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Ability of Gonococcal and Meningococcal Lipooligosaccharides To Clot *Limulus* Amebocyte Lysate

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We investigated whether the striking difference in severity of coagulopathy observed between bacterial sepsis involving *Neisseria meningitidis* and *Neisseria gonorrhoeae* species is related to species-dependent abilities to directly activate coagulation. Using lipooligosaccharide (LOS)-activated gelation of *Limulus* amebocyte lysate, we compared the relative abilities of outer membrane LOS of 10 *N. meningitidis* and 10 *N. gonorrhoeae* strains to initiate coagulation. A wide range of procoagulant potencies was observed for each species, and there was significant overlap of potencies between species. Relative biological activities did not correlate with the oligosaccharide components as defined by LOS molecular weight or specific antigenic epitopes. Purified lipid A of two LOS strains of different potency demonstrated relative procoagulant biological activities similar to those of their parent LOSs. When these lipid A preparations were further separated by thin-layer chromatography, the most polar component of each lipid A possessed the majority of the procoagulant activity. We conclude that the ability of neisserial LOS to initiate coagulation of *Limulus* lysate is a property of the lipid A portion of the molecule and is most likely determined by fine structural differences in the lipid A which are independent of species.

Neisserial organisms are major human pathogens, and much of the pathogenicity of both gonococcal (GC) and meningococcal (MGC) strains is attributable to their surface lipooligosaccharides (LOS) that structurally resemble and antigenically mimic human cell surface glycoproteins (20–22). GC LOS has been demonstrated to result in toxic effects on fallopian tube epithelium (5), possibly because of the lipid A portion of LOS (11), and antibody to LOS contributes significantly to effective serum bactericidal activity against both species (12, 13, 32, 39). MGC LOS have been implicated as neurovirulence factors (29), and positive correlations have been demonstrated between LOS liberation from cultured organisms and both invasiveness of nasopharyngeal organisms and severity of disseminated disease (1). MGC purpura also has been associated with LOS potency (6), and initiation of coagulation is commonly observed in the setting of endotoxemia in the absence of detectable bacteremia. MGC septicemia and endotoxemia are typically associated with a marked increase in concentration of several serum lymphokines (tumor necrosis factor alpha, interleukin-1, and interleukin-6) (40), and MGC LOS can trigger the rapid release of these lymphokines in in vivo experimental animal models (41). Since endotoxins play an important role in diseases caused by both neisseriae, it is curious that MGC septicemia commonly results in a fulminant endotoxemic syndrome of cardiovascular shock, disseminated intravascular coagulation, and multiple organ failure (3), whereas GC septicemia rarely is associated with shock. Additionally, organ involvement in disseminated GC disease, with the exception of arthritis, is typically of little clinical consequence.

Enteric endotoxins are known to vary widely in their procoagulant potencies (16, 42). Similar information for neisserial organisms would be potentially informative since the difference in the frequency with which MGC and GC septicemias activate coagulation and result in clinically significant disseminated intravascular coagulation is particularly striking. In order to investigate this major clinical difference, we compared the ability of MGC and GC LOS to activate coagulation in vitro. The abilities of purified LOS to initiate clotting of *Limulus* amebocyte lysate (LAL) (the basis for the LAL test) was used as a measure of their procoagulant biological potency. Endotoxins from neisserial species are readily detectable with the LAL test in clinical samples of cervical and urethral discharge (GC) (27, 34) and cerebrospinal fluid (MGC) (31), thus establishing that LAL activation is a feature of both species.

MATERIALS AND METHODS

Reagents. The reference endotoxin used for calculation of LOS endotoxin units per milliliter was *Escherichia coli* lipopolysaccharide (LPS) B, 055:B5 (Difco Laboratories, Detroit, Mich.). Sterile, endotoxin-free water and saline solutions were purchased from Travenol Labs (Deerfield, Ill.). All other chemicals used were reagent grade.

Glassware. All glassware was rendered endotoxin-free by being autoclaved for 45 min and then heated at 190°C in a dry oven for 4 h.

