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Specific Detection of *Haemophilus influenzae* Type b Lipooligosaccharide by Immunoassay

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Three monoclonal antibody (MAb)-based immunoassays were developed for specific detection of *Haemophilus influenzae* type b (Hib) lipooligosaccharide (LOS). (i) Hib LOS was captured onto microtiter plates by polyclonal Hib-directed antibodies and detected with MAbs to the oligosaccharide component of Hib LOS in an enzyme-linked immunosorbent assay. (ii) The high affinity of polymyxin B for lipid A was used to bind Hib LOS to microtiter wells, and the oligosaccharide-specific MAbs were used as the detection system in the polymyxin B-MAb assay. (iii) Hib LOS solubilized in detergent was captured by MAbs, and the immobilized LOS was detected with a chromogenic *Limulus* amebocyte lysate method in the immunolimulus assay. Endotoxin concentrations were measured in in vitro samples and cerebrospinal fluid samples from rabbits with experimental Hib meningitis. The results were compared with those obtained with the standard chromogenic *Limulus* amebocyte lysate assay. There were significant correlations between the results of all four assays. These new immunoassays provide methods for specific detection of Hib LOS in laboratory fluids and in research involving quantification of Hib endotoxin in experimental animal models.

The *Limulus* amebocyte lysate (LAL) assay is the classic method for detection of endotoxin in biological fluids (21). Endotoxin activates a protease system in LAL, an extract prepared from blood cells of the horseshoe crab *Limulus polyphemus*. The activated enzyme can initiate visible gel formation and also cleaves chromogenic *p*-nitroanilide substrates. This latter property of LAL was used in the development of a new chromogenic LAL (CLAL) assay (32). The usefulness of *Limulus* methods is, however, somewhat limited by nonspecific activators and inhibitors of LAL (3, 7, 33). The LAL assay also does not allow discrimination of different types of endotoxin, a fact that makes this assay vulnerable to trace amounts of contamination involving endotoxins from other bacteria. Furthermore, endotoxins from different bacterial species exhibit marked differences in their relative abilities to activate LAL (11).

Haemophilus influenzae type b (Hib) is the major cause of bacterial meningitis in the United States (2). The lipopolysaccharide (LPS) of Hib is located in the outer membrane of the bacterium but differs from the classic LPS molecule in that it lacks an O-antigen repeat unit and consists only of lipid A and a structure equivalent to the core oligosaccharide of enteric LPS molecules (10). Therefore, this endotoxin also has been termed lipooligosaccharide (LOS) (16). Recently, it has been shown that Hib LOS produces meningeal inflammation in experimental animals (35), and other findings suggest that LOS is actively involved in the ability of Hib to produce invasive disease in animal models (20, 45).

Several monoclonal antibodies (MAbs) to epitopes in the oligosaccharide portion of the Hib LOS molecule have been produced and characterized in our laboratory. Some of these LOS epitopes are highly conserved among Hib strains (14), a finding which suggested that these MAbs can be incorporated into immunoassays capable of detecting the LOS of any Hib strain. In this study, we combined the high sensitivity of the CLAL assay with the specificity of the LOS-specific MAbs to develop a new immunolimulus (IML) assay for specific detection of Hib LOS. The specificity and sensitivity of this new assay were compared with those of the CLAL method, an enzyme-linked immunosorbent assay (ELISA) involving both MAbs and polyclonal antibodies, and the recently developed polymyxin B sulfate (PMB)-MAb assay (27).

MATERIALS AND METHODS

Bacterial strains and endotoxins. Hib DL26 and DL42 have been described previously (14). Hib DL26 is the prototypic LOS antigenic group 1 strain, and DL42 is representative of LOS antigenic group 2 strains (14). For LOS immunoassay purposes and for intracisternal injection in the rabbit meningitis model, Hib strains were grown in brain heart infusion broth supplemented with Levinthal base as described earlier (15). Outer membrane vesicles (OMV) were prepared from Hib DL26 and DL42 by the lithium chloride-based extraction method of McDade and Johnston (26) as modified by Gulig et al. (13).

