

## Radioimmunoassay for Gram-Negative Bacterial Lipopolysaccharide O Antigens: Influence of Antigen Solubility

ROBERT S. MUNFORD\* AND CATHERINE L. HALL

*Departments of Internal Medicine and Microbiology, University of Texas Health Science Center, Dallas, Texas 75219*

Received for publication 2 July 1979

We have developed a solid-phase radioimmunoassay technique for specific gram-negative bacterial lipopolysaccharide (LPS) O antigens. The method exploits the high-titer, specific immunoglobulin M response of the rabbit to LPS immunization to measure as little as 5 ng of homologous LPS per ml with less than 0.5% cross-reactivity toward heterologous LPS or culture supernatants. We found that O antigen in complete LPS was less available for antibody binding than O antigen in the soluble polysaccharide derived by mild acid hydrolysis of LPS and that triethylamine-induced disaggregation of complete LPS increased its activity in the assay. Quantitation of O antigen with the assay was thus influenced by the physical state of LPS or "free" O antigen.

Although much has been learned about the chemistry of gram-negative lipopolysaccharides (LPS; endotoxins) in recent years, the absence of sensitive, quantitative immunochemical assays for these molecules has been a major impediment to further progress in understanding their biological role. At the present time, there is a sensitive, quantitative assay for endotoxin activity (the *Limulus* lysate test [15, 20]), but the specificity of this assay has been disputed (15).

There are at least three properties of LPS which present obstacles to an immunoassay. First, these are antigenically complex molecules which consist of three major structural regions. Lipid A, the moiety responsible for most biological activities of endotoxin, including *Limulus* lysate gelation (15), is attached by a region of R-core sugars to the O polysaccharide, a long polymer which gives serological identity to different gram-negative strains. Although it is possible to raise antisera to lipid A and R-core antigens (5, 26), the most immunogenic region of LPS is the O polysaccharide, and most antisera produced by heat-killed gram-negative bacteria or purified LPS are directed primarily to O-polysaccharide antigen(s). Second, in the rabbit and other species, the major antibody response to LPS is immunoglobulin M (IgM); IgG antibodies appear with prolonged immunization but have lower titer and less specificity for the immunizing O antigen (14, 23). Third, in aqueous solution LPS molecules exist in aggregates of variable size and solubility (3). It is thought that

the hydrophobic lipid A orients toward the interior, and the hydrophilic O polysaccharide orients toward the exterior, of the membranous or micelle-like structures observable by electron microscopy (7).

The insolubility of LPS and its tendency to aggregate discouraged us from attempting an antigen-binding assay, in which unlabeled LPS might be quantitated by its ability to inhibit binding of labeled LPS to antibody. Solid-phase "sandwich" methodology (22) seemed more suitable, since an LPS aggregate which attached to solid-phase antibody might present multiple binding sites to the second "indicator" antibody. However, solid-phase assays have previously used radiolabeled or (enzyme-linked) IgG as the indicator antibody (or capture antibody, or both); IgM, the major antibody produced in response to LPS immunization, is harder to purify and less stable in solution.

This report describes a sensitive, quantitative immunoassay method which we have used to measure three specific O-polysaccharide antigens. Our solid-phase sandwich technique is similar to that previously described for other antigens (22), modified to take advantage of the high titer and specificity of the IgM antibody response to LPS immunization. Microtiter wells are first coated with IgG obtained from a rabbit which has been immunized for 6 to 8 weeks with a heat-killed bacterial vaccine (capture antibody). The second layer is the sample containing LPS. Third, we add diluted early phase immune serum (IgM rich) from the same rabbit. Finally,

the amount of IgM sticking to the wells is detected with  $^{125}\text{I}$ -labeled sheep antibodies to rabbit IgM (indicator antibody).

We also present data to show that the assay is specific for the O-antigen moiety of LPS and that the solubility of the LPS molecule has an important influence on the measurement of O-antigen concentration.

#### MATERIALS AND METHODS

**Bacterial strains.** *Escherichia coli* O6 K13 was provided by James W. Smith and Bertil Kaijser; *E. coli* O55, O4, and O14:K7 were provided by the Bureau of Laboratories, Center for Disease Control; and *E. coli* O111 (J5 mutant) was provided by Abraham I. Braude. *Salmonella minnesota* R5 and R345 were provided by Otto Westphal, and *S. typhimurium* G-30 and GB-1 were obtained from M. J. Osborn and Paul Rick.

