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Turbidimetric Method for Quantifying Serum Inhibition of *Limulus* Amoebocyte Lysate

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This study describes a method to quantify the inhibition of lipopolysaccharide (LPS) activity by serum with a turbidimetric *Limulus* amoebocyte lysate assay. Assays were performed in multiwell microplates, and turbidity was measured as the optical density at 380 nm with a microplate spectrophotometer. LPS potency was measured as the 50% maximal *Limulus* amoebocyte response (LR_{50}) of LPS diluted with saline. By comparing LR_{50}s in saline, LPSs from various species of bacteria were standardized against the U.S. Reference Standard Endotoxin, lot EC-5. The potency of *Escherichia coli* O113 and O18 and *Serratia marcescens* LPSs was found to be equal to that of the reference standard EC-5, whereas LPSs from two salmonella species were half as potent. The least potent LPSs tested, obtained from *Klebsiella pneumoniae* and *E. coli* rough mutant J5, were 5- and 10-fold less potent, respectively, than EC-5. As a measure of inhibition, the LR_{50} of LPS in serum was compared to the LR_{50} of LPS in saline. Serum inhibited the potency of LPS 103- to 6,400-fold compared with saline. A positive correlation was found between standardized potency in saline and serum inhibition of the various LPSs tested. Thus, LPSs from *E. coli* O113, O18, and EC-5 and *S. marcescens*, which exhibited the highest potency in saline, were inhibited the most by serum. Likewise, *E. coli* J5 and *K. pneumoniae* LPSs, which were the least potent tested, were the least inhibited. The degree of inhibition of all types of LPS tested increased with increasing serum concentration.

Serum inhibition of lipopolysaccharide (LPS) detection with the *Limulus* amoebocyte lysate (LAL) tests has been a major obstacle for the routine use of LAL as an assay for LPS in serum, blood, and blood products (4–6, 8, 9, 13–16, 18, 19, 21, 24). Recent studies have been concerned with the removal of this inhibition and improvement of the methodology, sensitivity, and reliability of the LAL test for the detection of endotoxin (as LPS) in serum, plasma, or both, to diagnose bacteremia and endotoxemia (3, 12, 27, 30). None of these studies addressed the degree of LPS inhibition by serum or whether LPS from different bacterial species is inhibited to the same degree. We describe a method which uses LAL to compare and standardize potencies of purified preparations of LPS from different bacteria and to quantify serum inhibition of LPS activity. (Some of these results were presented at the 84th Annual Meeting of the American Society for Microbiology, March 1984, St. Louis, Mo.)

MATERIALS AND METHODS

Serum and plasma. Normal human serum was obtained from a volunteer. Serum from this donor was used to develop the methodology and to compare the LAL response of various species of LPS. Additional serum samples were obtained from healthy donors to assess the universality of the test. All serum samples were divided into 4-ml portions, frozen at −70°C, and thawed immediately before use. Samples were occasionally refrozen and thawed a second time before use. A single refreeze had no effect on the ability of serum to inhibit the LAL assay. All serum samples were diluted with 0.9% sodium chloride for injection, USP (SCI).

LPS. Purified LPS was obtained from several sources. In all cases, the dry LPS was reconstituted with sterile water for injection. LPS extracted from *Escherichia coli* O113 (U.S. Reference Standard Endotoxin, lot EC-5) was obtained from the Food and Drug Administration and reconstituted to 2,000 endotoxin units (EU) per ml. Associates of Cape Cod, Inc., (ACC) control standard LPS, *E. coli* O113 (ACC lot no. 20), was reconstituted to 25 μg/ml. After reconstitution, vials of LPS from both sources were stored refrigerated and were used repeatedly during the study. Results from this and previous studies (unpublished data) indicated biological activity of LPS was stable for several months when stored refrigerated as a concentrated solution. The LPS lots listed below were reconstituted and diluted to the concentrations indicated with sterile water for injection, separated into 0.5-ml portions, and frozen at −20°C until immediately before use. LPS was extracted from *E. coli* O18 by the phenol-water method of Westphal and Jann (31) as described by Rudbach et al. (20) and frozen at 0.1 mg/ml. All other LPSs were obtained from List Biological Laboratories, Inc., Campbell, Calif. These were reconstituted, portioned, and frozen at the following concentrations: *E. coli* J5, lot no. 2 (1 mg/ml); *Serratia marcescens*, lot no. 1 (0.1 mg/ml); *Klebsiella pneumoniae*, lot no. 2 (0.1 mg/ml); *Salmonella typhimurium*, lot no. 1 (0.1 mg/ml); and *Salmonella minnesota*, lot no. 1 (0.1 mg/ml). Immediately before assay, LPS was serially diluted in twofold increments with SCI as diluent; care was taken to vigorously vortex each dilution in the series to assure thorough and homogeneous dissolution of the LPS. Dilutions were prepared in disposable polystyrene test tubes (Evergreen Scientific/Scientific International, Inc., Los Angeles, Calif.), to which the LPS did not adhere (ACC, unpublished results).

