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Role of the Physical State of *Salmonella* Lipopolysaccharide in Expression of Biological and Endotoxic Properties

ALEXANDER SHNYRA,[1]† * KJELL HULTENBY,[2] AND ALF A. LINDBERG[1]

Department of Clinical Bacteriology F82[1] and the Clinical Research Centre,[2] Karolinska Institute, Huddinge Hospital, S-14186 Huddinge, Sweden

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Lipopolysaccharide (LPS) extracted from three strains of *Salmonella typhimurium*, i.e., the rough Re mutant SL1102, the rough Ra mutant TV119, and the smooth strain SH4809, was first electrodialyzed (eLPS) and then divalent cation deprived by EDTA treatment and finally made monomeric by deoxycholate solubilization. The removal of excess detergent by extensive dialysis in the absence of mineral cations resulted in the reassociation of LPS subunits into monodisperse micelles of reduced aggregation number (dLPS) as estimated by electron microscopy and gel filtration chromatography. For all LPS chemotypes tested, the developed procedure reproducibly results in stable and clear solutions of dLPS in concentrations of up to 100 mg/ml. The dLPS and eLPS preparations possessed the same reactivity with monoclonal antibodies (MAbs) raised against different LPS domains. The 100% lethal dose in galactosamine-sensitized mice of 0.01 μg for the smooth eLPS was from 10- to 100-fold lower than that of dLPS at 0.1 to 1.0 μg. dLPS from both the smooth strain and the Ra mutant had a significantly reduced capacity to activate the proenzyme cascade in the *Limulus* amoebocyte lysate assay in comparison with the slightly reduced activity of dLPS from the Re mutant. In contrast, dLPS as well as the deoxycholate-dispersed and then diluted eLPS from the smooth strain had a higher mitogenic activity on splenocytes than eLPS. The results indicate that the biological and endotoxic properties of LPS are significantly influenced by the physical state of its aggregates in aqueous solutions. The approach developed for production of a stable and dispersed form of LPS should further assist in investigation of LPS properties and interpretation of the data of endotoxic research.

The lipopolysaccharide (LPS) or endotoxin of gram-negative bacteria is an integral component of the outer membrane, which is a lipid bilayer with proteins interspersed between the phospholipid on the inner leaflet and the LPS on the exterior. LPS plays an important functional role in the outer membrane, providing it with a selective permeability, and as a major bacterial surface antigen, LPS determines the serological O specificity of gram-negative bacteria and their interaction with the host cells and bacteriophages (25). LPS consists of lipid A covalently bound via 3-deoxy-D-manno-octulosonic acid (Kdo) to a polysaccharide domain which in turn is subdivided into an oligosaccharide portion, the core, and a chain of polymerized repeating units of O antigen (22). LPS is an amphiphilic macromolecule with up to seven hydrophobic fatty acyl groups in the hydrophobic lipid A and with the hydrophilic polysaccharide part possessing negatively charged phosphate and carboxyl groups present mainly in the inner core polysaccharide. LPS is firmly bound to the surface of gram-negative bacteria, and lipid A is the membrane anchor of LPS in the bacterial envelope. Phospholipid-LPS and LPS-LPS hydrophobic bonds as well as the chelating effect of divalent cations such as Ca^{2+} and Mg^{2+} stabilize the outer membrane structure of gram-negative bacteria (10, 20).

LPS may be extracted from intact cells by different methods, including phenol-water and phenol-chloroform-petroleum ether procedures (9, 37). Because of self-aggregation, LPS has poor solubility in aqueous solutions and exists in a highly aggregated form as colloidal solutions or unstable suspensions (29). In fact, the growth conditions for the bacteria, the chemical composition, and the method of LPS isolation may affect heterogeneity of the macromolecular aggregates. The physical state of the LPS is dictated by the packing of the hydrophobic moiety of the molecules and dependent on the presence of inorganic cations and low-molecular-weight basic amines which neutralize negatively charged groups on LPS. Electrodialysis of LPS improves its solubility by reducing the amount of positively charged compounds bound to the LPS. The subsequent conversion of the acidic LPS into a triethylamine salt results in a preparation with a lower degree of aggregation (8).

Self-aggregation of LPS isolated from either smooth or rough bacterial strains may be prevented by surfactants, such as sodium deoxycholate, which disperse LPS and reduce its endotoxic activity (23). It has been assumed that deoxycholate-dispersed LPS exists in a dimer form (28). Removal of surfactant excess, however, results in the reaggregation of LPS and the appearance of macromolecular structures, suggesting that hydrophobic forces are important in determining the physical state and solubility of LPS.

When LPS is mixed with serum, the major part of it is complexed with high-density lipoprotein (HDL) which causes dispersion of LPS aggregates (33). This treatment diminishes some of the harmful biological effects of LPS such as fever, leukopenia, and complement activation, while activation of B lymphocytes and macrophages is less affected (34). Moreover, LPS released from bacteria treated with plasma was found in a monomeric form bound to different plasma proteins (32). The discrepancy in molecular size of LPS in aqueous solutions in vitro and LPS released from the bacteria in vivo upon exposure to plasma raises a fundamental question as to whether both forms of LPS have

* Corresponding author.

† Present address: Department of Emergency and Critical Care Medicine, Faculty of Medicine and Health Sciences, United Arab Emirates University, P.O. Box 17666, Al Ain, United Arab Emirates.

an equal potency to trigger a variety of biological responses in the body. A number of attempts have been made to determine the role of the physical state of LPS in interaction with target systems, including activation of complement (7) and B lymphocytes (31, 36), but the mechanisms which initiate and mediate the cellular effects of LPS remain poorly understood. It is not clear whether the profound changes in metabolism in response to intravenous LPS doses are mediated by nonspecific size-dependent interactions of LPS aggregates with cells of the reticuloendothelial system or whether LPS subunits are able to elicit endotoxic effects via receptor-mediated pathways.