**LPS preparations.** LPS from *E. coli* O6, *E. coli* O55, and *E. coli* O14 were prepared from cells grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) by the hot phenol-water method of Westphal and Jann (24). Subsequent purification steps (ether extraction, deoxyribonuclease and ribonuclease treatment, pronase treatment) were essentially as described by Romeo et al. (18). In addition, each preparation was ultracentrifuged twice ( $100,000 \times g$  for 3 h), and the supernatant was discarded. *S. minnesota* R5 and R345 LPS was prepared by the method of Galanos et al. (4) and purified in a similar manner. Preparations were ascertained to have less than 1% nucleic acid and less than 1.5% protein by ultraviolet absorbance.

Intrinsically labeled LPS was produced by *S. typhimurium* G-30, a mutant which lacks uridine 5'-diphosphate-galactose-4-epimerase and which incorporates exogenous D-galactose almost entirely into LPS (17). Cells were grown in 50 ml of PPBE (17) containing 0.5 mM D-galactose and 750  $\mu\text{Ci}$  of D-[1- $^3\text{H}$ ]galactose (New England Nuclear Corp., Boston, Mass.). Cells were harvested after four generations of growth, washed with saline, and stored at  $-70^\circ\text{C}$ . Before extraction of the LPS with the phenol-water method, labeled cells were combined with 1.5 g of lyophilized unlabeled cells grown under the same conditions. Purified LPS was hydrolyzed with 1% acetic acid at  $100^\circ\text{C}$  for 1.5 h, and the lipid A precipitate was removed by centrifugation at  $2,000 \times g$  for 20 min. The supernatant was lyophilized and then resuspended in pyridine-acetic acid for chromatography on Sephadex G-50 as described by Jann et al. (9). A single peak of  $^3\text{H}$  activity appeared just within the void volume of the column; these peak fractions were lyophilized and used as the acid-hydrolyzed polysaccharide. The polysaccharide had at least  $10^4$ -fold less activity than the original LPS in the *Limulus* lysate test (15).

For use in the radioimmunoassay, LPS preparations were suspended in pyrogen-free water at a concentration of 1 mg/ml, with 5  $\mu\text{l}$  of triethylamine (Eastman) per ml added to assist solubilization. Subsequent dilutions were in phosphate-buffered saline (PBS)-Tween (0.1 M potassium phosphate, 0.15 M NaCl, 0.02% sodium azide, 0.05% Tween-20, pH 7.4).

**Preparation of antiserum.** Bacteria from log-phase cultures in Trypticase soy broth were harvested by centrifugation, washed in saline, and heated at  $100^\circ\text{C}$  for 2 to 3 h. The cells were then washed and resuspended in saline before freezing at  $-70^\circ\text{C}$  (26). Rabbits weighing 4 to 6 pounds (ca. 1.8 to 2.7 kg) were immunized by intravenous injection of gradually increasing amounts (0.2 ml to 1 ml) of heat-killed bacterial suspension, administered two or three times each week. Rabbits were bled after 3 weeks of immunization ("early" serum), and then immunization was continued with once-weekly injections for 8 to 10 weeks. The rabbits were then bled again ("late" serum). Serum was stored at  $-70^\circ\text{C}$  until use.

Early antiserum titers were assessed by passive hemagglutination of LPS-sensitized human ( $\text{B}^-$ ) erythrocytes (16), and late antiserum titers were assessed by a bacterial agglutination test (19) with heat-killed bacterial cells diluted to an optical density of 0.78 (Coleman Jr. spectrophotometer, 1.2-cm tube diameter), in PBS containing 0.1% bovine serum albumin.

**Preparation of IgG (capture antibody).** Rabbit IgG was purified from late serum (see above) by fractionation of dialyzed (0.03 M potassium phosphate, pH 7.8) serum on DE-52 (Whatman) (2). Fall-through fractions were concentrated to approximately 10 mg/ml and stored in PBS at  $-70^\circ\text{C}$ .