Solubilization of LOS. Different detergent systems were tested for the ability to solubilize Hib LOS from OMV. OMV of Hib DL42 were suspended to a final concentration of 0.1 mg of protein per ml in 100 mM Tris hydrochloride (pH 7.8) containing: (i) 0.1 M octyl-β-D-glucopyranoside, (ii) 0.2% (wt/vol) sodium deoxycholate, or (iii) 150 mM NaCl–10 mM EDTA–1% (vol/vol) Triton X-100–0.1% (wt/vol) sodium dodecyl sulfate–0.2% (wt/vol) sodium deoxycholate (triple detergent). OMV samples were incubated for 1 h at 37°C with the detergents, and then a 100-μl volume of each OMV-detergent solution was centrifuged at 150,000 × *g* for 1 h at room temperature. The resultant supernatants and pellets were diluted in digestion buffer and heated at 100°C for 3 min before being subjected to sodium dodecyl sulfate-polyacry-

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lamide gel electrophoresis (20). Western blot analysis was then performed with MAb 4C4 as described previously (20).

Purified LOS and LPS. LOS was purified from strains DL26 and DL42 by the modified hot phenol-water method (19, 20, 43). Lyophilized Hib LOS was dissolved in pyrogen-free (pf) saline at a final concentration of 1 μg (dry weight) per ml and stored in multiple portions at −70°C. These portions were thawed immediately before use as standards in the different assays. LPS purified from *Escherichia coli* O111:B4 (L-33012) was obtained from Sigma Chemical Co., St. Louis, Mo., as were LPS preparations from *E. coli* O127:B8 (Sigma L-3137), *Klebsiella pneumoniae* (Sigma L-1770), and *Pseudomonas aeruginosa* (Sigma L-8643).

Antibodies. The two immunoglobulin G3 (IgG3) murine MAbs used in this study have been described previously (14). The MAb designated 4C4 is directed against an epitope in the oligosaccharide region of Hib DL42 LOS, whereas MAb 12D9 is directed against an oligosaccharide epitope in DL26 LOS. MAbs were purified from hybridoma culture supernatant fluid by affinity chromatography on protein A-Sepharose (Pharmacia, Piscataway, N.J.) by the method of Ey et al. (8).

Polyclonal antibodies to the LOS of Hib DL26 and DL42 were prepared by immunization of New Zealand White rabbits with OMV prepared from these two bacterial strains. For primary immunization with each OMV preparation, four rabbits were immunized by subcutaneous injection of 100 μg (protein content) of purified OMV suspended in 1 ml of complete Freund adjuvant (Difco Laboratories, Detroit, Mich.). The rabbits received a booster dose of 50 μg (protein) of OMV in 1 ml of incomplete Freund adjuvant (Difco) 1 month later and were then bled twice monthly. A second booster immunization was given 5 months later, and the rabbits were sacrificed 9 months after the primary immunization. The sera were stored in multiple portions at −70°C until pooled. Each antiserum had a titer of 5×10^{-6} when tested by ELISA with purified Hib LOS as the antigen as described below. These polyclonal antisera were further characterized by Western immunoblot analysis with Hib whole-cell lysates as the antigen as previously described (20). Other antibodies used in this study were alkaline phosphatase-conjugated goat anti-mouse IgG (Organon Teknika, West Chester, Pa.), affinity-purified goat anti-mouse IgG (a gift from Ellen Vitetta of this institution), and alkaline phosphatase-conjugated rabbit anti-goat IgG (Organon Teknika).