The present investigation was undertaken to improve LPS solubility in aqueous solutions and to determine whether a relationship exists between the size of LPS aggregates and its ability to activate different target systems. Electrodialyzed LPS (eLPS) was first depleted of inorganic cations by EDTA treatment and then dispersed with deoxycholate. Dialysis reassociated the LPS into aggregates of reduced size and heterogeneity (disaggregated LPS [dLPS]) in comparison with eLPS. The biological activities of dLPS and eLPS were comparatively examined by using D-galactosamine-treated mice, splenocytes isolated from C3H/HeN mice, and a quantitative *Limulus* amoebocyte lysate (LAL) assay. The studies show that the subunit form of LPS is able to activate specific host responses; however, the degree of LPS aggregation is important for the endotoxic activities.

MATERIALS AND METHODS

Animals. Eight-week-old C3H/HeN and C57BL/6 male mice were obtained from Bomholtgård Breeding and Research Centre Ltd., Ry, Denmark.

Bacteria and growth conditions. *Salmonella typhimurium* SL1102 (chemotype Re), TV119 (chemotype Ra), and SH4809 (smooth strain) were from the strain collection of the Department of Clinical Bacteriology, Karolinska Institute, Huddinge Hospital, Huddinge, Sweden. Bacteria were grown in a 12-liter fermentor (LKB, Bromma, Sweden) to the late log phase in L broth medium (Oxoid Ltd., Hampshire, England), washed, and lyophilized.

LPS. LPS from smooth *S. typhimurium* SH4809 was isolated by phenol-water extraction (37). LPS from the rough strains *S. typhimurium* SL1102 and TV119 were extracted by a phenol-chloroform-petroleum ether procedure (9). LPSs were obtained in the free acid form by electrodialysis and converted into their triethylamine salts (8). To remove contaminating phospholipids, all LPSs were reextracted with chloroform-methanol (2:1) (21).

Analytical procedures. The calcium and magnesium concentrations in LPS preparations were determined by the method of Savory et al. (26). Kdo was estimated by a thiobarbituric acid method described previously (11). Phosphate was determined as described previously (2). The total sugar content of LPS in chromatographic fractions was measured by the phenol-sulfuric acid method (3). LPS preparations were analyzed by thin-layer chromatography on silica gel plates (2.5 by 10 cm; Kieselgel 60; Merck, Darmstadt, Germany) after their conversion to monophosphoryl lipid A by treatment with 0.1 N HCl. Plates were developed with chloroform-methanol-water-ammonium hydroxide (50:31:6:2). To visualize chromatograms, the plates were sprayed with 10% phosphomolybdic acid and charred at 150°C.

Gel chromatography of LPS. Twenty milligrams of LPS in 2 ml of 50 mM borate (H_3BO_3) buffer, adjusted to pH 8.1 by tetramethylammonium hydroxide (Sigma Chemical Co., St. Louis, Mo.), was applied to a column (2.6 by 55 cm) prepacked with Sephacryl S-1000 (Pharmacia LKB Biotechnology AB, Sollentuna, Sweden) and equilibrated with the borate buffer. Chromatography was carried out at 50 ml/h, and 2-ml fractions were collected and assayed for Kdo and total sugar content as described above.

MAbs and ELISA. The mouse monoclonal antibodies (MAbs) 6B8 (binding to lipid A), MATy-1 [recognizing the α-D-GlcNAc-(1→2)-α-D-Glc disaccharide and the α-D-Gal-(1→6) branch residue in the core], and MAST-107 (binding to the O-polysaccharide chain of LPS isolated from the *S. typhimurium* strains) were available from previous studies (14, 15, 30). The enzyme-linked immunosorbent assay (ELISA) was carried out as described previously (15). Microtiter enzyme immunoassay plates (Costar, Cambridge, Mass.) were coated overnight at 4°C with 100 μl of either eLPS or dLPS dissolved in 50 mM carbonate buffer (pH 9.6) at a concentration of 10 μg of LPS per ml. After three washes with 200 μl of phosphate-buffered saline (PBS)–0.05% Tween (pH 7.5), the nonspecific binding was blocked with 0.2% bovine serum albumin (BSA) in PBS-Tween for 1 h at 37°C and 100-μl aliquots of threefold dilution steps of each MAb were added. The plates were incubated for 2 h at 37°C and washed several times as described above, and 100 μl of anti-mouse polyvalent immunoglobulins conjugated with alkaline phosphatase (Sigma) was added after a 1,000-fold dilution in PBS-Tween. After incubation for 2 h at 37°C, unreacted conjugates were decanted and the plates were again washed with PBS-Tween. The enzyme reaction was developed with *p*-nitrophenyl phosphate in 10% diethanolamine-HCl buffer (pH 9.8), and the A_{405} was measured after 30 min of incubation at 37°C.

The inhibition ELISA was carried out with the plates coated with either eLPS or dLPS from *S. typhimurium* SH4809. MAbs were diluted in PBS-Tween to yield a concentration of approximately 250 ng/ml. One hundred microliters of each diluted MAb was added in triplicate with 100 μl of the threefold serial dilutions of either eLPS or dLPS from *S. typhimurium* SH4809 to microtitration wells coated with LPS in the corresponding form, e.g., eLPS or dLPS. After 2 h of incubation at 37°C, the plates were washed and developed as described above. The optimal conditions of the assay producing a sigmoidal inhibition curve were predetermined in pilot experiments. The percentage of inhibition was calculated as a reduction in A_{405} compared with a control value without inhibitor. A plot of the arcsine of percent inhibition versus log inhibitory concentration was created as described previously (4).