**Indicator antibody.** Sheep anti-rabbit IgM (mu specific) was purchased from Cappell Laboratories, Cochranville, Pa. A 2-ml amount of reconstituted serum was treated twice with ammonium sulfate (8), dialyzed against normal saline, and stored in aliquots at  $-70^\circ\text{C}$ . A 40- $\mu\text{l}$  quantity of this globulin fraction (300  $\mu\text{g}$ ) was iodinated with 500  $\mu\text{Ci}$  of  $\text{Na}^{125}\text{I}$  (Amersham) by the chloramine-T method (6). The specific activity (assuming 100% recovery of labeled protein) was approximately 1.5  $\mu\text{Ci}/\mu\text{g}$ . The labeled material was stored in PBS with 0.5% bovine serum albumin at  $-70^\circ\text{C}$ . Before use in the assay, each portion was adsorbed at  $4^\circ\text{C}$  overnight with rabbit IgG-Sepharose (BioRad Immunobead R-1); 100  $\mu\text{l}$  of packed beads was added to a 500- $\mu\text{l}$  portion of labeled globulin. The adsorbed material was kept at  $4^\circ\text{C}$  until used in the assay.

**Immunoassay.** Polyvinyl microtiter wells (Dynatech Laboratories) were coated with rabbit anti-LPS IgG in 0.1 M sodium carbonate buffer, pH 9.5 (150  $\mu\text{l}$ /well). For each IgG preparation, the optimal coating concentration was determined to be the lowest concentration (from 20 to 100  $\mu\text{g}/\text{ml}$ ) which gave the maximal test/background ratio in the assay. Optimal coating occurred with incubation at  $37^\circ\text{C}$  for 3 h; the plates could then be left at  $4^\circ\text{C}$  for at least 24 h before use. Before adding samples containing antigen, the wells were washed twice with 200  $\mu\text{l}$  of PBS-Tween. Antigen was added in PBS-Tween (145  $\mu\text{l}$ /well), and the plates were incubated for at least 2 h at  $4^\circ\text{C}$ . The plates were washed again and then filled (140  $\mu\text{l}$ /well) with homologous rabbit antiserum (early serum) diluted in PBS-Tween containing 0.1% ovalbumin. A 1:400 or 1:500 dilution was chosen for most experiments. After at least 2 h of incubation at  $4^\circ\text{C}$ , the wells were washed again, and a 1:300 dilution of  $^{125}\text{I}$ -labeled sheep anti-rabbit mu was added (in 135  $\mu\text{l}$  of PBS-Tween-

ovalbumin [approximately 100,000 cpm] per well). The plates were then incubated for at least 2 h at 4°C, washed three times with PBS-Tween, and dried. The wells were removed from the plates with a Dynapunch (Dynatech Laboratories) and counted in a Beckman 4000 gamma counter. Standard deviations were less than 5% of the mean for 80% of 200 samples tested in quadruplicate (10 experiments, 0 to 10 µg of LPS per ml).

**Adsorption experiments.** Purified LPS (*E. coli* O6, O55) was attached to washed B<sup>-</sup> human erythrocytes after heating the LPS (1 mg/ml) in PBS at 100°C for 60 min. The sensitized erythrocytes were washed three times in PBS, and 100-µl volumes of the packed cells were used to adsorb a 1:100 dilution of heat-inactivated, homologous, early antiserum in PBS (0.03 M sodium phosphate, 0.15 M NaCl) containing 0.1% ovalbumin. After incubation at 4°C for 30 min, the erythrocytes were sedimented by low-speed centrifugation, the supernatant was transferred to another tube, and another portion of sensitized cells was added. This procedure was repeated six times. A similar dilution of antiserum was treated with unsensitized erythrocytes in a parallel fashion. The adsorbed sera were then tested in the immunoassay for activity toward homologous LPS and culture supernatants from homologous *E. coli*, with unadsorbed serum at a similar dilution as a control.

## RESULTS

**Antisera.** Early antisera had hemagglutination titers of 1:6,400 (*E. coli* O55), 1:25,600 (*E. coli* O6) and 1:6,400 (*S. typhimurium*). IgG purified from late antisera had bacterial agglutination titers of 1:800 (anti-*E. coli* O55 IgG, 10 mg/ml), 1:400 (anti-*E. coli* O6 IgG, 15 mg/ml), and 1:200 (anti-*S. typhimurium* IgG, 8 mg/ml).