Extraction and purification of LOS and lipid A. The LOS used in this study have been characterized previously and are as follows: MGC 6155, MGC 6275, MGC 35E, MGC M981, MGC 126E, MGC 89I, MGC M978 (25); GC PID2 (2); GC F62, GC JW31R, GC 220, GC 302, GC DOV, GC 56 (20); MGC 7880, MGC 7889 (14); GC 15253 (22); GC 1291 (8); MGC 8032 (17); GC FA5100 (33). LOS were extracted by hot phenol-water (43), and LOS was obtained by centrifugation. Lipid A was prepared from purified LOS by 1% acetic acid hydrolysis at 95°C (10) for 2 h and then by three aqueous extractions. Lipid A was then extracted from the aqueous pellet with chloroform-methanol-water-ammonium hydroxide (50:25:4:2 [vol/vol]) (36).

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TLC analysis. Analysis of purified lipid A was performed on thin-layer plates (Silica gel G60; E. Merck AG, Darmstadt, Germany) with a solvent system of chloroform-methanol-water-ammonium hydroxide (50:25:4:2 [vol/vol]). Plates were sprayed with 5% sulfuric acid in methanol and then heated for the detection of lipid A components. For estimation of relative amounts of lipid A components in the thin-layer chromatography (TLC) spots, relative areas of the charred components were determined by image analysis (Microcomputer Imaging Device; Imaging Research, Inc., St. Catherines, Ontario, Canada). Phospholipids were identified by a molybdenum blue spray reagent for phosphate esters (7). For isolation of lipid A components, the components were visualized with iodine vapor. Spots were scraped off the glass plate and extracted with the chromatography solvent, and the solvent was evaporated under nitrogen.

Monoclonal antibodies. Monoclonal antibodies 1-1-M, 3F11, and 6B4 were provided by Michael Apicella (State University of New York, Buffalo). Monoclonal antibodies D6A and 1-9C4 were provided by Jan Poolman (Bilthoven, The Netherlands), and monoclonal antibody 2-1-L8 was provided by Wendell Zollinger (Walter Reed Army Institute of Research, Washington, D.C.).

LAL. Amebocyte lysates were prepared from *Limulus polyphemus* (the horseshoe crab) by disruption of washed amebocytes in distilled water, as described previously (19). LALs were stored at 4°C.

Turbidimetric LAL test. Potency of each LOS was determined with the LAL test. LOS were suspended in endotoxin-free water by vortexing for 2 min, and serial 10-fold dilutions were prepared. A 7-log range of LOS concentrations (10 fg/ml to 100 ng/ml) was assayed for each sample. A total of 50 μl of LOS was added to 50 μl of LAL, and the mixture then was incubated for 4 h at 37°C (19), with visual inspections at 15-min intervals (first hour) and then at 30-min intervals. Samples were graded for flocculation, increased viscosity, and gelation, as previously described (18). For determination of relative potency of LOS, the least reactive LOS was defined as having a potency of 1. All other LOS were graded by comparison of their rates of gelation (at each of the concentrations prepared above) to the gelation rates of the reference (least reactive) LOS. Thus, if the gelation rate of a given LOS at a concentration of 10 fg/ml was identical to that of the reference LOS at 100 fg/ml, the given LOS demonstrated a relative potency of 10. Purified lipid A from two neisserial strains (MGC 6275 and GC 15253) was assayed for relative potency by comparison of the minimum time and concentration required to produce a gel.

Chromogenic LAL test. Lipid A components of MGC 6275, GC 15253, and GC F62 were obtained after TLC (described above), suspended in 1 ml of water with vigorous vortexing for 2 min, and then assayed for relative potency by using the chromogenic LAL test. Each lipid A component (0.05 ml) was incubated with 0.05 ml of LAL in borosilicate tubes (10 by 75 mm; VWR Scientific, San Francisco, Calif.) for 30 min in a 37°C water bath. Chromogenic substrate S-2423 (0.2 ml; Kabi Vitrum, Molndahl, Sweden) (0.25 mM stock solution in 20 mM sodium phosphate buffer, pH 7.6) was then added to each assay tube, mixed well by vortexing, and incubated for an additional 10 min at 37°C. Reactions were stopped by addition of 0.15 ml of 50% acetic acid, and A_{405} was measured in a Gilford model 2400-2 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). A standard curve was generated from a 6-log range of concentrations by using whole GC 15253 lipid A. Relative glycolipid concentrations were calculated for each extracted lipid A component by extrapolation from the standard curve. These relative glycolipid concentrations were expressed as relative potencies by comparison with the amount of background endotoxin activity, defined as a relative potency of 1, which was extracted from a blank section of the TLC plate. Background activity from the plate was the equivalent of approximately 100 ng of GC 15253 lipid A per ml.