Lymphocyte proliferation. Mitogenic responses of splenocytes to different LPS preparations from *S. typhimurium* SH4809 were measured by [^{3}H]thymidine incorporation (17). Spleen cells isolated from C3H/HeN mice and purified on Ficoll-Paque (1) were resuspended in RPMI 1640 (GIBCO, Paisley, Scotland) supplemented with glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. A total of 0.5×10^6 viable cells per well was inoculated into 96-microwell plates (Costar) and incubated in an atmosphere of 95% air and 5% CO_2 for 24 h. The serial fivefold dilutions of a 1-mg/ml stock suspension of eLPS and dLPS from *S. typhimurium* SH4809 in MilliQ water and the same eLPS in 0.4% deoxycholate were prepared in RPMI 1640 immediately prior to assay. One hundred microliters of each LPS dilution step was added in triplicate to the cells and incubated for an additional 24 h. Finally, the stimulated cells were pulsed with 0.5 μCi of [^{3}H]thymidine (20 Ci/mmol;

Amersham Sweden AB, Solna), cultivated for an additional 24 h, and harvested with a cell harvester (Inotech Biosystems International, Inc., Jaffrey, N.H.). The incorporated radioactivity was determined on a MiniGamma 1275 counter (LKB-Wallac, Turku, Finland).

Lethality test on D-galactosamine-sensitized mice. The lethal toxicity of LPS was estimated by using D-galactosamine-treated C57BL/6 mice (6). Briefly, serial 10-fold dilutions of a 1-mg/ml stock suspension of eLPS and dLPS from *S. typhimurium* SH4809 in MilliQ water and the same eLPS in 0.4% deoxycholate were prepared in pyrogen-free PBS with 20 mg of D-galactosamine-HCl (Sigma) per ml immediately prior to administration. A 0.5-ml volume of the different LPS dilutions was injected intraperitoneally into the mice, and deaths were recorded 24 h after the treatment. A group of 10 mice was used for each dose of the LPS preparations tested. In control experiments, mice were given either 1 μg of LPS or 20 mg of D-galactosamine-HCl alone and no deaths occurred.

LAL assay. The capacity of different LPS preparations to catalyze the proenzyme cascade in the LAL was evaluated with the chromogenic quantitative COATEST endotoxin kit (Chromogenix AB, Mölndal, Sweden) by following the manufacturer's instructions for the water method.

Electron microscopy. Carbon film was coated with LPS suspended in MilliQ water at a concentration of 1 mg/ml, and the specimens were negatively stained with 1% uranyl acetate at pH 3.5 as described previously (29). Dried samples were examined in a Philips 420 electron microscope at 100 kV.

RESULTS

Electrodialysis and disaggregation of LPS. The procedure used for electrodialysis and disaggregation of LPS is outlined in Fig. 1. LPS from smooth bacteria was isolated by phenol-water extraction, and the aqueous phase was lyophilized. Traces of contaminating nucleic acids and proteins were removed by phenol-chloroform-petroleum ether reextraction. Contaminating phospholipids in the LPS preparations were removed by chloroform-methanol extraction. The specimens were electrodialyzed and converted into a triethylamine salt. The concentration of Ca^{2+} and Mg^{2+} in all LPS preparations tested was reduced after electrodialysis, but the reduction never exceeded more than 50% of the starting content (Table 1). The eLPS was lyophilized and used in comparative investigations with EDTA–deoxycholate-treated LPS (i.e., dLPS; see below).

Further depletion of the divalent cations in the eLPS preparations was obtained by EDTA treatment. eLPS was reconstituted in 50 mM Tris-HCl (Sigma) buffer (pH 7.0) at 21°C containing 10 mM EDTA (Merck) and sonicated for 5 min for better dispersion. The release of divalent cations was achieved at room temperature by dropping the pH to 4.0 by the addition of 1.0 M HCl, incubation for 2 min, and then neutralization with 1 M NaOH. The resultant LPS was dialyzed at 4°C for 3 days against 5-liter daily changes of the Tris-EDTA buffer and, finally, against MilliQ water before lyophilization. The content of Ca^{2+} in EDTA-treated LPS was reduced to 4 to 13% in untreated LPS, and that of Mg^{2+} was reduced to ≤1% (Table 1). While the resultant EDTA-treated LPS had a reduced amount of the divalent cations, it appears that the procedure caused no chemical modifications of the LPS molecules as assessed by analysis of relative Kdo and phosphate concentrations (Table 1). EDTA-treated LPS and electrodialyzed LPS preparations were also analyzed by thin-layer chromatography and revealed a similar appearance in that monophosphoryl lipid A samples denerated from dLPS and control eLPS had equal R_f values (data not shown).