**Radioimmunoassay.** We used the same approach to develop immunoassays for LPS from *E. coli* O6, *E. coli* O55, and *S. typhimurium*. Each assay could detect as little as 1 ng of homologous purified LPS per ml. Assay sensitivity depended upon the dilution of the second (IgM-rich) antiserum (Fig. 1), and greater sensitivity might be achieved by using less dilute serum than we employed for this step.

The amount of measured LPS was significantly influenced by the solubility of the LPS preparations. Disaggregation of LPS with triethylamine increased the amount of LPS measured by approximately 1.5- to 2.0-fold (Fig. 2A). We studied the effect of LPS solubility further by comparing intrinsically labeled *S. typhimurium* LPS with its derivative polysaccharide, prepared as described in Materials and Methods. The polysaccharide had substantially greater activity in the assay and was detectable at 0.1 to 0.5 ng/ml (Fig. 2B). The two antigens were adjusted before assay to obtain the same <sup>3</sup>H counts per unit volume; since [<sup>3</sup>H]galactose is present only in the polysaccharide moiety of the labeled LPS

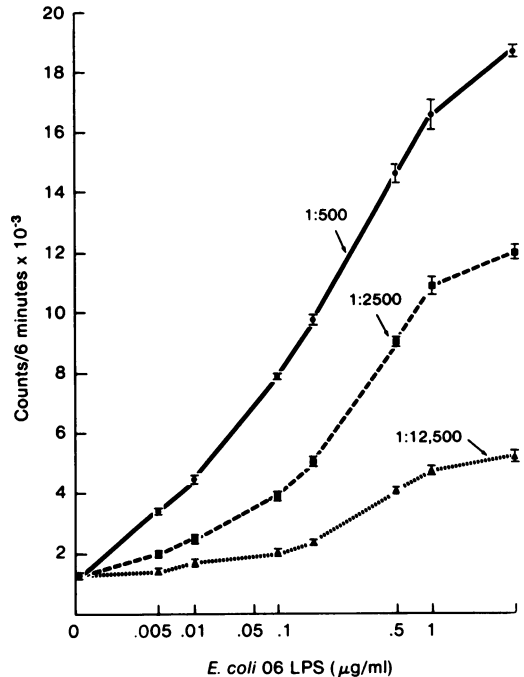


FIG. 1. Effect of second (IgM-rich) antiserum dilution on LPS immunoassay. Purified LPS standards were tested three times on the same microtiter plate with different dilutions of the second (IgM-rich) antiserum. The same amounts of capture (IgG) and indicator (<sup>125</sup>I-sheep anti-rabbit µ) antibody were added to all the wells. Quantitation of LPS with the assay was dependent upon the amount of IgM added. Points indicate the mean of four determinations ± standard deviation.

(17), the difference observed probably reflects a difference in the solubility of the LPS and its derivative polysaccharide and not in the number of antigen molecules.

We assessed the specificity of the assays in three ways. First, each assay was shown to have at least 10<sup>4</sup> greater activity toward homologous LPS than toward heterologous smooth and rough LPS preparations (Table 1) and at least 10<sup>2</sup>-fold greater activity toward homologous culture supernatants than toward supernatants of heterologous smooth or rough *E. coli* and *Salmonella* strains (Table 2). Second, each early immunization antiserum was adsorbed with erythrocytes which had been sensitized with homologous or heterologous LPS. After adsorption with homologous LPS, there was essentially complete loss of activity toward homologous stationary-phase culture supernatants, whereas adsorption with heterologous LPS or unsensitized erythrocytes caused minimal reductions in activity (Table 3). Taken together, these analyses indicate that the *E. coli* radioimmunoassays