TABLE 1. Relative potencies of LOS[a]

LOS[b]	Minimum endotoxin concn (ng) producing a gel	Gelation time (min)
MGC 6155	1	30
MGC 7889	1	30
MGC 6275	1	30
GC 1291	1	30
MGC 35E	1	30
GC F62	1	30
MGC 126E	1	60
MGC M981	1	60
GC JW31R	1	60
GC PID2	1	60
MGC 8032	1	90
MGC 7880	1	90
MGC 89I	1	90
GC 220	1	90
GC FA5100	1	90
GC 302	1	120
GC 15253	1	120
GC DOV	1	120
GC 56	1	>180
MGC M978	100	60[c]

[a] Relative potencies of LOS were established by determination of the minimum concentration of each LOS required to produce a gel and the length of time required to produce the gel, by using the LAL test.

[b] LOS are grouped on the basis of the gelation time produced by the concentration of endotoxin indicated. Further rank ordering of LOS (as they appear here and in Fig. 1) for relative potency was based upon the rate of progression of each sample through pregelation changes (flocculation and increased viscosity), as previously described (18).

[c] No gelation produced by 1 ng/ml.

RESULTS

Procoagulant potencies of LOS. A total of 10 LOS from MGC isolates and 10 LOS from GC isolates were tested for LAL potency. These LOS possessed carbohydrate chains of greatly differing structure, as shown by molecular weight analysis and their abilities to bind several monoclonal antibodies to the oligosaccharide moieties. Of 20 LOS, 19 produced a solid gel at a minimum concentration of 1 ng/ml, with rates of gelation varying from <30 min to >3 h (Table 1). The least potent LOS (M978) required 100 ng/ml to produce a solid gel and was thus designated the reference LOS and assigned a potency of 1. Six different potency groups were defined according to gelation time and minimum LOS concentration that produced a gel. When the remaining 19 LOS were compared for rate of gelation at multiple concentrations (over a 7-log range of concentration), their relative potencies varied from 2 to 500 times that of the reference LOS, M978 (Fig. 1). Both MGC and GC LOSs covered the full range of potencies (Fig. 1). Although the group of MGC species contained many more potent LOS than the GC isolates, the distributions of potencies between