Other reagents. Sterile polystyrene ELISA plates with flat-bottom wells (Corning Laboratory Sciences Co., Houston, Tex.) were used throughout the study. The following buffers were used for immunoassays. (i) A 0.1 M concentration of sodium carbonate buffer (pH 9.6) was used to coat antigens onto microtiter plates. (ii) The LOS standards or test samples were suspended in pH 7.2 pf phosphate-buffered saline (PBS) or pf PBS containing 0.05% (vol/vol) Tween 20 (PBS-Tw). (iii) The antibodies used in the detection layer were diluted with 1% (wt/vol) bovine serum albumin (BSA) in pf PBS-Tw. (iv) The alkaline phosphatase substrate (*p*-nitrophenylphosphate; Sigma 104) was diluted (1 mg/ml) in 10% (vol/vol) diethanolamine buffer (pH 9.8) containing 1 mM $MgCl_2$. All washes were performed three times with pf PBS-Tw unless otherwise stated.

LAL was obtained from Associates of Cape Cod, Inc., Woods Hole, Mass., and LAL substrate (*n*-benzoyl-Val-Gly-Arg *p*-nitroanilide hydrochloride) was from Sigma (B-4758), as was PMB. Each vial of LAL was reconstituted with 10 ml of pf water and stored in multiple portions at −20°C for less than 3 months.

CLAL assay. Endotoxin concentrations were measured by use of the CLAL assay (11), with some modifications for use with microtiter plates (27). Purified Hib DL42 LOS was used as the standard.

IML assay. Microtiter plates were coated with MAbs 4C4 and 12D9, both separately and together, at a 1:500 dilution overnight at room temperature. Three wells were used for each sample; two wells were coated with MAbs, and a third, uncoated well served as an internal control for specificity. After the wells were washed, the LOS standards and the test samples were diluted 1:1 in triple detergent and then incubated for 2 h at room temperature in the wells. In experiments involving rabbit cerebrospinal fluid (CSF) samples, the standards and the test samples were heated (in 200-μl volumes) at 80°C for 10 min, cooled at room temperature for 30 min, and diluted 1:1 with triple detergent before being loaded into the wells of microtiter plates. After the wells were washed five times with PBS-Tw, 50 μl of LAL (at a concentration 50% of that used in the CLAL assay) was incubated in the wells for 20 min at room temperature. The LAL chromogenic substrate was then added as in the CLAL assay, and the plates were incubated for 30 min before the optical density was measured. For calculation of the results, the optical density of the uncoated well was subtracted from the mean optical density of the MAb-coated wells. The uncoated wells routinely gave absorbances of less than 0.050.

PMB-MAb assay. The details of the PMB-MAb assay have been described earlier (27). Briefly, microtiter plates were coated with PMB in carbonate buffer. After washes, the diluted LOS standards and the test samples were each incubated in two PMB-coated wells and one uncoated well. After washing, MAbs were added and the plates were incubated overnight at 4°C. The plates were washed and goat anti-mouse IgG was added. After being washed, the wells were blocked with BSA-PBS-Tw and thereafter alkaline phosphatase-conjugated rabbit anti-goat antibodies were incubated in the wells. The wells were again washed, and phosphatase substrate was added. After incubation, the enzyme reaction was stopped by addition of 1 N NaOH and the optical density at 410 nm was measured. LOS standards were included on each plate, and the optical density-versus-concentration curve was plotted after subtraction of the absorbance of the uncoated well from the mean of the PMB-coated wells. The concentrations of LOS in the test samples were obtained from this standard curve.

ELISA. The microtiter wells were coated overnight at room temperature with a 1:1,000 dilution in carbonate buffer of rabbit polyclonal antiserum containing antibodies to Hib OMV. After being washed, the wells were incubated with BSA-PBS-Tw for 2 h at 37°C. The wells were again washed, and each test sample and the LOS standards, diluted in PBS-Tw, were incubated in three wells (two antibody coated, one uncoated) for 2 h at 37°C. After being washed, MAbs diluted 1:500 in BSA-PBS-Tw were incubated overnight in the wells at 4°C. After being washed again, the alkaline phosphatase-conjugated goat anti-mouse antibodies at a 1:500 dilution were incubated in each well for 2 h at 37°C. The wells were again washed, phosphatase substrate was added, and the rest of the assay was performed as described above for the PMB-MAb assay.