LAL-saline assay. LAL (ACC Pyrotell lot no. 96-03-265) was used for all assays. LAL was handled and reconstituted as recommended by the package instructions. The LAL assay employed was a modified turbidimetric microplate method (1). The assays were performed in sterile 96-well tissue culture multiwell plates (Linbro Division, Flow Lab-

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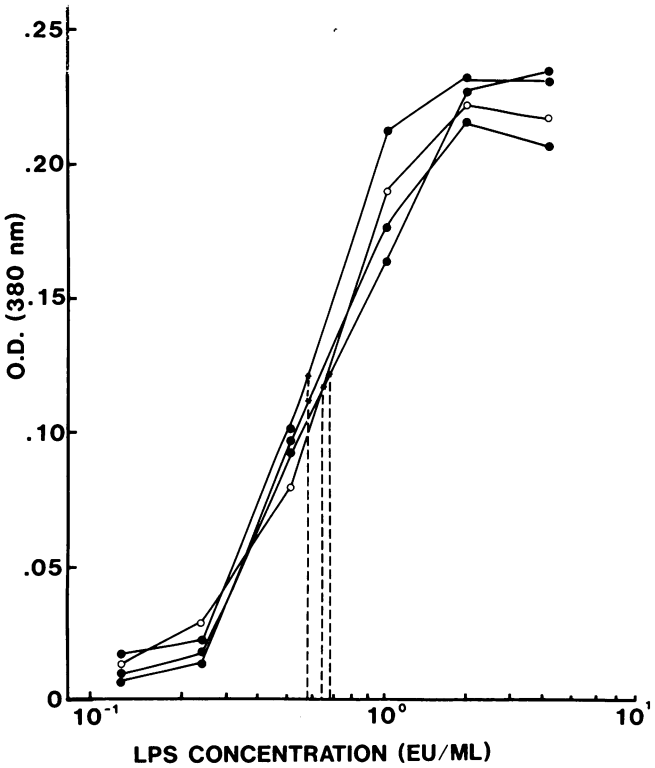

FIG. 1. Reproducibility of the OD curve of a turbidimetric LAL assay of LPS concentration in saline, with a comparison of frozen (○) and unfrozen (●) LPS samples. The LR_{50} was calculated as the LPS concentration at which the OD equals one-half the difference between the maximum OD increase and the saline control. The LPS used was *E. coli* 0113, ACC lot no. 20. The four trials were done in different microtiter plates, and all trials of unfrozen samples used the same LPS dilution series. There is no significant difference among the LR_{50}s of the four trials.

oratories, Inc., McLean, Va.). The optical density (OD) of all reactions were measured with a high-speed photometer (Flow Laboratories Multiskan TM). All assays were done in duplicate, and the values were averaged.

To each well of the multiwell plates were added, in order, 0.1 ml of LPS variously diluted in SCI and 0.1 ml of LAL. Care was taken to avoid contaminating neighboring wells during pipetting. The plates were covered with Parafilm (3M Co., St. Paul, Minn.), agitated for 15 s, and incubated at 37°C for 1 h. The OD of all reactions was determined in the photometer. One-half the maximum OD increase above the saline control was defined as the 50% LAL response (LR_{50}). Each LPS was assigned a potency value in EU by comparing LR_{50} values of LPS diluted in saline to the LR_{50} of the U.S. Reference Standard Endotoxin, EC-5. EC-5 was shown by collaborative assay to have a potency of 10 EU/ng (11).