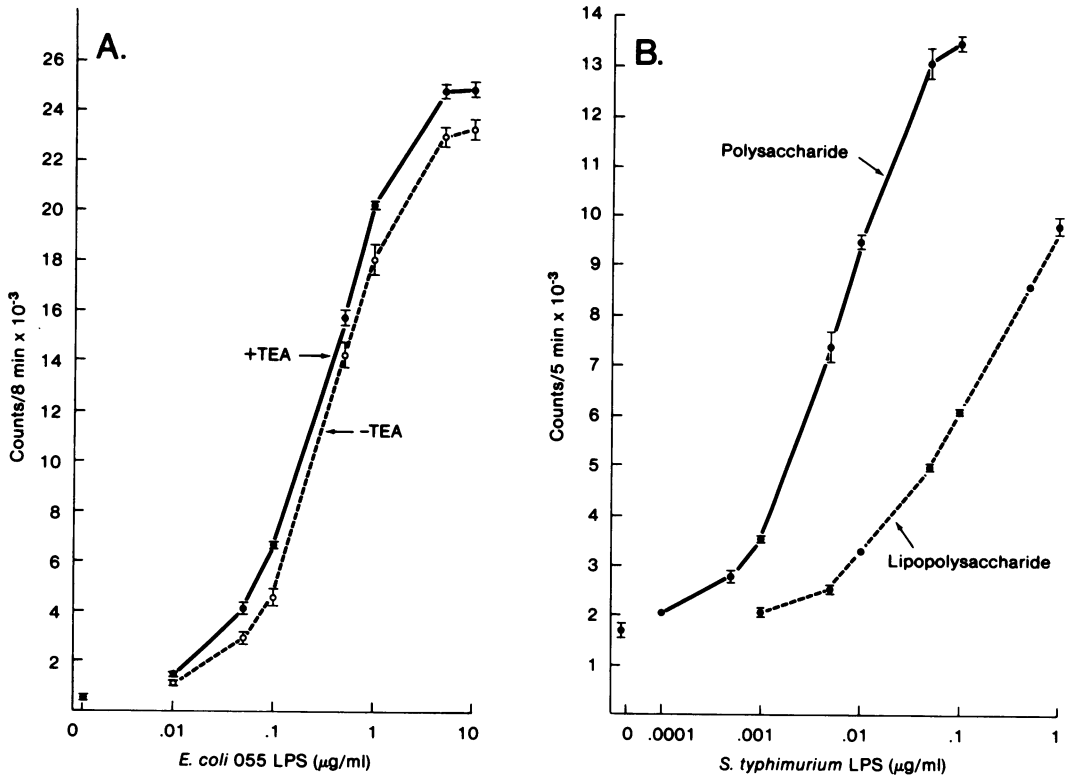


FIG. 2. Effect of increasing LPS solubility on the result of immunoassay. (A) Solubilization (disaggregation) of LPS with triethylamine (TEA). *E. coli* O55 LPS was suspended in pyrogen-free water (1 mg/ml) and divided into two aliquots. Triethylamine (5 µl/ml) was added to one aliquot, with mixing. The two preparations were then diluted in parallel and tested in the immunoassay (microtiter plate and other reagents were identical). In two separate experiments, addition of triethylamine significantly increased the amount of LPS measured in the assay. Points indicate mean of four determinations  $\pm$  standard deviation. (Note that the scale of the horizontal axis is logarithmic.) (B) Comparison of LPS with its derivative polysaccharide. *S. typhimurium* G-30, intrinsically labeled with D-[1-<sup>3</sup>H]galactose, was subjected to mild acid hydrolysis to cleave the polysaccharide moiety (containing O antigen) from lipid A. Polysaccharide and complete LPS were adjusted to the same counts per minute per milliliter of suspension and tested in parallel by the immunoassay. Data are expressed relative to the lyophilized dry weight of complete LPS. The derivative polysaccharide was substantially more active in the assay, as indicated by the greater sensitivity of the assay for the polysaccharide and by the steeper slope of the assay curve. Points indicate mean of two or three determinations  $\pm$  1 standard deviation.

are specific for homologous O antigen. Although we cannot exclude small amounts of anti-R-core antibody in the antiserum, the absence of assay activity toward rough LPS and supernatants of rough mutants suggests that this cross-reactivity is minimal.

The specificity of the *S. typhimurium* assay was also tested by measuring the amount of LPS released into culture supernatants of *S. typhimurium* G-30 grown with and without D-galactose in the culture medium. This mutant incorporates D-galactose exclusively into the R-core and O regions of LPS (17). The supernatant of the culture grown without galactose had only 2.0% (average of two experiments) of the LPS

detected with the immunoassay in the supernatant of the culture grown with 1.0 mM D-galactose to the same point in the growth curve; with the *Limulus* lysate test (15), in contrast, three-fold more LPS (lipid A) activity was measured in the culture grown without galactose. When the same experiment was repeated with *S. typhimurium* GB-1 (a derivative of G-30 which is additionally blocked in O-antigen synthesis, and which thus incorporates D-galactose only into R core [17]), the immunoassay detected less than 0.5% of the culture supernatant LPS measured for G-30 grown with galactose. The *S. typhimurium* immunoassay is thus directed toward the O antigen. We believe that the small