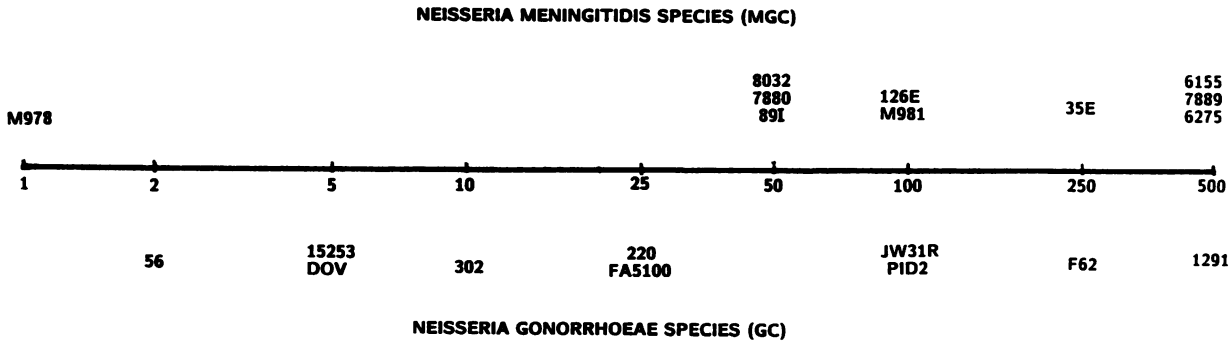

FIG. 1. Relative abilities of 20 MGC and GC LOS to clot LAL were determined (Table 1); the LOS demonstrating the least biological activity (M978) was assigned a relative potency of 1. Relative potencies of the other LOS were derived from their rates of gelation of amebocyte lysate, as described in Materials and Methods. The MGC and GC LOS tested included a diverse selection of oligosaccharide chain length, chemical composition, and antigenic characteristics. The range of relative potencies was similar for both species. Distributions of potencies between the two species demonstrated that the populations were statistically indistinguishable (Mann-Whitney U test).

the two groups were not statistically distinguishable (Mann-Whitney U test).

Relative potencies of the 20 LOS did not correlate with LOS molecular weight. Potency also did not correlate with antigenic reactivities of the LOS, as determined by using six monoclonal antibodies with specificities for a variety of chemically distinct oligosaccharide structures (44–46) (Table 2). We were thus unable to correlate LOS LAL potency with any general characteristics of the carbohydrate moieties of individual LOS. Importantly, appreciable *Limulus* potency was demonstrated for GC FA5100 (relative potency of 25), an LOS containing only lipid A and a 2-keto-3-deoxyoctulosonic acid [$(dOclA)_2$] heptose core oligosaccharide without additional oligosaccharide elongation (33), suggesting that the oligosaccharide portion of LOS may not contribute appreciably to activation of coagulation by *Neisseria* spp.

TABLE 2. LOS potencies and O chain carbohydrate structures[a]

LOS	*Limulus* activity[b]	Identification by following monoclonal antibody (M_r [10^3] of band identified):					
		1-1-M (5.4)	6B4 (4.8)	3F11 (4.8)	D6A (3.6)	2-1-L8 (3.6)	1-9C4 (3.6)
MGC 6155	500	−	+	+	−	+	+
MGC 7889	500	−	−	−	+	+	−
MGC 6275	500	−	+	−	+	+	+
GC 1291	500	+/−	+	+	+	−	+
MGC 35E	250	−	+	−	−	−	−
GC F62	250	+	+	+	+/−	−	+/−
MGC 126E	100	−	−	−	+	+	−
MGC M981	100	−	+/−	+/−	−	−	−
GC JW31R	100	−	−	−	−	−	−
GC PID2	100	+	+	+	−	−	−
MGC 8032	50	−	+	+	−	−	−
MGC 7880	50	−	−	−	+	+	−
MGC 891	50	−	+	−	−	−	−
GC 220	25	−	+	+	+	+	+
GC FA5100	25	−	−	−	−	−	+/−
GC 302	10	−	+	+	+	+	−
GC 15253	5	−	−	−	+	+	−
GC DOV	5	+	+	+	−	−	−
GC 56	2	+	+	+	−	−	−
MGC M978	1	−	+	−	+	+	+

[a] O chain antigenic structures are identified by a series of monoclonal antibodies.

[b] *Limulus* activity is expressed as relative potency of each LOS, as shown in Fig. 1.