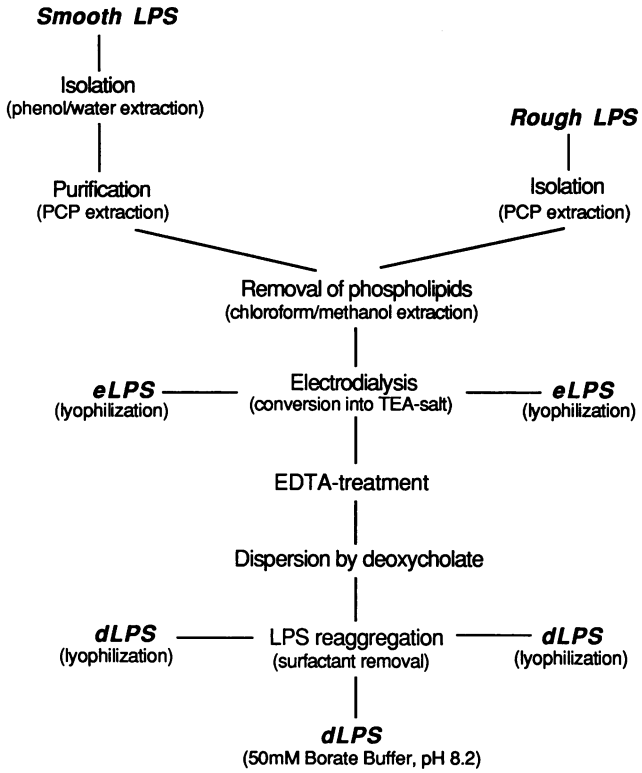

FIG. 1. Schematic representation of the purification and dispersion procedure developed for smooth and rough LPS. TEA, triethylamine; PCP, phenol-chloroform-petroleum ether.

Since EDTA treatment results in a substantial decrease of Ca^{2+} and Mg^{2+} content in the LPS preparations, we expected that the LPS would have altered physical properties. EDTA-treated LPS was prepared in mineral cation-free 50

TABLE 1. Comparative chemical analysis of LPS preparations

LPS prepn	Amt (μmol/mg) in LPS prepn[a]			
	Ca^{2+}	Mg^{2+}	Kdo	Phosphate
S. typhimurium SL1102				
Untreated	0.078	0.119	0.098	1.526
eLPS[b]	0.070	0.078	0.096	1.502
dLPS[c]	0.010	0.001	0.104	1.488
S. typhimurium TV119				
Untreated	0.105	0.210	0.049	1.473
eLPS	0.058	0.148	0.050	1.497
dLPS	0.008	0.002	0.047	1.489
S. typhimurium SH4809				
Untreated	0.143	0.169	0.043	1.729
eLPS	0.120	0.094	0.039	1.711
dLPS	0.005	0.002	0.038	1.758

[a] Each value depicts the mean of triplicate determinations from three separate LPS batches. Standard deviations were less than 10%.
[b] eLPS, electrodialyzed LPS.
[c] dLPS, electrodialyzed, EDTA-treated LPS.

mM borate buffer adjusted to pH 8.1 by tetramethylammonium hydroxide. The LPS was dispersed into a subunit form by 0.4% deoxycholate. Subsequent dialysis of the samples against borate buffer (pH 8.1) at 4°C by using Spectra/Por 6 dialysis tubing (molecular weight cut-off, 2,000) allowed the LPS subunits to reaggregate in the absence of divalent cations. The resultant preparations had a reduced size of LPS aggregates in comparison with that of eLPS (see below) and were designated dLPS.

Electron microscopy of LPS preparations. Figure 2a, c, and e shows negatively stained eLPS isolated from *S. typhimurium* SL1102, TV119, and SH4809, respectively. eLPSs were mostly found in the form of rods and spherical particles with an apparent heterogeneity. Electron microscopy of dLPS from the rough strains *S. typhimurium* SL1102 and TV119 revealed a lamellar network structure of tiny rods of homogeneous size (Fig. 2b and d), while smooth dLPS from *S. typhimurium* SH4809 reassociated into small spherical particles (Fig. 2f). We surmise that the observed differences in the shape of the resultant dLPS aggregates reflect the relative balance of the hydrophobic lipid A region and the hydrophilic polysaccharide region in the LPS molecules.

Physicochemical properties of disaggregated LPS. Aqueous suspensions of eLPS from the deepest rough mutants *S. typhimurium* SL1102 and *Salmonella minnesota* R595 are opalescent and unstable at a concentration of 1 mg/ml. In contrast, the dLPS suspensions of the same chemotype in MilliQ water or mineral cation-free borate buffer are stable and clear preparations of low viscosity and opalescence at a concentration of 30 to 50 mg/ml and even at 100 mg/ml (R595). To quantitatively characterize this difference, the eLPS and dLPS suspensions of various chemotypes were subjected to filtration through a 0.45-μm-pore-size filter (Acrodisc; Gelman Sciences GmbH, Dreieich, Germany), and the LPS concentration was assessed by Kdo determinations (Table 2). Regardless of dLPS chemotype tested, the loss of the material on the filter was less than 5%, compared with 22 and 29% for those of eLPS isolated from *S. typhimurium* SL1102 and TV119, respectively, while the retention of smooth eLPS from *S. typhimurium* SH4809 was initially low (<5%). The findings support the hypothesis that depletion of divalent cations is a prerequisite for improved LPS dispersion subsequently achieved by the dissociation-reassociation procedure.