LAL-serum assay. The LAL-serum assay was conducted in a manner similar to the LAL-saline assay, except that to each well of the multiwell plates were added, in order, 0.05 ml of serum (undiluted or variously diluted in SCI) and 0.05 ml of LPS solution. The plates were covered with Parafilm to prevent evaporation, agitated on a mechanical vibrating platform for 15 s, and incubated at 37°C in a convention oven for various times. Multiwell plates were then uncovered, and 0.1 ml of LAL was added to each well. The plates were subsequently handled and read as described for the LAL-saline assay.

RESULTS

LPS potency in saline. The interaction of LAL with a range of LPS concentrations generated a sigmoid curve when the OD was plotted against the LPS concentration. Typical curves (Fig. 1 and 2) show how the LR_{50}, as EU/ml or ng/ml, was determined from the OD values obtained. The relative potency in saline (as LR_{50}) varied among LPSs extracted from different bacteria (Table 1). Interassay variation was in general very low (Table 1). LPS potency ranged from 10 EU/ng (*E. coli* wild types and *Serratia marcescens*) to 1 EU/ng (*E. coli* J5). No change in LPS potency was observed when reconstituted vials were sampled repeatedly over 1 month. Also, little difference in potency was found between LPS samples that had been reconstituted and frozen in high concentrations and unfrozen samples (Fig. 1).

Serum assay. When the OD was plotted against the LPS concentrations incubated in serum for 3 h (Fig. 2), a sigmoid curve was obtained similar to that of the OD versus the LPS in saline, except that the LR_{50} in serum was always greater than that in saline. There was little difference between the OD plots of serum collected at two different times within 24 h from the same volunteer, at least for LPS extracted from *E. coli* O113 (Fig. 2). Serum was also found to inhibit all eight types of LPS tested (Table 2).

Incubation period. Varying the time that LPS was incubated in the presence of serum increased the degree of inhibition as LR_{50}. The effect of serum inhibition of LPS was very rapid, and a 10-fold increase in LR_{50} was observed in 15 min (Fig. 3). In a similar 24-h experiment (data not shown), the LR_{50} continued to increase up to 6 h and at 24 h had not increased further. Although it would have been better to conduct future experiments by using a time period in which the serum-LPS reaction was maximized, the longer incubation time presented additional problems. Condensation on the Parafilm covering the plate caused unequal loss of volume in the wells and introduced the possibility of bacte-

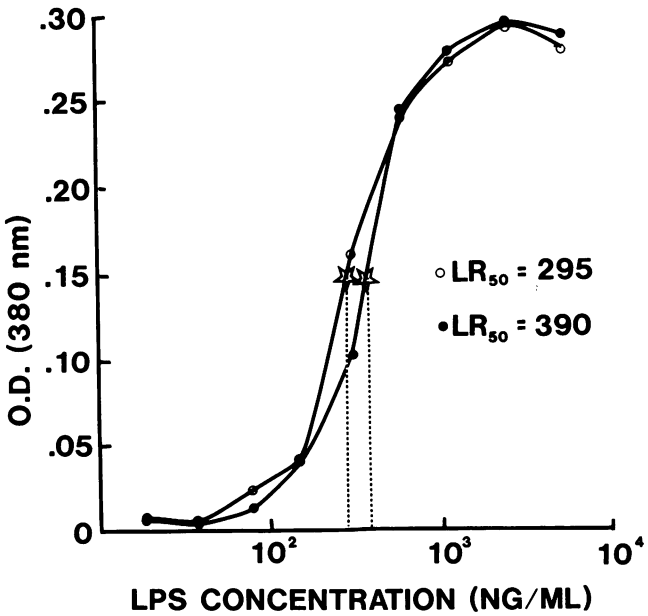

FIG. 2. Comparison of LR_{50}s (☆) using two serum samples (diluted twofold) from one volunteer. The LPS used was *E. coli* O113, ACC lot no. 20.