Rabbits. A model of experimental meningitis described by Dacey and Sande (4) was used in a modified form with Hib (31). The animals were inoculated intracisternally with Hib

DL42 organisms (10^6 CFU). CSF samples (0.2 ml), collected before inoculation and at 6 and 22 h after injection of bacteria, were analyzed for leukocyte content and then centrifuged. The resultant supernatant fluids were immediately frozen at −70°C until tested. The number of CFU of bacteria in the CSF was determined by plating serial 10-fold dilutions of the CSF onto chocolate agar plates.

Statistical analysis. The strength of the relationship between the results of the CLAL assay and the immunoassays were assessed by Pearson's correlation coefficient.

RESULTS

IML assay. The working principle of the IML assay involves solubilization of the Hib LOS in outer membranes and subsequent binding of the LOS onto the microtiter plates by using MAbs to the oligosaccharide moiety of the LOS molecules. After removal of the detergent and nonspecifically bound molecules by extensive washing, the lipid A component of the LOS is detected by the CLAL assay.

Several detergent systems were tested for efficacy in extraction of Hib LOS from outer membranes as described in Materials and Methods. Triple detergent, containing the ionic detergents sodium dodecyl sulfate and sodium deoxycholate and the nonionic detergent Triton X-100, solubilized essentially all of the LOS in Hib OMV (data not shown). Possible release by triple detergent of the capture MAbs from the microtiter plates was tested by ELISA. The wells were coated with MAb 4C4, washed, blocked with BSA-PBS-Tw, and then incubated for 2 h with triple detergent. Alkaline phosphatase-conjugated goat anti-mouse antibodies at a 1:1,000 dilution were then incubated in the wells for 2 h, the wells were washed, substrate was added, and the reaction was measured by ELISA as described in Materials and Methods. The decrease in absorbance values was only 8% compared with wells incubated with PBS instead of triple detergent.

Several different LOS solubilization conditions were tested in preliminary studies. Preincubation of test samples from broth cultures with triple detergent at 37°C did not increase the sensitivity of the assay compared with results obtained by incubation of these samples at room temperature. Incubation of the test samples in MAb-coated wells with triple detergent at 37°C for 2 h significantly decreased the sensitivity of the assay.

In early experiments, some of the CSF samples from rabbits with Hib meningitis were totally negative in the IML assay but then gave positive results after being heated at 80°C for 10 min before use in the assay system. Therefore, this procedure was used for all rabbit CSF samples tested later with this assay and reported here. Heating had to be accomplished before dilution of the samples with triple detergent, because the combination of high temperature and detergent totally destroyed the ability of the samples to activate LAL in the second step of the assay. Agitation of the plates with the test samples or during LAL incubation was not advantageous.

Sensitivities and specificities of the different assays. The conditions for each of the immunoassays were optimized by evaluating different concentrations of antibodies, PMB, and CLAL reagents. The optimal working dilutions for the different antibodies (data not shown) were 1:500 for MAbs (corresponding to final concentrations of 4 μg/ml for MAb 4C4 and 3 μg/ml for MAb 12D9), 1:1,000 for goat anti-mouse IgG (corresponding to a final concentration of 1 μg/ml), and 1:500 for the commercially obtained enzyme-antibody conjugates. In the PMB-MAb assay, a 1-mg/ml concentration of PMB proved most effective when used for coating of the plates (27), and in the ELISA, the optimal dilution of the polyclonal Hib antisera used for coating was 1:1,000. Western blot analysis of these polyclonal sera to Hib OMV showed that these sera contained high levels of antibodies to Hib LOS, as well as antibodies to various Hib outer membrane proteins (data not shown).