To further examine the physicochemical properties of the eLPS and dLPS preparations, they were subjected to Sephacryl S-1000 gel chromatography (Fig. 3). The rough eLPS from *S. typhimurium* SL1102 and TV119 eluted as a broad peak in the early fractions corresponding to a high molecular weight (Fig. 3A and B). Three major peaks were evident when the eLPS from the smooth strain *S. typhimurium* SH4809 was chromatographed, suggesting a polydispersity of eLPS aggregates (Fig. 3C). In contrast, all of the corresponding dLPS eluted much later as a single sharp peak, indicating a reduced and uniform size of dLPS micelles. All three dLPS preparations had approximately the same elution volume. Calibration of the column with molecular standards of known size demonstrated that they eluted from the column after human low-density lipoprotein and in front of blue dextran (molecular weight, 2,000,000). In addition, the eLPS from the rough strain *S. typhimurium* SL1102 had a consistently lower chromatographic recovery than that of dLPS. With equal amounts of this LPS applied to the column, approximately 20 and 80% of the starting eLPS and dLPS preparations, respectively, were recovered (Fig. 3A). The recoveries of eLPS and dLPS from both *S. typhimurium* TV119 and SH4809 were similar and represented 75 to 80% of LPS applied.

To support an idea that the removal of divalent cations such as Ca^{2+} and Mg^{2+} plays an important role in disaggregation of LPS, dLPS from the smooth strain *S. typhimurium* SH4809 was incubated with 10 mM $CaCl_2$ at 37°C for 4 h before chromatography (Fig. 3D). The $CaCl_2$-treated dLPS eluted from the column in the early fractions, suggesting a cation-induced reaggregation of dLPS.

Immunological reactivity of LPS preparations. Possible chemical modifications and/or conformational changes in the LPS preparations resulting from the EDTA-deoxycholate treatment were evaluated by using a panel of well-characterized MAbs raised against the different LPS domains, including lipid A (6B8), the core (MATy-1), and O antigen (MAST-107). No difference in reactivity of the dLPS preparations from three *Salmonella* strains with the antibodies was demonstrated on a direct ELISA in comparison with that of eLPS (data not shown). Figure 4A shows the results of inhibition ELISA carried out with eLPS and dLPS from *S. typhimurium* SH4809 as competitors of MAST-107 MAb binding to the O chain of corresponding LPS on the surface. Presenting these data as a plot of the arcsin of percent inhibition versus the logarithm of inhibitory concentration shows a 1,000-fold increased sensitivity of the immunoassay with dLPS (Fig. 4B). Similarly, dLPS from *S. typhimurium* SH4809 had at least a 10-fold-greater inhibitory effect on the binding of MATy-1 MAb to the core epitopes than eLPS (Fig. 5C and D). An inhibitory assay using 6B8 MAb and dLPS from *S. typhimurium* SH4809, however, did not reveal any difference in assay sensitivity (data not shown). A two- to threefold increase in sensitivity of inhibitory ELISA, however, was demonstrated with dLPS of Ra and Re chemotypes and MATy-1 or 6B8 MAb in comparison with the eLPS assay (data not shown).

Lethal toxicity of LPS. The comparative lethality of *S. typhimurium* eLPS and dLPS of three chemotypes was investigated by using D-galactosamine-treated C57BL/6 mice. Table 3 summarizes the results obtained with eLPS and dLPS from *S. typhimurium* SH4809. All mice in the control groups treated with either 20 mg of D-galactosamine per mouse, 1 μg of LPS per mouse, or a 1,000-fold-diluted 1% deoxycholate survived the 24-h observation period (data not shown). The result for the mice given dilutions of eLPS from *S. typhimurium* SH4809 in 20 mg of D-galactosamine per ml was 100% lethality with a dose of ≥0.01 μg. The *S. typhimurium* SH4809 dLPS caused 70 and 100% mortality with 0.1 and 1 μg per mouse, respectively. Thus, the lethal toxicity of dLPS was approximately 10- to 100-fold less than that of eLPS. Similar changes in lethal toxicity were also manifested by eLPS and dLPS preparations of Re and Ra chemotypes (data not shown). That eLPS and dLPS prepa-

FIG. 2. Electron micrographs of negatively stained LPS preparations from different bacterial strains. Bars, 0.1 μm. (a) eLPS from the rough mutant *S. typhimurium* SL1102 (Re chemotype); (b) dLPS from the same bacterial strain as that in panel a; (c) eLPS from the rough strain *S. typhimurium* TV119 (Ra chemotype); (d) dLPS from the same bacterial strain as that in panel c; (e) eLPS from *S. typhimurium* SH4809 (wild type); (f) dLPS from the same bacterial strain as that in panel e.