TABLE 1. Comparison of the potencies of LPSs extracted from different bacteria

LPS source	Treatment	LR_{50} (EU/ml)		LR_{50} (ng/ml)		EU/ng
		Mean ± SD	*n*	Mean ± SD	*n*	
E. coli O113, RSE[b] lot no. EC-5	FA	0.64 ± 0.037	5			
	URV	0.61 ± 0.058	3			
	Both	0.63 ± 0.041	8			10
E. coli O113, ACC lot no. 20	FA			0.071 ± 0.027	2	
	URV			0.062 ± 0.004	5	
	Both			0.064 ± 0.011	7	10
E. coli O18	FA			0.064 ± 0.021	6	10
Serratia marcescens	FA			0.057 ± 0.024	3	10
Salmonella typhimurium	FA			0.130 ± 0.006	3	5
Salmonella minnesota	FA			0.120 ± 0.007	2	5
Klebsiella pneumoniae	FA			0.305 ± 0.151	8	2
E. coli J5	FA			0.573 ± 0.057	4	1

[a] FA, Frozen aliquot; URV, unfrozen reconstituted vial.
[b] RSE, U.S. Reference Standard Endotoxin.

rial contamination. Since the reaction between LPS and serum was very rapid during the first hour of incubation, reproducibility was improved with the longer, 3-h incubation time, and this time was chosen for subsequent experiments.

Serum dilution. The LR_{50} increased rapidly with increasing serum concentration (Fig. 4). The LR_{50} values of LPS from two different organisms, *E. coli* O113 and *K. pneumoniae*, although quantitatively different, followed parallel curves when plotted against the higher range of serum concentrations. These values converged as the serum concentration approached zero. Because of experimental design, all serum samples were diluted (by the addition of LPS) at least twofold (50% serum concentration). For experiments on the single-donor serum, a twofold dilution yielded acceptable results (Fig. 2). However, when several other donor samples were tested with this high serum concentration, it was often difficult to calculate an LR_{50} due to deviation of the response from the typical sigmoid curve (data not shown). Therefore, most samples were tested at a fourfold dilution or a concentration of 25% serum.

Reproducibility of the LR_{50} in serum. LR_{50} assays in serum were more variable than those in saline. A single serum sample was portioned and frozen at −60°C. During the next month, the LR_{50} of this serum was assayed 32 times. The average of these determinations was 162 ng/ml, with a standard deviation of 39 ng/ml.

LR_{50} of LPS in serum. Inhibition of different LPSs in the serum samples followed a pattern which was roughly the reverse of the relative potency of the LPS in saline (Table 2). LPSs extracted from *K. pneumoniae* and *E. coli* J5 were relatively less inhibited by serum (LR_{50} = 91 and 59 ng/ml, respectively) than LPSs from other *E. coli* strains, *Salmonella* spp., and *Serratia marcescens* (LR_{50} range = 193 to 410 ng/ml. The observation that the LPSs of *E. coli* J5 and *K. pneumoniae* were less inhibited than other LPS species was duplicated by using a mixed serum sample. (15 pooled normal serum samples).

The LR_{50}s of these LPSs in serum were compared to the LR_{50}s obtained in saline by plotting the value in serum (nanograms per milliliter) against the value in saline (nanograms per milliliter). Figure 5 shows a range of inhibition of 103-fold for the LPS of *E. coli* J5 to 6,400-fold for the LPS of *E. coli* O18.

Variation of serum LR_{50}. The LR_{50}s of 25% serum obtained from 26 healthy donors varied from 50 to 615 ng/ml (data not shown). The LR_{50}s of 15 of these serum samples

TABLE 2. LR_{50}s of various LPSs in serum[a]

LPS	LR_{50}					
	ng/ml				EU/ml	
	Serum A		Serum pool		Serum A	Serum pool
	n	Mean	*n*	Mean		
E. coli J5	2	59	2	37	59	37
Klebsiella pneumoniae	6	91	2	68	182	136
E. coli O113, RSE lot no. EC-5	1	193			1,930	
Salmonella typhimurium	2	300	1	140	1,500	700
Serratia marcescens	1	330			3,300	
E. coli O113, ACC lot no. 20	7	339	2	235	3,390	2,350
Salmonella minnesota	2	370	2	440	1,850	2,200
E. coli O18	1	410	1	410	4,100	4,100

[a] EU was defined directly for *E. coli* O113, RSE lot no EC-5. For the other LPS types EU/ml were calculated on the basis of the relative potencies in saline shown in Table 1. Serum A was from a single volunteer; the serum pool was made up of 15 normal serum samples. RSE, U.S. Reference Standard Endotoxin.

