Structural Features of *Salmonella Typhimurium* Lipopolysaccharide Required for Activation of Tissue Factor in Human Mononuclear Cells

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ABSTRACT Activation of mononuclear cell tissue factor was examined utilizing lipopolysaccharides obtained from wild-type and both R_c and R_e mutants of *Salmonella typhimurium*. Wild-type (smooth) lipopolysaccharide, galactose-deficient (R_c) lipopolysaccharide, heptose-deficient (R_e) lipopolysaccharide, and lipid A preparations were all active in their ability to generate tissue factor activity in human mononuclear cells grown in tissue culture. Polymyxin B has been reported to prevent some of the lethal effects of endotoxin in vivo, and the drug reportedly binds to the 2-keto-3-deoxyoctulosonate-lipid A region of the lipopolysaccharide molecule. Polymyxin B was effective in inhibiting the tissue factor generating activity of wild-type lipopolysaccharide, R_e lipopolysaccharide, and lipid A in a dose-dependent fashion. Treatment of lipid A preparations with mild alkali abolished the ability of these preparations to activate tissue factor in cells. Analogous to many of the other biologic properties of lipopolysaccharide, tissue factor activation in human mononuclear cells appears to depend upon the integrity of the lipid A portion of the molecule.

INTRODUCTION

Tissue factor, the lipid-dependent protein which initiates the extrinsic system of coagulation, appears to reside between the surface coat and plasma membrane of a variety of mammalian cells, including fibroblasts (1–3), granulocytes (4, 5), mononuclear cells (6–10), and endothelial cells (11–13). Unperturbed cells manifest little, if any, tissue factor activity, and "activation" by endotoxin, antigens, mitogens, or enzymes is required for expression of latent coagulant activity (3, 6). A central role has been suggested for white cell tissue factor in the initiation of intravascular coagulation secondary to progranulocytic leukemia (14–16) and Gram-negative sepsis (17–20). Recent evidence has also suggested that tissue factor activity generated by immunocompetent cells may play a role in the deposition of fibrin seen in association with delayed hypersensitivity reactions (21, 22). Specific or nonspecific activation of cell-bound tissue factor may be important, therefore, in the pathogenesis of both intravascular and extravascular coagulation.

Maynard and his colleagues (3) have demonstrated that trypsin, chymotrypsin, pronase, papain, and a bacterial protease activate tissue factor in fibroblasts and cells derived from human amnion tissue. They have interpreted these findings to mean that the latency of tissue factor activity may represent a protective mechanism. In this way, tissue factor can be in proximity to flowing blood for use in the defense against trauma without producing intravascular coagulation. The mechanism of this activation reaction and the step or steps involved, therefore, become of central importance to the understanding of hemostasis and thrombosis.

The lipopolysaccharide (LPS)[1] of Gram-negative bac-

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[1] *Abbreviations used in this paper*: APTT, activated partial thromboplastin time test; BSA, bovine serum albumin; HSA, human serum albumin; KDO, 2-keto-3-deoxyoctulosonate; LPS, lipopolysaccharide.

teria is a potent activator of tissue factor when introduced into suspensions of leukocytes (4, 5, 8–10, 17–20). The multiple biologic properties of LPS obtained from a variety of microorganisms are well known and have recently been reviewed (23, 24). Availability of purified preparations of chemically modified LPS (lipid A) and incomplete (rough) LPS from mutant strains of *Salmonella typhimurium* has permitted us to examine the structural characteristics of the LPS molecule requisite for activation of mononuclear cell tissue factor.