Procoagulant potencies of lipid A. Since the differences we observed in LAL test potency could not be attributed to the oligosaccharide moieties, we compared the *Limulus* reactivities of purified lipid A prepared from an LOS of low potency (GC 15253) and lipid A from an LOS of high potency (MGC 6275). GC 15253 is an isolate from a disseminated GC infection, and MGC 6275 is an isolate from an epidemic disseminated MGC infection. GC 15253 lipid A required 300 ng/ml to produce a gel, whereas MGC 6275 lipid A produced a gel at a minimum concentration of 3 ng/ml. Although both lipid A preparations demonstrated less potency than their intact LOS molecules, the relative potencies of these two lipid A preparations matched the relative potencies of their respective LOS. We compared these lipid A's by TLC in an attempt to identify a candidate lipid A component that could potentially account for the observed potency difference. Three major components were seen in both lipid A preparations, while minor GC 15253 components with R_fs of 0.27 and 0.38 which were not present in MGC 6275 were observed (Fig. 2). All of the TLC spots were examined for ability to activate coagulation with the chromogenic LAL test. Total recovered biological activity (as determined both by the sum of individual spot activities and by the activity of a mixture of all the extracted spots) was approximately 25% of that applied. For both species, half or more of the biological activity was observed in the most polar of the major lipid A components (each demonstrated an R_f of 0.20) (Fig. 2). These lipid A components with the majority of biological activity contained phosphoryl groups, whereas phosphate was not detected in the more hydrophobic components. In a comparison of the estimated quantity of glycolipid in each of the polar (R_f = 0.20) spots of Fig. 2 with their biological activities, the relative procoagulant potencies of the two components were similar (the polar component of MGC 6275 had 1.4-fold the potency of GC 15253 on a weight basis). In contrast, the most nonpolar components (R_f = 0.45) of the two species had vastly different potencies. The nonpolar MGC 6275 component had approximately 39-fold greater biological activity than the similarly migrating component of GC 15253, a relationship similar to that observed for their whole lipid A mixtures and also for the intact LOS. The two minor lipid A components of GC 15253

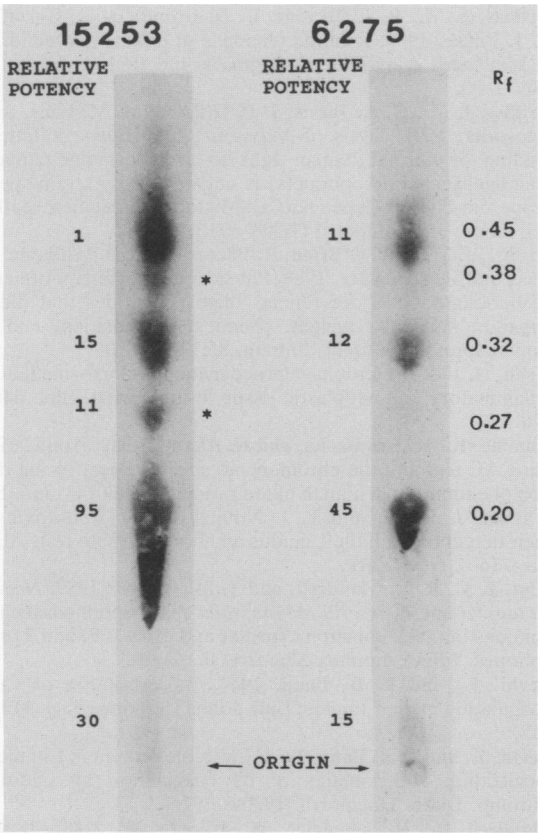

FIG. 2. TLC analysis of lipid A from GC 15253 and from MGC 6275. Minor components detected in GC 15253 but not in MGC 6275 (*) have R_f values of 0.27 and 0.38. Relative potency in the LAL test of each lipid A component was determined by extracting spots which were detected by iodine vapor from other lanes of the thin-layer chromatograph (see Materials and Methods). Relative potencies for the major components of each lipid A and for the material remaining at the origin are indicated. The majority of the procoagulant potency for each lipid A was associated with the prominent spot at $R_f = 0.20$.

that were absent in MGC 6275 did not demonstrate any appreciable biological activity.

Structural compositional data have been reported for the lipid A components of GC F62 (36); therefore, we also examined these components for procoagulant biological activity. TLC analysis showed four major components, which is similar to the pattern seen with GC 15253. Also similar to the data obtained with GC 15253 was the fact that the majority of the biological activity (90% of the recovered activity in the chromogenic LAL test) resided in the polar lipid A component (data not shown). This component has been shown previously (36) to migrate on TLC as diphosphoryl lipid A.