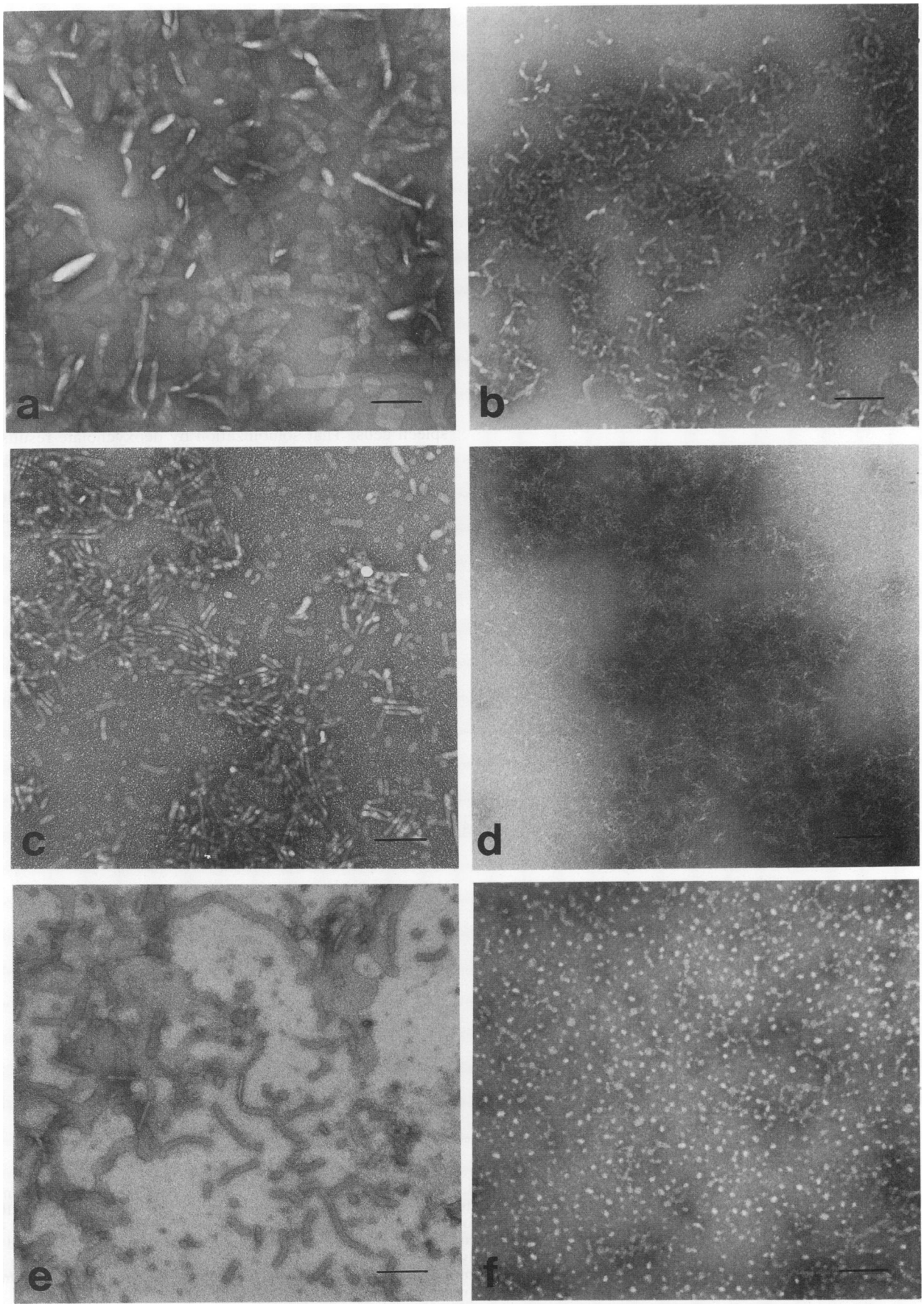
a
b
c
d
e
f

TABLE 2. Changes in concentration of LPS preparations after filtration via a 0.45-μm-pore-size membrane filter

LPS	OD_{548} (mean ± SD)[a]		Yield (%)[b]
	Before filtration	After filtration	
S. typhimurium SL1102			
eLPS	1.472 ± 0.09	1.146 ± 0.06	77.7
dLPS	1.349 ± 0.06	1.264 ± 0.05	95.7
S. typhimurium TV119			
eLPS	0.722 ± 0.04	0.512 ± 0.04	70.9
dLPS	0.738 ± 0.03	0.723 ± 0.03	98.0
S. typhimurium SH4809			
eLPS	0.616 ± 0.02	0.593 ± 0.02	96.2
dLPS	0.609 ± 0.03	0.583 ± 0.01	95.8

[a] LPS concentrations were estimated by Kdo determinations. OD_{548}, optical density at 548 nm.
[b] The values before filtration are taken as 100%.

rations were found to differ only in the size of their aggregates suggests that disaggregation of LPS diminishes endotoxic lethal activity. When *S. typhimurium* SH4809 eLPS was mixed with 0.4% deoxycholate at a concentration of 1 mg/ml and diluted in PBS before injection, 80 and 100% lethalities were seen at 0.1 μg and 1.0 μg per mouse, respectively. It is likely that the presence of dLPS in a highly disaggregated form rather than the loss of active subcomponents during EDTA-deoxycholate treatment of eLPS determines changes in the lethal toxicity of the former preparation.

Mitogenic activity of LPS. We next investigated the mitogenic capacity of *S. typhimurium* eLPS and dLPS of different chemotypes by using splenocytes isolated from LPS-responsive C3H/HeN mice. In general, dLPS preparations exhibited slightly higher mitogenic potency at low concentrations than did corresponding eLPS. However, only the difference in the activity between dLPS and eLPS from *S. typhimurium* SH4809 was statistically significant (Fig. 5). The role of the eLPS and dLPS macromolecular state in their differential mitogenic activity was further supported by using *S. typhimurium* SH4809 eLPS solubilized in 0.4% deoxycholate at a concentration of 1 mg/ml. Diluted immediately prior to a [^{3}H]thymidine incorporation assay, this LPS preparation triggered the highest proliferative response of spleen cells. That solubilization by deoxycholate results in the appearance of eLPS in a subunit form representing an LPS dimer and that subsequent dilution of the sample to a concentration of 100 ng/ml or less would probably exclude reassociation of the LPS subunits into larger aggregates