TABLE 1. *Immunoassay activity toward homologous and heterologous purified LPS*

LPS <sup>a</sup>	Amt (μg/ml) detected by immunoassay for:		
	<i>E. coli</i> O6	<i>E. coli</i> O55	<i>S. typhimurium</i>
<i>E. coli</i> O6	10	≤0.01	≤0.005
<i>E. coli</i> O55	≤0.01	10	≤0.005
<i>E. coli</i> O26 (Difco)	≤0.01	≤0.01	≤0.005
<i>E. coli</i> O127 (Difco)	≤0.01	≤0.01	0.008
<i>S. enteritidis</i> (Difco)	≤0.01	≤0.01	≤0.005
<i>S. typhimurium</i>	NT <sup>b</sup>	NT	10
<i>E. coli</i> O14	≤0.01	≤0.01	≤0.005
<i>S. minnesota</i> R345	≤0.01	≤0.01	NT
<i>S. minnesota</i> R5	≤0.01	≤0.01	≤0.005

<sup>a</sup> Tested at 10 μg/ml.<sup>b</sup> NT, Not tested.TABLE 2. *Immunoassay activity toward homologous and heterologous culture supernatants<sup>a</sup>*

Culture supernatant (stationary phase)	Amt (μg/ml) detected by immunoassay for:		
	<i>E. coli</i> O6	<i>E. coli</i> O55	<i>S. typhimurium</i>
<i>E. coli</i> O6	7	<0.05	≤0.05
<i>E. coli</i> O55	<0.05	52	≤0.05
<i>E. coli</i> O4	<0.05	<0.05	≤0.05
<i>E. coli</i> O14	<0.05	<0.05	<0.05
<i>E. coli</i> J5	<0.05	<0.05	<0.05
<i>S. typhimurium</i>	<0.05	<0.05	25

<sup>a</sup> Cultures were centrifuged (12,000 × *g*, 15 min) and the supernatant was recentrifuged before dilution (1:10 or greater) in PBS-Tween for assay.

amount of O antigen in the cultures of G-30 grown without added galactose is synthesized from traces of galactose already present in PPBE media (12).

## DISCUSSION

Immunization of the rabbit with LPS, whether in purified form or as heat-killed whole cells, mainly raises IgM antibodies to the O antigen (14, 23). With prolonged immunization, significant titers of IgG are also produced to the O antigen. We chose to use IgG, isolated from late immunization serum, as the capture antibody for our assay because it was easy to purify and stable after adsorption to microtiter wells. We initially attempted to use radiolabeled IgG as the indicator antibody as well (as has been described by Leibowitz et al. [13]), but in our hands there was significant assay activity toward heterologous LPS with this approach. By following the antigen with IgM-rich early immunization

TABLE 3. *Adsorption of second (IgM) antiserum with LPS-sensitized erythrocytes*

Antiserum	Adsorption	Activity <sup>a</sup>	
		Purified LPS <sup>b</sup>	Culture supernatant <sup>b</sup>
Anti-O55	None	1.0	1.0
	Unsensitized RBC <sup>c</sup>	0.98	.98
	O6 LPS-RBC	0.94	.80
	O55 LPS-RBC	≤0.0004	≤0.0004
Anti-O6	None	1.0	1.0
	Unsensitized RBC	0.89	1.0
	O55 LPS-RBC	1.0	.86
	O6 LPS-RBC	≤0.005	≤0.005

<sup>a</sup> Amount measured in radioimmunoassay. Unadsorbed serum value = 1.0.<sup>b</sup> Homologous LPS or supernatant.<sup>c</sup> RBC, Erythrocytes.

serum and then adding radiolabeled sheep anti-rabbit mu-chain indicator antibodies, we were able to achieve greater sensitivity while gaining a high degree of specificity for the immunizing O antigen. Another approach used by others to assay *E. coli* heat-labile enterotoxin (25) is to use antiserum from one species as the capture antibody, follow the antigen with antiserum from a second species, and use labeled antibody to the second species' globulins as the indicator antibody. Our method allows antiserum from a single rabbit to function as the first and second antibodies in the assay and exploits the difference in antibody class used for the two "layers" by using a mu-specific antiserum as the indicator antibody.