METHODS

Mononuclear cell cultures. Human mononuclear cells were obtained from heparinized peripheral blood by the Ficoll-Hypaque separation technique previously described (6) utilizing lymphocyte separation medium (Bionetics Laboratories, Kensington, Md.). Cell suspensions obtained in this manner consisted of 98±2% mononuclear cells, of which 70–90% were lymphocytes and 10–30% were monocytes. Lymphocytes and monocytes were quantified by their appearance after Wright staining and ingestion of latex particles (Difco Laboratories, Detroit, Mich.), respectively. Cell viability was determined by exclusion of trypan blue dye (6), and suspensions containing less than 90% viable cells were discarded. Viability averaged 96±3%. Cell cultures consisted of 1×10^6 mononuclear cells/ml resuspended in RPMI 1640 tissue culture media (Gibco, Grand Island Biological Co., Grand Island, N. Y.) and were supplemented with L-glutamine (1%) and penicillin-streptomycin (0.5%). Autologous serum (10%) was utilized only in those cultures assessed for DNA synthesis and was routinely heated at 56°C for 30 min before use. Cultures evaluated for procoagulant activity were harvested between 16 and 24 h after stimulation which corresponded to the peak time of tissue factor generation in previous experiments (25). Individual cultures were harvested for the determination of procoagulant activity by sonication at 4°C for 20 s (Biosonic IV, VWR Scientific, Inc., Rochester, N. Y.; microprobe at 60-low). Although supernates, obtained by centrifugation of stimulated cells, contained tissue factor (26), more consistent results were obtained by assaying disrupted whole cells as previously described (6). Sonicates were stored briefly at 4°C during the tissue factor assay or frozen immediately at −70°C for assay at a later date. Stability of the tissue factor in cell sonicates permitted storage at −70°C for up to 3 yr without demonstrable loss of procoagulant activity. DNA synthesis was evaluated in the serum-supplemented cultures at 72 h after stimulation by preparations of LPS or the standard mitogen phytohemagglutinin (Difco Laboratories). Triplicate samples of stimulated and control cells were pulsed for 4 h with 2 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Mass., 6.7 Ci/mM) and TCA-insoluble material was collected on millipore filters (Millipore Corp., Bedford, Mass.). Filters were dried overnight in scintillation vials and 10 ml of Econofluor (New England Nuclear) was added. Radioactivity of the samples was measured as cpm in a Nuclear Chicago model Isocap/300, liquid scintillation system, and expressed as the arithmetic mean±1 SEM of triplicate samples.

Tissue factor assay. Cell sonicates were evaluated for tissue factor activity by a modification of the one-stage activated partial thromboplastin time test (APTT) described previously (6). Fresh-frozen normal human plasma (kindly supplied by Dr. Frederick Katz, Connecticut Chapter, American National Red Cross, Farmington, Conn.) was pooled from three or more donors and subjected to celite-exhaustion as described by Ratnoff (27) to deplete factors XII and XI. This material, frozen in 1-ml aliquots at −70°C, was utilized as substrate plasma to avoid the complex interaction of lipopolysaccharide directly with Hageman factor (28). Automated APTT reagent (General Diagnostics Laboratories, Morris Plains, N. J.) was used as the source of mixed brain lipids. The cell suspension (0.1 ml) was preincubated (37°C) with the substrate plasma (0.1 ml) for 2 min. Autonated APTT reagent (0.1 ml) was then added and the reagents incubated for a further 6 min at 37°C. Calcium chloride (0.025 M, 0.1 ml) was then added and the clotting time was measured. Arbitrary units of tissue factor were established as noted in Fig. 1. Rabbit brain thromboplastin (Dade Div., American Hospital Supply Corp., Miami, Fla.) was used as an external tissue factor standard to determine the slope of the normal curve. Because of the day-to-day variability of the clotting times obtained when utilizing stimulated mononuclear cells of various normal donors, an internal reference standard was used which consisted of a "maximally stimulated culture" (1.0 μg/ml of *Escherichia coli* endotoxin, 026B6, control no. 581232, Difco Laboratories; reference 25). The clotting times of serial twofold dilutions of each culture were determined automatically on a Coagulyzer (Sherwood Medical Industries, St. Louis, Mo.). Experimental results were discarded if the slope of the cell suspension curve was not parallel to that of the brain thromboplastin curve. Tissue factor units were determined utilizing the clotting time of the undiluted *E. coli*-stimulated culture as the arbitrary reference point representing 100 U/10^6 cells. Utilization of this standard, stimulated culture (Fig. 1)