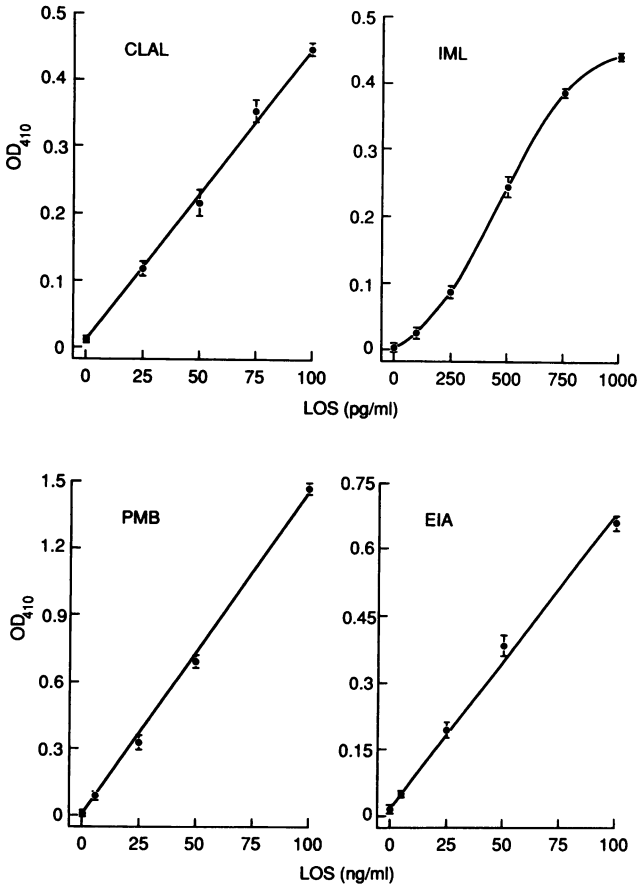

FIG. 1. Quantification of Hib DL42 LOS by CLAL, IML, and PMB-MAb assays and ELISA (EIA). Results are expressed as means ± the standard deviations. OD_{410}, Optical density at 410 nm.

The standard curves generated with the CLAL assay and three immunoassays are shown in Fig. 1. The sensitivities (the sensitivity cutoff point corresponds to the mean + 2 standard deviations of the absorbance of the diluent buffer) of the assays were 2 pg of Hib LOS per ml for the CLAL method, 100 pg of LOS per ml for the IML assay, and 1 ng/ml for both the ELISA and the PMB-MAb assay.

MAbs 12D9 and 4C4 are specific for two different Hib LOS molecules which are collectively common to all Hib strains (14). When these two MAbs were used in the IML assay, each MAb detected its own homologous antigenic type of LOS (DL26 and DL42, respectively) (Fig. 2). When the two MAbs were pooled (1:1) and then used in the assay, they bound to their homologous epitopes, giving results consistent with those obtained when each MAb was used by itself. When LPS (in five different concentrations ranging from 1 ng/ml to 10 μg/ml) from four different types of other gram-negative bacteria (*E. coli* O127:B8 and O111:B4, *P.*

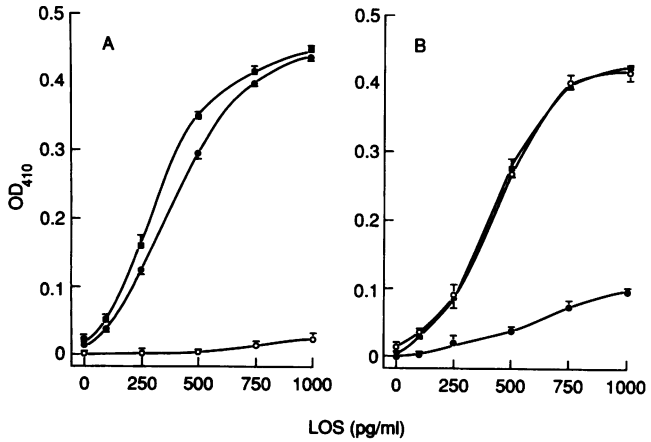

FIG. 2. IML-based quantification of Hib DL26 LOS (A) and DL42 LOS (B) with MAbs 12D9 (●), 4C4 (○), or both MAbs 12D9 and 4C4 in a 1:1 combination (■). OD, Optical density at 410 nm.

aeruginosa, and *K. pneumoniae*) were tested with MAb 4C4 in the IML assay, no endotoxin activity was detectable.