We found that the amount of purified LPS estimated by the assay could be increased (1.5- to 2.0-fold) by increasing the solubility of the LPS with triethylamine, a compound which reduces the size of LPS aggregates (3). Even more striking was the difference in assay activity found for LPS and its derivative soluble polysaccharide (Fig. 2B). Significant differences in the result of the immunoassay might therefore be caused by variations in the degree of aggregation of LPS molecules in a sample or by factors otherwise affecting solubility (such as the relative lengths of hydrophilic O and hydrophobic lipid A regions), without actual differences in the amount of O antigen present. Similar problems doubtless exist with the *Limulus* lysate assay, which detects lipid A activity (20) and which thus could be influenced by the degree of exposure of lipid A moieties to the *Limulus* proclotting enzyme. The dependence of these assays upon the solubility (availability) of the relevant LPS moieties may mean that purified complete LPS is not the optimal assay standard

for "natural" LPS (or O antigen) released from gram-negative bacteria. Studies to examine these relationships are in progress.

How might assays for LPS O antigens be used? First, they should provide a new dimension to studies of the release of LPS (and possibly O polysaccharide) from gram-negative bacteria in vitro. Second, purification of bacterial components, especially membrane proteins, is frequently hampered by contaminating LPS; these assays may allow the presence of such contamination to be recognized and monitored more easily. Third, the assays may allow more specific evaluation of the role of endotoxin in experimental animal models of gram-negative bacterial infection. Fourth, in certain clinical situations in which the *Limulus* lysate test has been used to detect specific endotoxins (such as *Shigella dysenteriae* endotoxemia [11], bacterial meningitis [10], and septic arthritis [21]), these assays might allow an independent estimation of the presence of the specific endotoxin(s) in plasma or other body fluids.

The methodology developed for this assay may also be useful for the quantitation of other bacterial polysaccharides (for example, *E. coli* K antigens; meningococcal, pneumococcal, and *Haemophilus influenzae* polysaccharides). IgM also accounts for the major antibody response to immunization with these antigens. Previous assays for these polysaccharides have been semi-quantitative (counterimmunoelectrophoresis, latex agglutination) and have had less sensitivity than the present method. A recently described solid-phase enzyme-linked immunoassay for *H. influenzae* b capsular polysaccharide (1) with hyperimmune burro antiserum indicates that the solid-phase approach is applicable to these antigens. Our data suggest that because of their relative solubility these polysaccharides may possibly be quantitated even more accurately, and with greater sensitivity, than LPS.

#### ACKNOWLEDGMENTS

We thank Morton N. Swartz and James W. Smith for helpful advice and Leon Eidels, Emil C. Gotschlich, and Richard A. Finkelstein for reading the manuscript.

This work was supported by Public Health Service Young Investigator Research Grant 1-R23-A114942-01 from the National Institute of Allergy and Infectious Diseases.