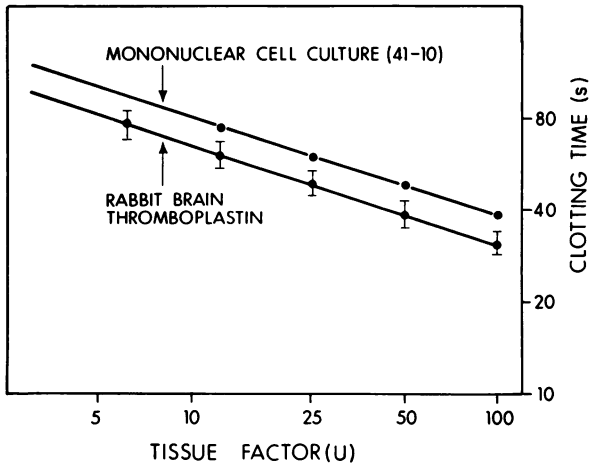

FIGURE 1 Assay system for measuring tissue factor activity. The clotting times (±1 SEM), as determined in the one-stage system (see Methods), are plotted on the vertical axis, arbitrary tissue factor units are plotted on the horizontal axis. A standard curve is established for each experiment by plotting the clotting times of twofold dilutions of rabbit brain thromboplastin (lower curve), and a clotting time of 28–32 s is arbitrarily designated as equivalent to 100 U of tissue factor. To ensure day-to-day reliability of the assay, a maximally stimulated mononuclear cell culture (1.0×10^6 cells stimulated with 1.0 μg of *E. coli* endotoxin), was diluted similarly and clotting times established in parallel. A representative culture (41-10) is depicted in the upper curve.

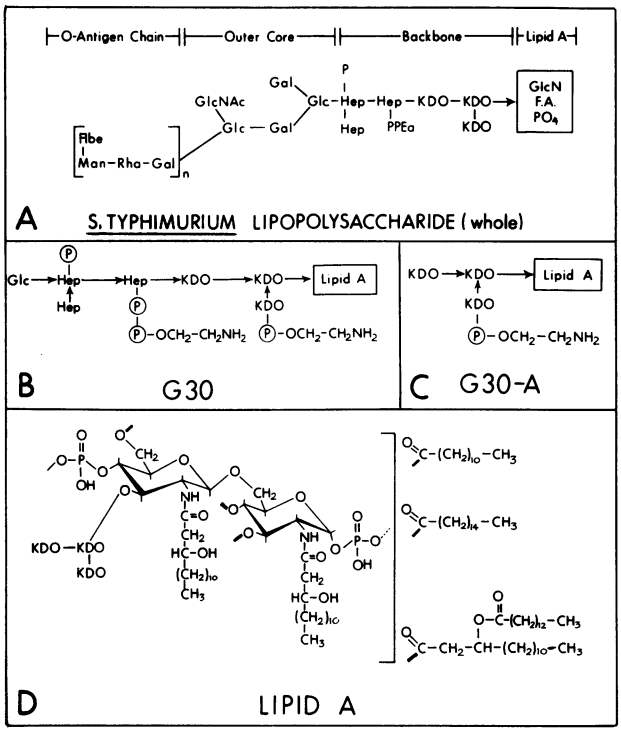

FIGURE 2 Structures of lipopolysaccharide (LPS) obtained from *S. typhimurium*. The structure of wild-type or "whole" LPS is shown in panel A. Panels B and C represent the structures of the galactose-deficient LPS obtained from strain G30 and the heptose-deficient LPS obtained from strain G30-A, respectively. Panel D shows the structure of the lipid A unit of *S. minnesota*, R595 with an attached KDO trisaccharide as proposed by Lüderitz et al. (23). The three ester-linked fatty acid residues shown at the right (from top to bottom: lauric, palmitic, and 3-myristoxymyristic acid) are linked to the hydroxyl groups of the glucosamine residues at positions 3, 4, and 6′. However, the distribution of these fatty acids among these hydroxyl groups is not known (see reference 23 for further details). It is assumed that the structure of lipid A from *S. typhimurium* is identical. Abe, abequose; Man, mannose; Rha, rhamnose; Gal, galactose; GlcNAc, *N*-acetyl glucosamine; Glc, glucose; Hep, heptose; P, phosphate; PPEa, pyrophosphoryl-ethanolamine; KDO, ketodeoxyoctonate; F.A., fatty acid.

allowed better comparison of results when different cell donors were employed from day to day. Specificity of this assay for tissue factor has been previously established (6). No other known procoagulants (e.g., specific zymogens or activated coagulation factors) are generated in this cell system, and the two-stage assay, which is specific for tissue factor, has been used previously to demonstrate that the procoagulant activity detected by the one-stage assay is indeed the result of tissue factor generation (6). In addition, procoagulant activity generated by mononuclear cells in this culture system was neutralized by a rabbit antibody to purified human placental tissue factor, which was prepared by Dr. Frances A. Pitlick (Yale University School of Medicine, New Haven, Conn.). This antibody has been characterized previously and does not inhibit any coagulation factors other than tissue factor (6).