DISCUSSION

Bacterial endotoxin is capable of initiating both the extrinsic pathway of blood coagulation, via cellular mechanisms (e.g., induction of macrophage tissue factor) (15), and the intrinsic pathway, via direct activation of factor XII (26). The clinical observation that coagulopathy is frequent, and often severe, in MGC disease yet absent or subclinical in GC disease might reflect a species-specific difference in procoagulant potency of the neisserial endotoxins. Therefore, our study was developed to examine whether LOS from MGC and GC species differ in their abilities to directly activate coagulation. We used activation of the sensitive model system LAL to measure direct procoagulant potency of LOS. Activation of LAL is a good marker for MGC septicemia and purpura fulminans (40). Our findings demonstrated that neisserial LOS exhibit a wide range (500-fold) of procoagulant potencies. LAL activation was not species specific. Furthermore, when MGC and GC isolates were selected such that they had similar oligosaccharide chain lengths and antigenic structures, their relative potencies varied. Our findings thus suggested that LAL test potency is not related to the oligosaccharide components but instead may be related to the constituents of lipid A.

Since neisserial lipid A preparations are heterogeneous (as demonstrated by TLC), we determined LAL test potency for each of the major lipid A components. The majority of the procoagulant activity for both species was identified after TLC analysis to be within the most polar component. This component, though not yet examined for total chemical composition, was shown to contain a phosphoryl group(s). Our results thus suggest that fine chemical or structural features within the most polar lipid A components of neisserial LOS may be responsible for their relative procoagulant potencies. However, it is interesting that, on an equal weight basis, the most nonpolar component of MGC 6275 lipid A was 39 times more potent than the corresponding component of GC 15253 lipid A. It is possible that the nonpolar components of lipid A may have a significant role in LOS potency, but that they require the presence of the polar components in order to maximally interact with LAL in aqueous solution. Endotoxins generally have poor aqueous solubility, and it is possible that nonpolar components of high potency have limited ability to interact with the *Limulus* coagulation cascade without the presence of the more polar components to improve their bioavailability.

Compositional data for neisserial LOS (23, 24, 32, 36) have demonstrated considerable differences among strains for phosphoryl content and fatty acid heterogeneity. Detailed structural information on neisserial lipid A's (9, 36) is limited, however, and structure/function studies have not been reported. In contrast, there is a substantial literature on structure/function relationships of native lipid A from enteric (*E. coli* and *Salmonella* species) organisms as well as for well-defined synthetic lipid A's. For enteric endotoxin-induced LAL coagulation, evidence exists that both the disaccharide backbone of lipid A and the monosaccharide backbones of lipid A precursors lipid X and lipid Y can activate LAL (28, 37, 38). Maximal LAL activation requires both ester-linked fatty acids (35, 37) and branched acyloxyacyl fatty acids (9, 35). Finally, LAL activation by enteric LPS also requires at least one phosphoryl group (28, 35, 37, 38); the phosphoryl group has similarly been proposed to be critical for the direct activation of factor XII (Hageman factor) by LPS (26). This requirement for the phosphoryl group is consistent with our finding that phosphorylated, polar components of lipid A demonstrated the greatest relative biological potency in the activation of LAL. Interestingly, structural requirements of enteric glycolipids for other biological activities (e.g., pyrogenicity, complement activation, and chicken embryo lethality) (35, 37) are not necessarily the same as those for LAL activation.

Thus, the importance of both fatty acyl and phosphoryl groups of lipid A support the concept that lipid A procoagulant activity in vitro is related to the amphipathic nature of

the molecule. The amphipathic components of lipid A may contribute a critical catalytic structure(s); alternatively, procoagulant activity may be related to the effect of lipid A on glycolipid solubility or the physical state of LOS in aqueous solution (i.e., aggregation state). For enteric LPS, the amphipathic nature of lipid A, and in particular the highly ordered hydrophobic fatty acyl region, has been implicated for critical activities of LPS within the bacterial membrane (e.g., permeability barrier, cell shape, membrane fluidity, and expression of protein receptor activity) (30). In vivo, fine structural differences between LOS may affect LOS biological reactivity or may affect other variables such as their bioavailability or rates and mechanisms of LOS clearance. Differences in shedding of LOS from organisms in vivo, similar to differences in vitro of neisserial endotoxin liberation (4), may also be a factor in the differential pathogenicity of the neisserial species. LOS bioreactivity and bioavailability, as well as different bacterial growth rates, may all contribute to the major clinical differences in severity of coagulopathy observed between GC and MGC endotoxemias.

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