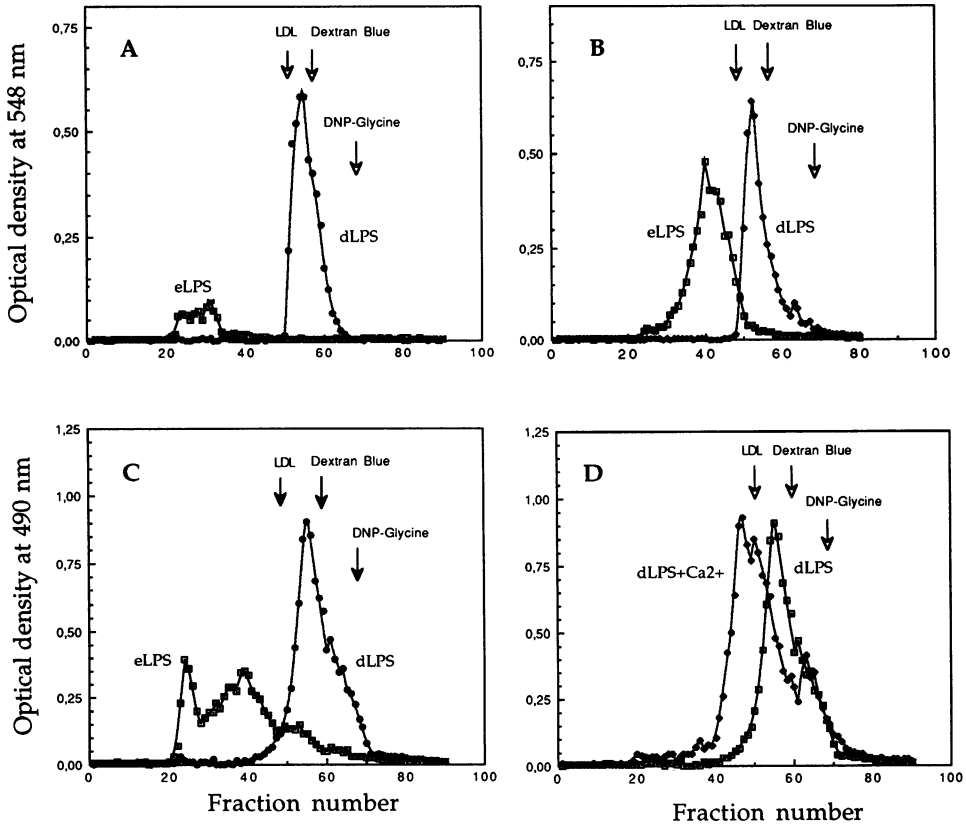

FIG. 3. Gel filtration chromatography of LPS on Sephacryl S-1000. eLPS and dLPS from different bacterial strains were prepared in 50 mM borate buffer (pH 8.1) at a concentration of 10 mg/ml. Two milliliters of LPS preparations was chromatographed on a Sephacryl S-1000 column as described in Materials and Methods. Kdo and total sugar content in the chromatographic fractions were measured at 548 and 490 nm, respectively. A representative elution profile of four to five separate experiments performed with different LPS batches is presented for each eLPS and dLPS preparation. DNP-glycine, 2,4-dinitrophenyl-glycine; LDL, low-density lipoprotein. (A). eLPS and dLPS from the rough mutant *S. typhimurium* SL1102; (B) eLPS and dLPS from the rough mutant *S. typhimurium* TV119; (C) smooth eLPS and dLPS from *S. typhimurium* SH4809; (D) dLPS and $CaCl_2$-treated dLPS from *S. typhimurium* SH4809.

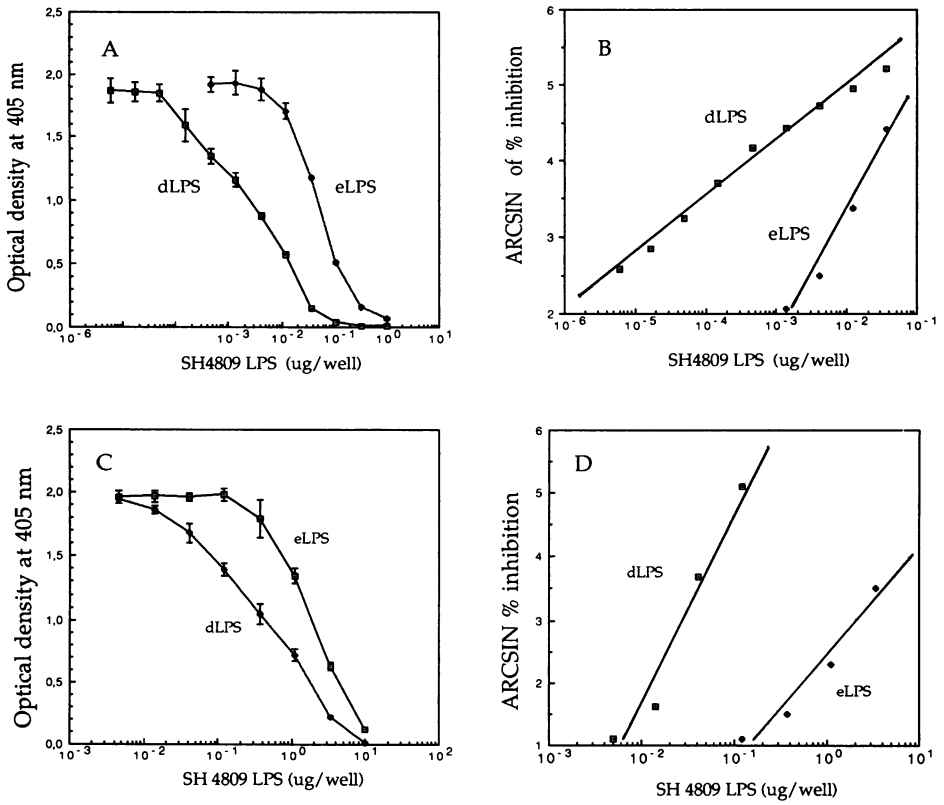

FIG. 4. Inhibition ELISA. The inhibition immunoassay was done with eLPS and dLPS from *S. typhimurium* SH4809 and MAST-107 MAb against the O-polysaccharide chain of *S. typhimurium* strains or MATy-1 MAb binding to the LPS core (see Materials and Methods). A representative inhibition curve of three to four separate experiments with three parallels utilizing different LPS batches is shown for each LPS-MAb combination tested. (A) ELISA using MAST-107 MAb and eLPS or dLPS from *S. typhimurium* SH4809; (B) a linear transformation of the data given in panel A as a plot of the arcsine of percent inhibition versus the logarithm inhibitory concentration; (C) ELISA using MATy-1 MAb and eLPS or dLPS from *S. typhimurium* SH4809; (D) the semilogarithmic representation of the result given in panel C as a plot of the arcsine of percent inhibition versus the logarithm inhibitory concentration.