Bacteria and media. All bacterial strains were obtained from Dr. M. J. Osborn, Department of Microbiology, University of Connecticut Health Center, Farmington, Conn. Wild-type *Salmonella typhimurium* LT-2 has been previously described (29). Strain G30 is a mutant of *S. typhimurium* LT-2 which lacks the enzyme UDP-galactose-4-epimerase (29). Strain G30-A is a mutant of G30 which produces a heptose-deficient LPS. Cultures were grown at 37°C with vigorous aeration in protease-peptone beef extract medium (30). The structure of wild-type LPS and LPS from strains G30 and G30-A, as well as that of the lipid A portion of the molecule, are shown in Fig. 2.

Lipopolysaccharides and lipopolysaccharide derivatives. Wild-type LPS was isolated from strain LT-2 and purified according to Romeo et al. (31). Rough LPS was extracted from strains G30 and G30-A and subsequently purified employing the method of Galanos et al. (32). Lipopolysaccharide from another heptose-deficient mutant, *S. minnesota* R595, was kindly supplied by Dr. David C. Morrison, Scripps Clinic and Research Foundation, LaJolla, Calif.

Lipid A was prepared by mild acid hydrolysis of G30-A LPS as described by Galanos et al. (33). These preparations were free from detectable heptose or 2-keto-3-deoxyoctulosonate (KDO) when assayed by the cysteine-H_2SO_4 (34) and thiobarbituric acid (34) procedures, respectively. Lipid A preparations were complexed to either bovine serum albumin (BSA) or human serum albumin (HSA) before their assay for tissue factor generating activity (33, 35). Briefly, lipid A (2 mg) in water (1.0 ml) was solubilized by addition of triethylamine (10 μl) followed by vigorous stirring. The resulting solution was added to 1.0 ml of a 0.25% solution of either BSA or HSA. The solutions were mixed thoroughly and evaporated to dryness in a rotary evaporator at 30°C. The resulting complex was dissolved in water (2.0 ml). The preparation of lipid A and subsequent complex formation were carried out by using pyrogen-free glassware and reagents.

The polysaccharide portion of wild-type LPS (0-antigen plus core region minus acid-labile KDO residues) was obtained by mild acid hydrolysis of strain LT-2 LPS. Briefly, 25 mg of wild-type LPS was suspended in 5.0 ml of 0.1 *N* acetic acid and incubated in a sealed tube at 100°C for 1 h. The suspension was then cooled and centrifuged at 39,000 $\times$ *g* for 30 min. The pellet was washed twice with 5 ml water and the supernatant fractions were pooled and lyophilized. The lyophilized residue was solubilized in water (5.0 ml) and applied to the bed of a Sephadex G-50 (medium) column (1 $\times$ 60 cm). The column was eluted with 0.22 *N* ammonium formate buffer (pH 2.5), and fractions were assayed for total carbohydrate employing the phenol-H_2SO_4 method (36). The peak fractions were pooled, lyophilized and solubilized in water. Alkaline treatment of lipid A was carried out as described by Galanos et al. (33).

Contamination of the chemically altered materials, reagents, or bound fractions by exogenous whole LPS was monitored by the use of the Limulus assay (kindly performed by Dr. Jack Levin, the Johns Hopkins University School of Medicine, Baltimore, Md.), using both wild-type *S. typhimurium* LPS and *E. coli* LPS (Difco Laboratories) as reference standards (25). Polymyxin B-treated LPS was prepared as described by Jacobs and Morrison (37) and utilized in tissue culture after overnight dialysis against pyrogen-free water or, polymyxin B itself (Aerosporin, Burroughs Wellcome Co., Research Triangle Park, N. C.) was added, uncomplexed, to LPS-stimulated cultures to achieve a final concentration of 10 μg/ml. All materials were handled with pyrogen-free plastic pipettes (Falcon Plastics, Oxnard, Calif.) and serial log-dilutions of LPS (10 μg/ml to 10^{-5} μg/ml) were made in pyrogen-free plastic tubes (Falcon Plastics),

using sterile, pyrogen-free tissue culture media (RPMI-1640).

RESULTS

Lipopolysaccharides isolated from R_e (G30-A) and R_c (G30) mutants of *S. typhimurium* lack all portions of the polysaccharide region distal to the KDO residues and the first glucose residue, respectively (Fig. 2 B and C). The LPS molecules isolated from these mutants proved capable of activating tissue factor in cultures of human mononuclear cells (Fig. 3). Although the dose-response curve revealed statistically significant differences in the activity of these polymers, LPS from the heptoseless mutant (G-30A) generated nearly as much tissue factor as wild-type LPS at the higher doses. These data suggest that the O-antigen and outer core regions, as well as the heptosyl portion of the backbone region, are not required for the activation of tissue factor. This conclusion was supported by the fact that the polysaccharide portion of wild-type LPS, obtained by treatment of LPS with mild acid, failed to activate tissue factor in this system (Table I). However, because of the acid lability of the KDO ketosidic linkages, the polysaccharide obtained in this manner contains only one KDO residue located at the reducing terminus.