Detection of Hib LOS in in vitro cultures. Hib DL42 was grown in broth culture to the mid-logarithmic phase. Thirty minutes after inoculation of the bacteria into the growth medium, a sample (0.3 ml) was withdrawn from the broth and immediately diluted 1:10 with PBS supplemented with 0.2% (wt/vol) sodium azide to prevent further growth of the bacteria. Similar samples were collected 1, 2, and 3 h postinoculation. The samples were stored on ice until being tested on the same day by means of the ELISA and the IML, CLAL, and PMB-MAb assays. The LOS concentrations and the corresponding viable numbers of bacteria obtained from this culture are shown in Fig. 3.

Detection of LOS in CSF from rabbits with Hib meningitis. Ten rabbits received intracisternal injections of 10^6 CFU of Hib DL42. The CSF samples obtained from these rabbits 6 h later exhibited indications of meningitis, with 3.0×10^7 CFU of Hib per ml (mean) and 1,000 leukocytes per μl (mean). At

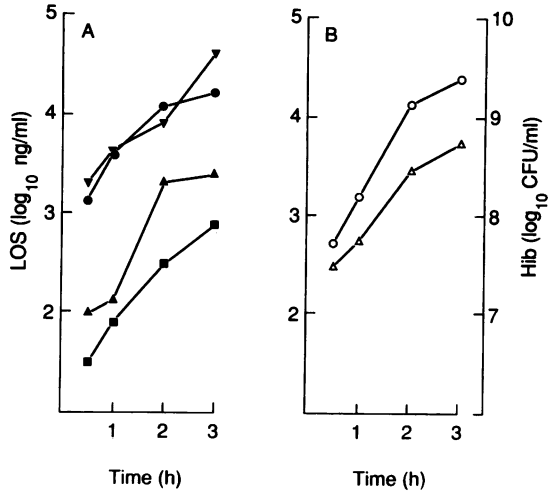

FIG. 3. (A) Quantification of LOS by IML (■), PMB-MAb (▲), and CLAL (●) assays and ELISA (▼) in samples from an in vitro culture of Hib DL42. (B) Mean concentrations of LOS (from panel A) (△) and CFU of Hib DL42 (○) are shown. Each point represents the arithmetic mean of concentrations from two experiments.

TABLE 1. Concentrations of Hib LOS, Hib bacteria, and leukocytes in CSF of rabbits after intracisternal injection of Hib DL42[a]

Assay	Mean ± SEM concn of Hib LOS after infection for:[b]	
	6 h	22 h
CLAL	9.0 ± 2.2	65 ± 5.3
IML	1.2 ± 1.2	23 ± 11.3
PMB-MAb	11.4 ± 3.4	102 ± 31.3
ELISA	2.3 ± 1.2	22 ± 9.1

[a] Ten rabbits were injected with 10^6 CFU of Hib.

[b] After infection for 6 and 22 h, the Hib concentrations were $(3.0 \pm 0.7) \times 10^7$ and $(2.7 \pm 1.9) \times 10^8$ and the concentrations of leukocytes in CSF were $(1.0 \pm 0.3) \times 10^3$ and $(1.9 \pm 1.2) \times 10^3$, respectively.