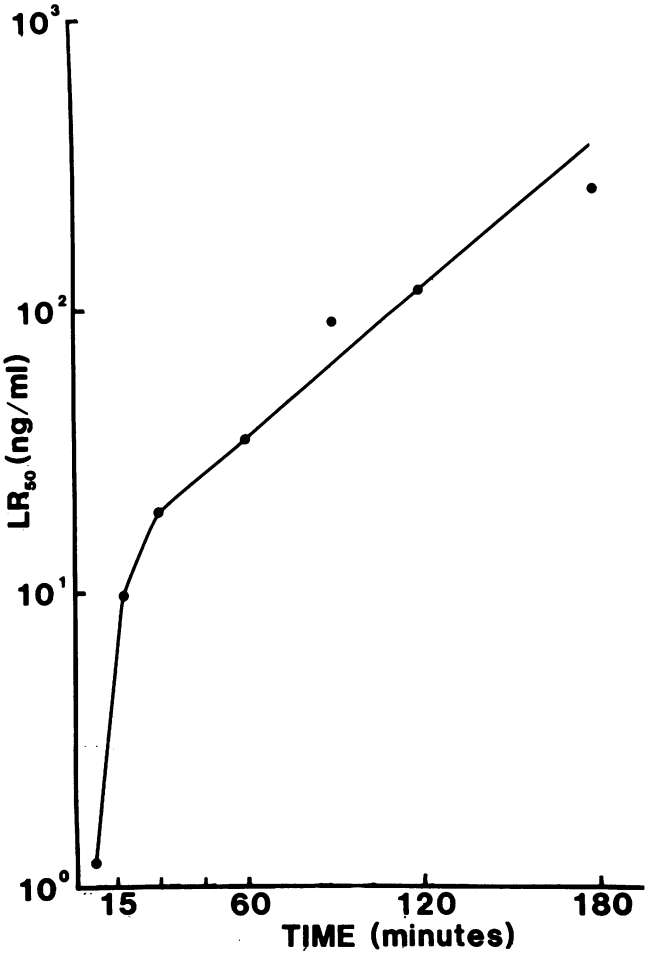

FIG. 3. Effect of incubation time on the LR_{50} of LPS extracted from *E. coli* O113, ACC lot no. 20, with the serum diluted fourfold.

ranged between 50 and 100 ng/ml, whereas only 4 were above 200 ng/ml. This data indicates a 12-fold variation. More samples will be required to determine whether a normal distribution exists.

DISCUSSION

The LR_{50} was found to be a convenient and precise way to compare potencies of various types of LPS and to quantify inhibition in the LAL assay. Because of the steep slope and linear central portion of the OD response (Fig. 1 and 2), the LR_{50} in saline was relatively reproducible (Table 1). It was also shown (Fig. 1) that freezing and thawing LPS standards had no effect on their potency.

The LR_{50} in serum was not as reproducible as that in saline. This difference could be attributed to errors in serum dilution and the rapidity of the LPS-serum interaction.

The LR_{50}s of various LPS species in serum samples rapidly approached the LR_{50}s of LPS in saline as the dilution of serum increased (Fig. 4). Virtually all serum inhibition, more than 99.9%, was lost upon 10-fold dilution. This finding supports the use by others of 10-fold dilutions to partially eliminate serum inhibition of LAL (9, 15). Because of the experimental design of this study, we were unable to test undiluted serum. It is not clear at this time whether inhibition by using *E. coli* O113 or *K. pneumoniae* and undiluted serum could be predicted by extrapolation of the curve in Fig. 4. We also encountered problems obtaining typical sigmoid responses when certain donor serum samples were tested at a twofold dilution (50% serum). It is possible that the greater viscosity of these more concentrated samples interfered with the development of turbidity in the assay. Some improvement was obtained when the multiwell plates were mixed for a longer time after the addition of serum and LPS or when the mixture was repeatedly withdrawn and dispensed with a mechanical pipetter before incubation. To avoid this problem, we used a serum concentration of 25% for most tests.