The above findings suggested that the ability of LPS to activate tissue factor might be ascribed to the lipid A region. Thus, lipid A was prepared by mild acid treatment of G30-A LPS as described in Methods. The insoluble nature of lipid A preparations required that they be solubilized by complexing with protein carriers. Our initial experiments employed commercially available BSA for this purpose. However, all preparations of BSA tested were variably contaminated with exogenous endotoxin as detected by the Limulus assay. Therefore, lipid A was complexed to pyrogen-free HSA under sterile conditions. As shown in Fig. 4, these preparations were capable of activating mononuclear cell tissue factor. The relative decrease in potency of lipid A can probably be attributed to degradation, which most likely occurs during the acid hydrolysis procedure employed for its preparation. In contrast, mild alkaline-treated lipid A was unable to activate tissue factor when added to cell cultures (Table I). Treatment with mild alkali results primarily in the loss of the ester-lined fatty acyl substituents of lipid A, but other structural modifications may also occur. The biological activity of endotoxin preparations in a variety of systems is reduced or abolished after treatment with mild alkali (23, 38, 39).

In an attempt to alter the biological activity of LPS without modifying the covalent structure of the molecule, we examined the effects of the cyclic polypeptide antibiotic polymyxin B on this system. Polymyxin B is bactericidal for most Gram-negative bacteria, and it has been reported to prevent the lethal endotoxin activity of LPS (40–44) as well as to alter the physical structure of LPS (37, 45). The ability of either wild-type LPS or galactose-deficient LPS (G30) to activate tissue factor was unaffected by poly-

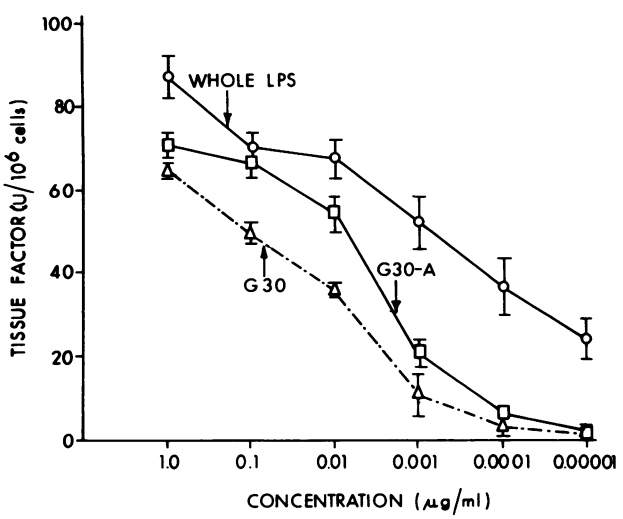

FIGURE 3 Tissue factor generation in human mononuclear cell cultures stimulated by LPS from *S. typhimurium* and its mutant strains. Tissue factor units (±1 SEM) are plotted on the vertical axis, decreasing concentrations of LPS are plotted on the horizontal axis. Open circles represent the mean tissue factor units generated in at least eight separate experiments by wild-type or "whole" LPS. Open squares and triangles represent, respectively, the mean tissue factor units generated in at least six separate experiments by the LPS from G30-A and G30 mutants.

TABLE I
Tissue Factor Activity Generated by LPS Variants

Material	Tissue factor
1 μg/ml	*U/10⁶ cells**
Lipid A‡	44.0±3.5
Polysaccharide‡	3.3±1.5
Lipid A (Alkali treated)	<1.0

* Each value represents the mean±1 SEM of at least eight experiments.

‡ Polysaccharide preparations and BSA, to which lipid A was initially complexed, were both found to be contaminated with exogenous endotoxin (0.01 μg/ml, as determined by the Limulus assay—see Methods and Results). Therefore, these values have been corrected for the amount of tissue factor generated by the concentration of exogenous endotoxin found in the BSA preparations. The polysaccharide used in these experiments was prepared as described in Methods and represents the entire structure of LPS as shown in Fig. 2 A (from left to right) up to the first KDO residue.

