*, the LPS-induced activation inhibition response is shown for three concentrations of Hageman factor. There appears to exist a stoichiometric relationship between the amount of Hageman factor present and the amount of LPS required to inhibit. For example, when the amount of Hageman

⁵ As LPS-II has an approximate sedimentation value of 11S and Hageman factor about 4.5S, the binding of Hageman factor to the LPS molecule would not be expected to substantially change the position of the LPS peak.

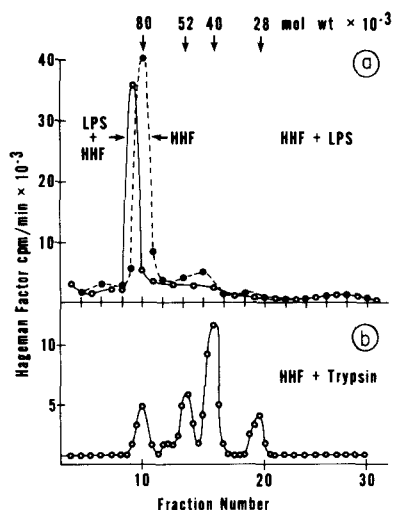


FIG. 7. SDS acrylamide gel electrophoresis of activated Hageman factor. Samples of ^{131}I -labeled human Hageman factor were activated by either trypsin or by LPS. After electrophoresis, gels were sectioned into 1 mm fractions and the radioactivity in each section determined. (a) The solid phase activation of Hageman factor by LPS. (b) The fluid phase activation of Hageman factor by trypsin.

factor is increased by 2.5 or 6.3, the amount of LPS required to give an inhibition of 50% is increased from 50 μg to 120 μg and 300 μg , respectively, suggestive that the LPS may be directly inhibiting Hageman factor activation.

In a second experiment, the amount of Hageman factor was maintained constant and two concentrations of purified prekallikrein were used. As shown in Figure 8 *b*, an increase in the amount of prekallikrein added to the incubation mixture partially reversed the inhibitory effect of LPS. However, at the higher concentrations of LPS, as the reversal of inhibition was not stoichiometrically correlated with the amount of prekallikrein added (twofold increase in rate with fourfold increase in prekallikrein), some inhibitory effect of LPS on prekallikrein is also suggested.

To examine further the nature of the inhibition of Hageman factor activation at high concentrations of LPS, an experiment was performed to determine whether the inhibited Hageman factor could still be activated by fluid phase activators. Human Hageman factor was incubated with various concentrations of LPS for 20 min at 37°C, after which the incubation mixture was divided into two aliquots. The Hageman factor in one aliquot was then further activated with trypsin for 10 min at 37°C, after which the reaction was stopped with OMTI. The other aliquot was treated simultaneously with trypsin and trypsin inhibitor. Both aliquots were then tested for their ability to activate prekallikrein. The results of this experiment, shown in Table I, suggest that noninhibitory concentrations of LPS (10 μg) do not inhibit further activation of

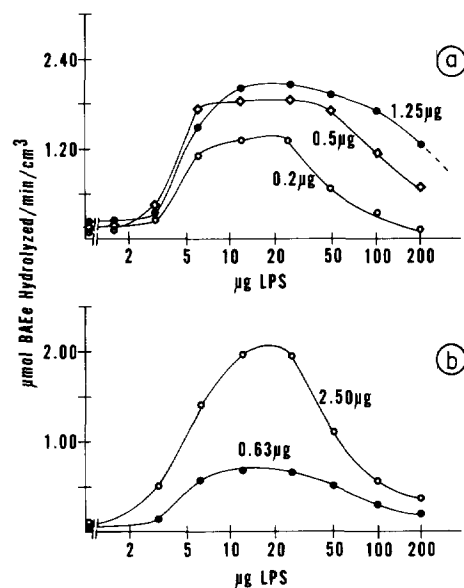


FIG. 8. The activation/inhibition of Hageman factor as a function of LPS concentration. The capacity of either Hageman factor or prekallikrein to reverse the inhibition observed at high concentrations of LPS was examined. The experimental details were as noted in the legend to Fig. 1 except where noted. (a) Activation/inhibition as a function of Hageman factor concentration. Increasing concentrations of LPS-II were incubated with 0.2 μg , 0.5 μg , and 1.25 μg of purified human Hageman factor in a final vol of 100 μl . Then 1.25 μg of purified rabbit prekallikrein in 100 μl were added and activation determined as described in Fig. 1. (b) Activation/inhibition as a function of prekallikrein concentration. Approximately 0.3 μg of purified human Hageman factor were incubated with increasing concentrations of LPS-II. Then 0.63 and 2.5 μg of purified rabbit prekallikrein in 200 μl were added and activation determined as described in Fig. 1.