Because of technical considerations, a 3-h incubation of LPS and serum was chosen for this study. Although differences in the ability of various LPSs to react with serum were seen with the 3-h incubation, further study will be needed to determine whether these differences were due to the rate of interaction between LPS and serum components or a quantitative difference in the amount of interactive serum components present.

Although this study provides a means of measuring the amount of LAL inhibition by serum, the mechanism of inhibition is still unclear. Several studies have shown interaction of LPS with serum components (17, 22, 29, 33). It is likely the LAL assay is inhibited due to a lack of availability

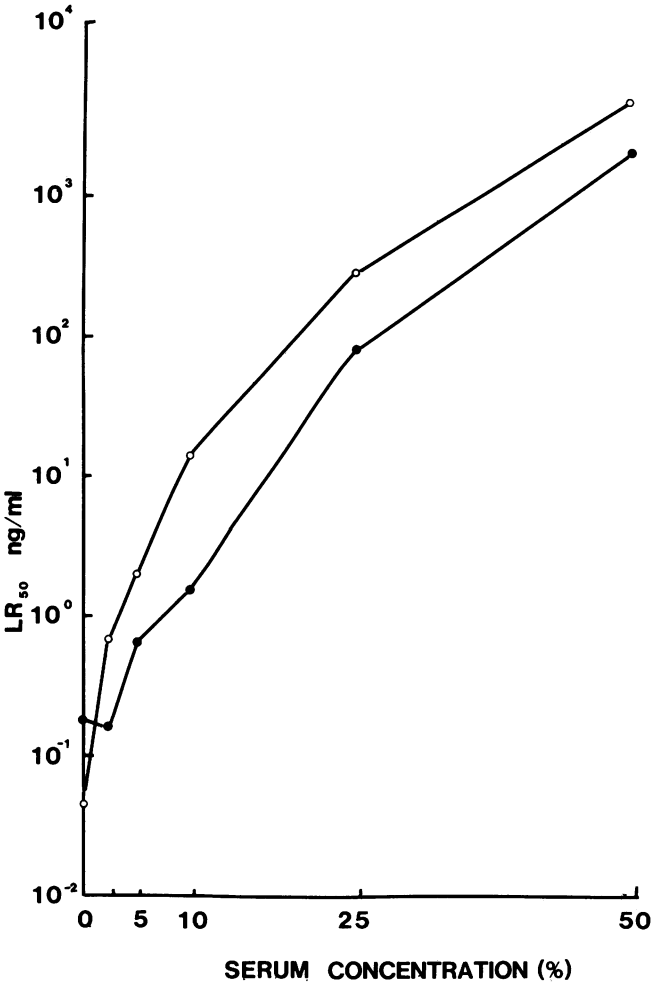

FIG. 4. Effect of serum dilution on the LR_{50} of LPS extracted from two different bacteria, *Klebsiella pneumoniae* (●) and *E. coli* O113, ACC lot no. 20 (○).

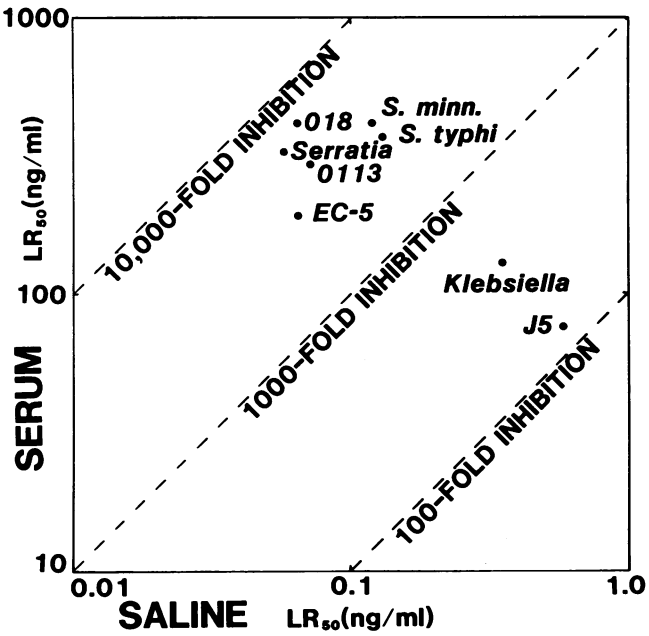

FIG. 5. Comparison of the LR_{50}s (ng/ml) of various LPSs as determined in 25% serum and saline. Lines delineating 100-fold, 1,000-fold, and 10,000-fold inhibition in serum as compared with saline are shown.