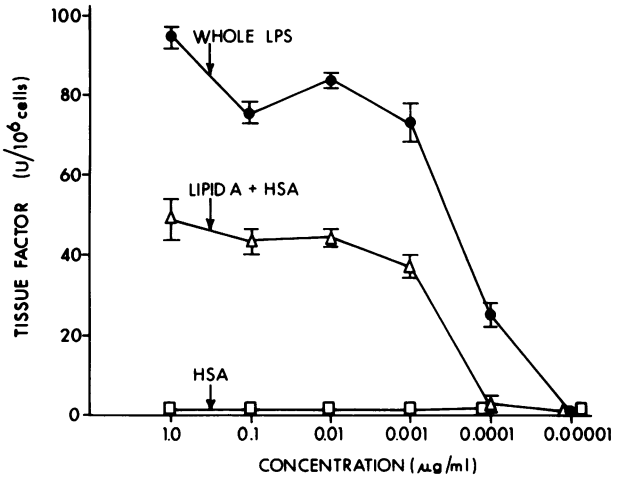

FIGURE 4 Tissue factor generation in human mononuclear cell cultures stimulated by LPS from *S. typhimurium* and lipid A. HSA was used to solubilize the lipid A (see Methods) and was free of contaminating exogenous LPS. Units of tissue factor (±1 SEM) are plotted on the vertical axis and decreasing concentrations of LPS or the carrier HSA are plotted on the horizontal axis. Each point represents the mean of at least six separate experiments.

myxin B (Fig. 5). However, activation by both heptoseless LPS (G30-A) and lipid A, although not abolished, was significantly decreased by preincubation with the antibiotic. The antibiotic alone had no effect on either the viability of the cultured cells (As determined by trypan blue exclusion) or the assay for tissue factor. At high LPS or lipid A concentrations, polymyxin B failed to completely inhibit the ability of these preparations to activate tissue factor. However, dose-response curves demonstrated that polymyxin B almost completely inhibited these preparations when they were present at lower concentrations (Fig. 6). This inhibitory effect could be demonstrated either by incubating polymyxin B with the cultured cells in the presence of the LPS preparation or by using LPS that had been pretreated with polymyxin B and from which excess antibiotic had been removed by exhaustive dialysis. Similar results were obtained when the LPS from the rough mutant *S. minnesota* R595 was substituted for the analagous LPS isolated from *S. typhimurium* G30-A.

DISCUSSION

Tissue factor is a potent procoagulant which may be important in the pathogenesis of intra- and extravascular coagulation in a variety of disorders (14–22, 46). Recent evidence has suggested that tissue factor is present in cell membranes in an inactive form which is "activated" by an unknown mechanism as a result of the interaction of the cell with various agents (3, 6). The location of tissue factor in the surface coat of endothelial cells and fibroblasts provides a ready source of procoagulant material for intravascular thrombus formation, while its presence in mononuclear cells (monocytes and lymphocytes) may explain the prominence of fibrin in many inflammatory disorders (22, 47, 48). We have suggested that antigens or antigen-antibody complexes, such as those elaborated during the renal allograft rejection reaction, can activate mononuclear cell tissue factor (7). Alternatively, other stimuli such as LPS, which has been shown to activate tissue factor, may do so secondary to binding to cell membranes (49–51). Characterization of this activation process, therefore, becomes important in order to assess the role of tissue factor in thrombosis and inflammation.

We have demonstrated previously (25) that activation of mononuclear cell tissue factor by LPS does not require DNA synthesis. In addition, we have confirmed the observation of Rivers et al. (9) that the bulk of the activity in the LPS-stimulated cultures is supplied by monocytes although we demonstrated that B-type lymphycytes are also active. T cells appear to lack the ability to generate tissue factor activity (10). In the current study we have focused our attention on the specific structural requirements for LPS activation of tissue factor. Inasmuch as LPS activation of tissue factor in a variety of cells may be important in the pathophysiology of disseminated intravascular coagulation associated with Gram-negative sepsis (17–20), further characterization of the properties of LPS necessary for this biological role seemed warranted.