22 h postinoculation, the mean concentration of bacteria in the CSF had increased about 10-fold. The different immunoassays were used to measure LOS concentrations in these CSF samples diluted serially in PBS. In these assays, the LOS standards (1 to 100 ng/ml) were diluted correspondingly with PBS containing 10% (vol/vol) normal rabbit CSF. The LOS concentration results obtained with the CLAL assay and the three immunoassays are shown in Table 1. When the results were evaluated with Pearson's correlation test, there were significant correlations between results from all of the assays ($P < 0.01$). The specific correlations were as follows: ELISA versus CLAL, 0.609; IML versus CLAL, 0.562; PMb-MAb versus CLAL, 0.727; IML versus ELISA, 0.937; PMB-MAb versus ELISA, 0.973; PMb-MAb versus IML, 0.931.

DISCUSSION

Assays for endotoxin in biological fluids need to be specific for LPS, capable of detecting the LPS of all gram-negative pathogens, sufficiently sensitive to detect low amounts of LPS, and quantitative. Knowing whether the measured LPS is bioactive is also desirable if the assay is to be useful clinically. Unfortunately, none of the reported assays for LPS meets all of these requirements.

There are several factors which affect measurement of endotoxin concentrations in biological samples. Some of these involve the physical properties of the endotoxin molecule, some are dependent on host factors, and still others are related to the assays themselves. Lipid A is common to the LOS or LPS molecules of all gram-negative bacteria and is responsible for the endotoxic properties of these molecules. This part of the LPS-LOS molecule is extremely hydrophobic, is inserted deep into the outer membrane of gram-negative bacteria, and is generally considered not to be exposed on the surface of intact bacteria (24, 25). To become biologically active, endotoxins probably have to be liberated from the outer membrane. During growth in vitro, bacteria constantly shed outer membrane fragments (17) and the rate of this vesicle or bleb formation varies in different bacterial strains (5). Bacterial death and subsequent cell lysis may result in liberation of outer membrane fragments of different sizes, as well as free LPS molecules or their aggregates (30). These different physicochemical forms of endotoxin all probably have variable effects on the assays used to measure LPS-LOS concentrations.

Equally important in this regard is the fact that endotoxin and lipid A can bind to several substances present in body fluids, such as serum albumin, acute-phase and other pro-

teins, high- and low-density lipoproteins, and complement (40, 41). Some of these interactions decrease the biological activity of endotoxin, whereas others do not (9). The possible presence of natural antibodies to different parts of the LPS-LOS molecule also represents an important problem common to many types of antigen detection immunoassays. Gagliardi et al. found that the clinical usefulness of a sensitive MAb-based assay for lipid A in serum was hindered by lipid A-directed antibodies in test samples (12). Antibodies to lipid A occur naturally in humans and were found in up to 73% of individuals tested (23). Antibodies to the oligosaccharide moiety of Hib LOS have been found in about 70% of children with Hib meningitis when acute-phase serum samples were tested by Western blot analysis (34).

Each of the assays for endotoxin has its own idiosyncrasies. The CLAL test, an exquisitely sensitive assay that measures bioactive LPS, may lack specificity for LPS. Activation of LAL is not totally endotoxin specific because it has, in addition to an endotoxin-sensitive C factor, a G factor which is activated by (1–3)-β-D-glucan and some polysaccharides (33). Different activators and other inhibitors have also been described (3) (e.g., material used for purification of gamma globulin gives false-positive results in the CLAL assay when endotoxins are measured in blood of patients receiving gamma globulin products [18]). Additionally, the color (e.g., icteric plasma) and turbidity of the test material can have an effect on the interpretation of CLAL assay results. The assay is also very sensitive to small amounts of LPS contamination, which is common in reagents and other laboratory materials.