#### LITERATURE CITED

- Crosson, F. J., J. A. Winkelstein, and E. R. Moxon. 1978. Enzyme-linked immunosorbent assay for detection and quantitation of capsular antigen of *Haemophilus influenzae* type b. *Infect. Immun.* **22**:617-619.
- Fahey, J. L., and E. W. Terry. 1973. Ion exchange chromatography and gel filtration, chapter 7. *In* D. M. Weir (ed.), *Immunochemistry*. Blackwell Scientific Publications, Oxford.
- Galanos, C., and O. Luderitz. 1976. The role of the physical state of lipopolysaccharides in the interaction with complement. *Eur. J. Biochem.* **65**:403-408.
- Galanos, C., O. Luderitz, and O. Westphal. 1969. A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* **9**:245-249.
- Galanos, C., O. Luderitz, and O. Westphal. 1971. Preparation and properties of antisera against the lipid A component of bacterial lipopolysaccharides. *Eur. J. Biochem.* **24**:116-122.
- Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of <sup>131</sup>I-labeled human growth hormone of high specific radioactivity. *Biochem. J.* **89**:114-123.
- Hannecart-Pokorni, E., D. Dekegel, and F. Depuydt. 1973. Macromolecular structure of lipopolysaccharides from Gram-negative bacteria. *Eur. J. Biochem.* **38**:6-13.
- Hebert, G. A., P. L. Pelham, and B. Pittman. 1973. Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse, and goat antisera. *Appl. Microbiol.* **25**:26-36.
- Jann, B., K. Jann, and G. Schmidt. 1970. Immunochemical studies of polysaccharide surface antigens of *Escherichia coli* O100:K?(B):H2. *Eur. J. Biochem.* **15**:29-39.
- Jorgensen, J. H., and J. C. Lee. 1978. Rapid diagnosis of gram-negative bacterial meningitis by the *Limulus* endotoxin assay. *J. Clin. Microbiol.* **7**:12-17.
- Koster, F., J. Levin, L. Walker, K. S. K. Tung, R. H. Gilman, M. M. Rahaman, M. A. Majid, S. Islam, and R. C. Williams, Jr. 1978. Hemolytic-uremic syndrome after shigellosis: relation to endotoxemia and circulating immune complexes. *N. Engl. J. Med.* **298**:927-933.
- Kulpa, C. F., Jr., and L. Leive. 1976. Mode of insertion of lipopolysaccharide into the outer membrane of *Escherichia coli*. *J. Bacteriol.* **126**:467-477.
- Leibowitz, A. I., A. O. Vladutiu, and J. P. Nolan. 1979. Immunoradiometric assay of endotoxin in serum. *Clin. Chem.* **25**:68-70.
- Möller, G., O. Sjöberg, and J. Andersson. 1973. Immunogenicity, tolerogenicity, and mitogenicity of lipopolysaccharides. *J. Infect. Dis.* **128**:S52-S56.
- Munford, R. S. 1978. Quantitative *Limulus* lysate assay for endotoxin activity: aggregation of radioiodinated coagulogen monomers. *Anal. Biochem.* **91**:509-515.
- Neter, E. 1956. Bacterial hemagglutination and hemolysis. *Bacteriol. Rev.* **20**:166-188.
- Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. *J. Biol. Chem.* **247**:3962-3972.
- Romeo, D., A. Girard, and L. Rothfield. 1970. Reassembly of a functional membrane enzyme system in a monomolecular film. *J. Mol. Biol.* **53**:475-490.
- Smith, J. W., J. A. Barnett, R. P. May, and J. P. Sanford. 1967. Comparison of the opsonic activity of  $\gamma$ -G- and  $\gamma$ -M-anti-*Proteus* globulins. *J. Immunol.* **98**:336-343.
- Sullivan, J. D., F. W. Valois, and S. W. Watson. 1976. Endotoxins: the *Limulus* amoebocyte lysate system, p. 217-236. *In* A. W. Bernheimer (ed.), *Mechanisms in bacterial toxicology*. John Wiley & Sons, Inc., New York.
- Tuzaon, C. U., A. A. Perez, R. J. Elin, and J. N. Sheagren. 1977. Detection of endotoxin in cerebrospinal and joint fluids by *Limulus* assay. *Arch. Intern. Med.* **137**:55-56.
- Voller, A., D. Bidwell, and A. Bartlett. 1976. Microplate enzyme immunoassays for the immunodiagnosis of viral infections, p. 506-512. *In* N. H. Rose and H. Friedman (eds.), *Manual of clinical immunology*. American Society for Microbiology, Washington, D.C.

23. Weidanz, W. P., A. L. Jackson, and M. Landy. 1964. Some aspects of the antibody response of rabbits to immunization with enterobacterial somatic antigens. *Proc. Soc. Exp. Biol. Med.* 116:832-837.
24. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides. *Methods Carbohydr. Chem.* 5:83-91.
25. Yolken, R. H., H. B. Greenberg, M. H. Merson, R. B. Sack, and A. Z. Kapikian. 1977. Enzyme-linked immunosorbent assay for detection of *Escherichia coli* heat-labile enterotoxin. *J. Clin. Microbiol.* 6:439-444.
26. Ziegler, E. J., H. Douglas, J. E. Sherman, C. E. Davis, and A. I. Braude. 1973. Treatment of *E. coli* and *Klebsiella* bacteremia in agranulocytic animals with antiserum to a UDP-Gal epimerase-deficient mutant. *J. Immunol.* 111:433-438.