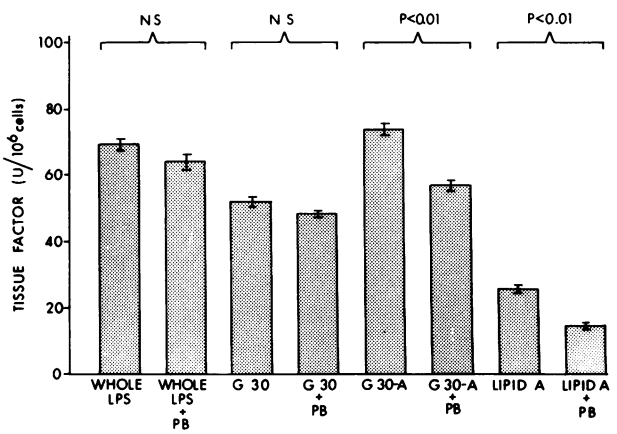

FIGURE 5 The effect of polymyxin B on tissue factor generation in human mononuclear cell cultures stimulated by LPS from *S. typhimurium*, mutant strains, and lipid A. Tissue factor units ±1 SEM are plotted on the vertical axis. PB, polymyxin B (10 μg/ml); G30, LPS from the galactose-deficient mutant (Fig. 2 B); G30A, LPS from the heptoseless mutant (Fig. 2 C); lipid A, LPS after acid hydrolysis (see Methods and Fig. 2 D). All preparations of LPS were tested at 1.0 μg/ml. A minimum of 15 paired experiments are represented by each set of bars (with and without PB) and a two-tailed student's *t* test was used to determine the significance of the differences between pairs. NS, not significant ($P > 0.05$).

The evidence presented here clearly indicates that the lipid A region of Gram-negative LPS is the biologically active portion of the molecule required for the generation of tissue factor activity by human mononuclear cells. In support of this conclusion, we found that LPS molecules lacking the O-antigen and much of the outer core and backbone regions (Fig. 2) were still potent in their ability to activate tissue factor. Furthermore, the polysaccharide region of wild-type LPS, purified from mile acid hydrolysates of LPS, was found to be inactive in this system. In contrast, lipid A preparations uncontaminated by exogenous LPS remained capable of activating tissue factor. The decrease in potency of lipid A relative to wild-type LPS may be the result of degradation which probably occurs during the mild acid hydrolysis procedure employed for preparation of this material.

Treatment of lipid A with mild alkali abolishes its ability to activate tissue factor. Similarly, a variety of other biological activities attributed to lipid A or endotoxin preparations have been reported to be abolished by alkaline treatment (23, 33, 38, 39). Although mild alkaline treatment is believed to result primarily in the loss of the O-fatty acyl substituents of lipid A (23), it is recognized that other structural modifications may also occur. For example, it has been suggested that alkaline hydrolysis might result in the cleavage of pyrophosphoryl bridges, thereby destroying the proposed polymeric nature of the lipid A backbone (52). In addition, the conversion of lipid A to a more hydrophilic molecule by alkali may result in the alteration of physical properties of this glycolipid which are important for its biological activity. However, the specific structural or physiochemical properties of lipid A required for biological activity are not known.

Recent studies by Nowotny et al. (53) indicated that the polysaccharide fraction obtained by mild acid hydrolysis of *S. minnesota* LPS is active in the stimulation of mouse bone marrow cell colony formation and in the protection of mice against lethal irradiation. Lipid A preparations were found by these investigators to be either much less active or completely inactive in the above two assays. In addition, Morrison et al. (54) have reported that the polysaccharide portion of LPS plays an important role in the activation of the alternative pathway of complement. However, to our knowledge, these reports are the first to attribute in vivo biological activity to portions of the LPS molecule other than the lipid A region.

Similar dissociations of the properties of LPS have been demonstrated after interaction with the cyclic peptide antibiotic, polymyxin B, in vitro (37, 40–45). Polymyxin B-treated LPS, though no longer mitogenic (37) nor anticomplementary (45), still retains its ability to generate an immune response when complexed with a hapten (37). As demonstrated in Fig. 5, polymyxin B-treated whole LPS and G30 were still capable of activating tissue factor, but tissue factor generation in response to LPS obtained from the heptoseless mutant (G30-A) and lipid A was significantly reduced by polymyxin B, particularly when these LPS fractions were used at lower concentrations (Fig. 6). Essentially complete inhibition of tissue factor activation was made possible when polymyxin B was added to lower concentrations of LPS or lipid A and subsequently reacted with the cells (Fig. 6).