Unlike the ELISA and, probably, the PMB-MAb assay, the CLAL assay detects one bioactivity of LOS, i.e., the ability to activate a protease system in LAL that can generate a colored reaction product. It should be noted, however, that studies with lipid A analogs indicate that several biological activities of lipid A (pyrogenicity, lethal toxicity, anticomplement activity, gelation of LAL, and LAL protease activation) are separable entities (36, 38). Many lipid A analogs that lack toxicity (in assays such as the Shwartzman reaction and chicken embryo lethality) are still able to activate *Limulus* clotting (37). Recently, it has been shown that the presence of 3-hydroxyacyl groups on the bisphosphorylated(β1-6)-D-glucosamine disaccharide backbone of lipid A is the prerequisite for effective activation of the clotting enzyme cascade of LAL, while the presence of an adequate number of 3-acyloxyacyl groups on the disaccharide bisphosphate backbone is needed for full pyrogenicity (36). Therefore, extrapolation even from LAL test positivity to biological activity in clinically relevant bioassays is questionable, especially in situations in which the LOS may have undergone partial degradation (e.g., deacylation in vivo [28]).

Although more specific than the CLAL assay, an immunoassay such as the O-antigen radioimmunoassay (29, 30) or the ELISA used in this study detects antigenic epitopes that may or may not be part of bioactive LPS. In the PMB-MAb assay (27), the lipid A region of LOS presumably binds to PMB on the solid phase, leaving the oligosaccharide epitopes available for antibody detection. The assay is specific for the LOS recognized by the antibody, yet the biological potency of the molecules detected in this manner is uncertain.

In this study, we sought to combine the high specificity of the MAbs with the sensitivity of the CLAL assay in a new hybrid test, the IML assay, so that a positive result would indicate detection of a specific bioactive endotoxin. Although we tested this approach by using Hib LOS and thoroughly tested MAbs, it seems possible that this approach might be extended to detection of other LPS molecules by using highly cross-reactive antibodies or antibody combinations for the antigen capture step.

This study shows a high degree of correlation among the ELISA and the IML and PMB-MAb assays in the quantification of LOS in CSF. The correlations between the results of these three immunoassays and those of the CLAL assay were also significant. In addition, the findings indicate that quantitative assays based on detection of different components of the LOS molecule can give different results, even when the same purified preparation of Hib LOS is used as the standard for the assays. This is in agreement with earlier findings in which *E. coli* LPS was quantified by using both the LAL assay and an O-antigen-based solid-phase radioimmunoassay to analyze in vitro-grown culture samples and CSF samples (29, 30).

The results also show that the immunoassays with selected MAbs described herein are highly specific for Hib LOS but are less sensitive than the CLAL assay. The detection thresholds for the immunoassays used in this study were between 0.1 and 1 ng of LOS per ml when purified Hib LOS was measured in laboratory fluids but differed substantially when LOS liberated into broth cultures or into CSF was measured. In contrast, the CLAL test detected the lowest concentrations of LOS in both in vitro- and in vivo-derived samples. The relative loss of sensitivity observed when native LOS (i.e., LOS present in bacteria or OMV) was quantified probably reflects differences among the assay systems with regard to dependence on the physical state of the LOS molecule. In particular, solubilization of the native LOS with triple detergent, with concomitant disruption of the bacterial membrane structure, may have decreased the ability of LOS to activate LAL in the IML assay (39). Each of these assays may require the presence of LOS in aggregate form for maximal detection sensitivity.

In conclusion, we compared three new immunoassays with a standard CLAL method for measurement of Hib LOS in in vitro samples and in CSF samples from rabbits with experimental Hib meningitis. The results indicated that although there was a significant correlation among all of the assays, the quantitative results were not similar from assay to assay. Nonetheless, these immunoassays provide new methods for specific detection of Hib LOS in laboratory fluids and in research involving quantification of Hib endotoxin in experimental animal models. Determination of whether these assays will have clinical utility will require further studies. The results from this study also support the idea that the exquisitely sensitive CLAL assay is the most useful assay for clinical purposes (1, 6, 22, 42, 44). However, the immunoassays described herein are potentially useful tools for measurement of endotoxins with great specificity, especially in experimental systems in which the concentrations of these molecules exceed 1 ng/ml.

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