of the lipid A moiety of LPS, which is believed to be responsible for LAL activation (32). Masking, or chemical modification of this portion of LPS should therefore "inhibit" the LAL test (28, 30). It is also possible however that a serum component(s) or chemical agent(s) used in the collection of blood samples acts on LAL directly. For example, it has been shown that the LAL reagent can be inhibited by agents that chelate divalent cations that are essential for the complete activation of the LAL proclotting enzyme (25, 26). These agents, EDTA and citrate, variously used in the collection of plasma and other blood components, inhibit LAL, albeit at concentrations greater than those used for blood collection (unpublished data). Since no chemical agents were used to collect the serum used in this study, and since an increase in the time LPS was incubated in the presence of serum before LAL assay resulted in greater inhibition, we believe the inhibitory action of serum can be explained by the interaction of serum with LPS.

The differences in serum LR_{50}s among *E. coli* J5, *K. pneumoniae*, and *E. coli* O18 may reflect the difference in LPS chemistry (including solubility and aggregate molecular weight). *E. coli* J5 is a mutant of *E. coli* O111 that lacks the enzyme uridine diphosphate glucose 4-epimerase and produces an incomplete LPS deficient in glactose (2). *K. pneumoniae*, on the other hand, possesses a complete LPS (10) similar to the other LPS types studied. It is not clear why its inhibition in serum compared with saline appeared lower than others with complete LPS types.

To accurately assess the ability of serum to inhibit LPS, all LPS preparations were first compared with a reference standard in saline, and potency values in EU were assigned. It was hoped that this standardization would eliminate biological differences that arise when endotoxins are compared on a weight rather than an activity (potency) basis. The potency in saline of some of the LPS species used in this study agreed with those obtained by others (7) who found *Klebsiella* LPS to be less potent than *E. coli* LPS, and *E. coli* LPS to be similar in potency to the LPS from *Serratia marcescens*.

Contrary to expected results, however, apparent differences in the inhibition of LPS in serum increased when the relative potencies of the LPSs used were compared by using EU rather than a weight measure. These findings suggest that perhaps because of aggregation and solubility factors, LPS potency is valid only within the medium in which it is determined. When the inhibition of LPS in the presence of serum was compared by using different types of LPS, the more potent LPS types (determined in saline) were inhibited to a greater degree than the less potent ones. Thus, *E. coli* J5 LPS, which was the least potent LPS (1 EU/ng in saline), was also the least inhibited by serum, and *E. coli* O18, which was one of the most potent LPS (10 EU/ng in saline), was the most inhibited. The reason for this remains unclear. It is possible that the availability of the lipid A moiety of the various LPS species tested is not only related to the potency of the LPS but also to the degree of binding (resulting in serum inhibition). Thus, *E. coli* J5, which shows low potency (has fewer lipid A moieties available for LAL reactivity), is also the least inhibited by serum (has fewer lipid A groups available for serum binding). It remains to be seen whether differences in the ability of serum to inhibit LPS has any correlation with the ability of the parent bacterium to cause bacteremia or endotoxic shock.

Since the interaction of serum or plasma and LPS can be quantified with LAL, it is now possible to examine the antiendotoxin activity of serum or plasma from healthy and high-risk patient groups with a view to determining the clinical significance of this phenomenon. As we have shown, the amount of inhibition varies at least 12-fold in a normal donor population. Although the number of donors in this study was low, a trend to a normal distribution was indicated. In confirmation of this, 145 human recovered plasma samples and 212 fresh-frozen plasma samples have been tested by using the LR_{50} assay and *E. coli* O113 LPS. A 100-fold range of LR_{50} was found, with a normal distribution curve for these samples (H. S. Warren, T. J. Novitsky, P. A. Ketchum, P. F. Roslansky, S. A. Kania, and G. R. Siber, manuscript in preparation).

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