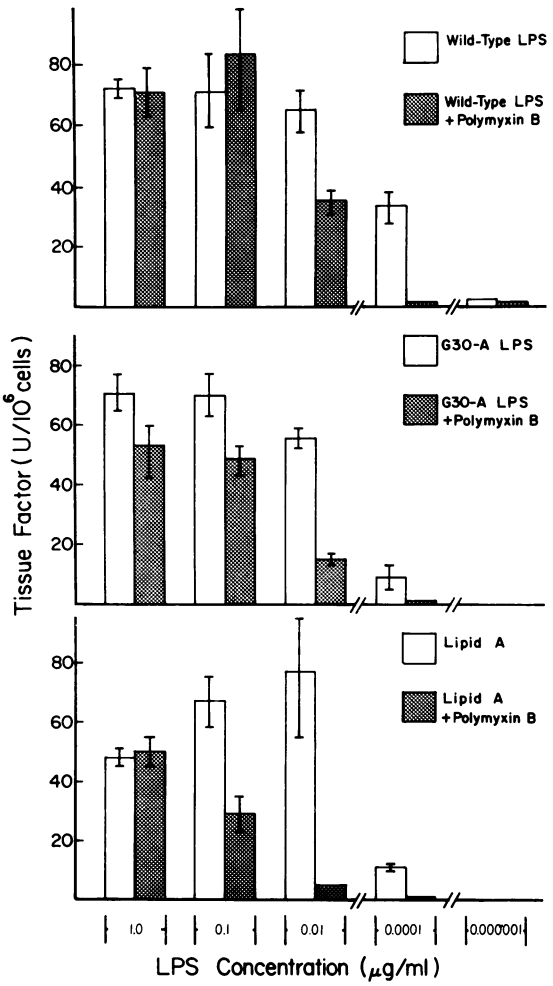

FIGURE 6 The effect of polymyxin B on tissue factor generation in human mononuclear cell cultures stimulated by decreasing concentrations of LPS from *S. typhimurium*, mutant strains, and lipid A. Tissue factor units ±1 SEM are plotted on the vertical axis. Concentrations of LPS or lipid A are plotted on the horizontal axis. PB, polymyxin B (10 μg/ml); wild-type LPS refers to the whole LPS as in Fig. 2 A, whereas G30-A and lipid A are as depicted in Figs. 2C and D. Each set of bars represents a minimum of four paired experiments. Inhibition of tissue factor generation by polymyxin B was as follows: (*a*) wild-type (whole) LPS—45% at 0.01 μg/ml and 100% at 0.0001 μg/ml and 0.000001 μg/ml; (*b*) G30-A—28% at 1.0 μg/ml, 30% at 0.1 μg/ml, 73% at 0.01 μg/ml, and 100% at 0.0001 μg/ml; (*c*) lipid A—57% at 0.1 μg/ml, 93% at 0.01 μg/ml, and 100% at 0.0001 μg/ml.

Although our results with LPS preparations are compatible with the suggestion by Morrison and Jacobs (45) that LPS and polymyxin B form a stable molecular complex via binding to the lipid A-2-keto-3-deoxy-octulosonate region of LPS, the complete absence of KDO residues in our preparations of lipid A suggests that KDO is not necessary for the binding of polymyxin B to LPS. Recent evidence published by Morrison and his co-workers (55) and Sultzer and Goodman (56) suggests that LPS preparations are contaminated to varying degrees with a low molecular weight protein which prevents the binding of polymyxin B to the lipid A region. Hot phenol extraction appears to remove the putative protein and renders the resultant LPS susceptible to polymyxin B action. It is of interest that both groups of investigators have found the contaminant to be itself mitogenic for spleen cells from the C_3H/HeJ strain of mice, whereas the resultant LPS after extraction is nonmitogenic, thus explaining disparate results from several laboratories using this genetically resistant strain of mice. It seems possible, therefore, that the contaminating protein, which copurifies with LPS, may be responsible for variable inhibition of LPS preparations by polymyxin B. Polymyxin B has been shown to prevent the development of the generalized Shwartzman reaction in rabbits and the subsequent development of disseminated intravascular coagulation (42, 43). Niemetz and others (17–19) have suggested that leukocyte tissue factor plays a central role in the mediation of this lesion, and it is conceivable, therefore, that the inhibitory activity of polymyxin B demonstrated in vitro in our experiments accounts for some of the protective effect of the antibiotic in vivo (43).

The understanding of the activation of tissue factor on the surface of a variety of cells, therefore, may be enhanced by further evaluation of the interaction of the lipid A moiety of LPS with mononuclear cell membranes. Experiments are also in progress to evaluate the role of the LPS-related protein (55–57) in the activation of tissue factor.

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