TABLE I

0111:B4 - LPS II	$\mu\text{mol BAEe hydrolyzed/min/cm}^3$	
	+ Trypsin	- Trypsin
0	5.59	0
10 μg	5.22	3.08
50 μg	0.52	0.16
100 μg	0.20	0.28

Hageman factor by trypsin but that inhibitory concentrations of LPS (100 μg) interfere with the action of trypsin on the Hageman factor molecule.

DISCUSSION

We have demonstrated that Hageman factor can be activated by incubation with purified preparations of LPS to initiate both the intrinsic clotting and the

kinin-forming pathways. As has been demonstrated for most of the other biological properties of LPS, capacity to activate Hageman factor appears to reside in the lipid A region of the molecule (see Fig. 2). We have demonstrated this by showing (a) that preparations of LPS rich in lipid A have a greater potential for activation of Hageman factor than those poor in lipid A; (b) that LPS mutants, lacking the complete carbohydrate moiety, but possessing the lipid A moiety still have the capacity to activate Hageman factor; (c) that the time-course of removal of lipid A from the polysaccharide backbone was approximately paralleled by loss of ability to activate Hageman factor; and (d) that isolated purified lipid A is an extremely potent activator of Hageman factor.

High concentrations of LPS have been demonstrated to inhibit the activity of Hageman factor-related systems, and the results of several experiments suggest that the LPS-induced inhibition may be the result of a direct interaction between LPS and Hageman factor. In addition to the observation that high concentrations of LPS can inhibit two distinct functions mediated by Hageman factor, e.g. initiation of clotting and activation of prekallikrein, this latter inhibition can be stoichiometrically correlated to the ratio of Hageman factor to LPS. The model of direct inhibition of Hageman factor is further suggested by experiments which show that LPS-inhibited Hageman factor is incapable of being further activated in the fluid phase by trypsin, whereas Hageman factor which has been only partially activated by LPS may still be further activated by trypsin. The concept of the inactivation of Hageman factor by LPS through direct contact is supported by the observation that Hageman factor forms a complex with LPS.

It has been known for some time that Hageman factor may be activated by contact with negatively charged surfaces such as glass, kaolin, celite, etc. LPS has been shown to have a net negative charge at physiological pH (26). In addition, the lipid A region, which consists of a diglucosamine with both ester-, and amide-linked fatty acids, also contains negatively charged pyrophosphate groups (Fig. 9) (27). It is, therefore, attractive to consider these charged phosphates as being responsible for the activation of Hageman factor. Alternatively, preparations of fatty acids have also been shown to affect Hageman factor (28-30) and as the lipid A region is rich in fatty acids, it may be this portion of the lipid A which is responsible for the activation.

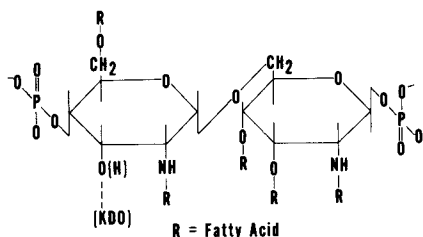


FIG. 9. Diagrammatic structure of lipid A. As defined by Reitschel et al. (27).

The demonstration of a stable complex between the Hageman factor molecule and the LPS molecule suggests that this mechanism of activation is analogous to the solid-phase activation as compared to the fluid-phase activation initiated by enzymatic cleavage of the molecule (25). The experimental data showing no significant change in the molecular weight of the Hageman factor molecule after LPS activation support the hypothesis originally proposed by Hardisty and Margolis (31) that binding of Hageman factor to negatively charged surfaces causes a conformational change in the molecule which then renders it enzymatically active. In the case of LPS it may be the lipid A region of the LPS molecule (and perhaps the negatively charged phosphate groups of this portion of the LPS molecule or the covalently linked fatty acids) which can bind the Hageman factor. This binding then causes a conformational change, but not covalent cleavage, of the molecule which is then rendered capable of at least two activities, initiation of clotting and activation of prekallikrein.

These results support the concept that bacterial endotoxins are capable of initiating activity of the intrinsic clotting system as well as the kinin-forming and perhaps also the fibrinolytic systems. The soluble LPS used in the present experiments was found to be one of the most active agents we have encountered among solid-phase activators in its ability to convert Hageman factor to an active state. The participation of this mechanism in disseminated intravascular coagulation remains to be defined.

SUMMARY

Purified precursor Hageman factor has been demonstrated to bind to soluble bacterial lipopolysaccharide (LPS, endotoxin) isolated from *Escherichia coli* 0111:B4, and this complex has been shown to have the capacity to convert prekallikrein to its active form. In addition, LPS-activated Hageman factor substantially reduces clotting times in XII-deficient plasma. The capacity to activate Hageman factor has been demonstrated to reside in the lipid A region of the LPS molecule. Activation of Hageman factor by LPS contrasts with fluid-phase activation (e.g., by kallikrein or trypsin) in that no cleavage to lower molecular weight fragments occurs. High concentrations of LPS inhibit the activity of Hageman factor, probably by a direct LPS-Hageman factor interaction.

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