make us postulate that the LPS subunit might be the active structure responsible for the proliferative activation of splenocytes.

Assessment of endotoxic activity of LPS on LAL assay. LPS is known to be a potent trigger of the coagulation cascade in LAL (13). We found that the macromolecular state of LPS in aqueous solutions influenced its endotoxic activity as estimated in the LAL assay (Fig. 6). The reducing size of dLPS aggregates parallels the decrease of endotoxic potency to catalyze the enzymatic cascade. A 10-fold difference between eLPS and its dispersed form, dLPS, was demonstrated with the preparations from *S. typhimurium* SH4809 (145.0 versus 15.2 endotoxin units [EU]/pmol). The endotoxic activity of dLPS from the rough strain *S. typhimurium* TV119 was only 50% decreased in comparison with that of eLPS (194.9 versus 92.8 EU/pmol). The EDTA-deoxycholate treatment of the eLPS from the deep rough mutant *S. typhimurium* SL1102 had only a marginal effect (264.4 EU/pmol for eLPS versus 229.9 EU/pmol for dLPS).

DISCUSSION

LPSs are a class of compounds distinguished by their amphiphilic properties. Each LPS molecule contains both a hydrophilic polysaccharide part and a hydrophobic, lipid A moiety. The hydrophobic interactions are the primary driving forces for the formation of LPS micelles in aqueous

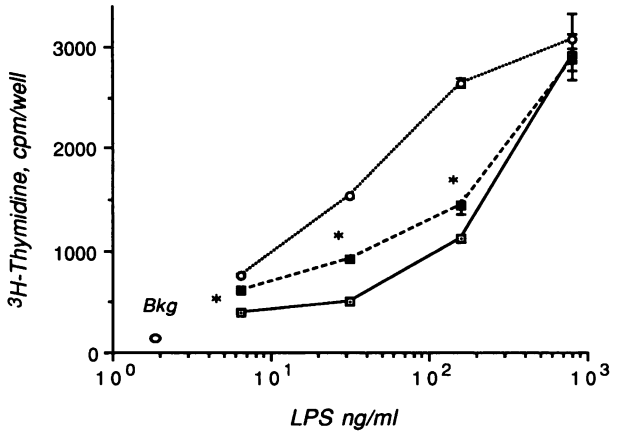

FIG. 5. Proliferative response of C3H/HeN spleen cells to different LPS preparations from *S. typhimurium* SH4809. The mitogenic response of splenocytes from C3H/HeN was assessed by [^{3}H]thymidine incorporation (see Materials and Methods). Each point represents the mean counts per minute ± standard deviation of five separate experiments utilizing different eLPS (⊡) and dLPS (■) batches and the eLPS in 0.4% deoxycholate (○) in which triplicate cultures were performed in each experiment for each LPS preparation. *, by Student's *t* test, $P < 0.05$, for dLPS versus eLPS.

TABLE 3. Lethal toxicity of LPS preparations from *S. typhimurium* SH4809 in D-galactosamine-sensitized C57BL/6 mice[a]

LPS (μg/mouse)	Lethality (no. of dead mice/total no.)		
	eLPS	dLPS	eLPS-DOC[b]
1.0	10/10	10/10	10/10
0.1	10/10	7/10	8/10
0.01	10/10	0/10	0/10
0.001	0/10	0/10	0/10
0.0001	0/10	0/10	0/10

[a] D-Galactosamine (12.5 mg per mouse) and LPS at the concentrations given were injected intraperitoneally as a mixture in 0.5 ml of endotoxin-free PBS.

[b] eLPS was dispersed in 0.4% deoxycholate at a concentration of 1 mg/ml. The appropriate dilutions of the resultant solution were made in PBS, and the aliquots were administered with D-galactosamine (see text for details).

solutions (8, 29). These relatively weak but abundant interactions lead to self-aggregation of LPS subunits, which ultimately results in the poor solubility of LPSs. While the micellar structure adopted by a particular LPS depends on the chemical composition of its monomers and on the relative ratio of hydrophobic to hydrophilic regions, it has been shown that divalent cations are important by providing an additional stabilization of LPS aggregates by means of low energy bonds of a chelating nature (27). The poor solubility and varying aggregation of LPS have inadvertently resulted in different interpretations of data in endotoxic research. Therefore, the fundamental questions as to whether a physical state of LPS can affect the biological properties of LPS and how it can be related to the activity of LPS released during an infectious process in the host were raised. By disaggregation and improved LPS dispersion in aqueous solution, we have attempted to shed more light on the influence of the size of the micelles on endotoxic activity.

A number of different attempts to improve dispersion of LPS in aqueous solutions have been tried, e.g., binding and complexing of LPS to carriers (31) or dispersion by detergents (23, 28, 36), electrodialysis, and subsequent conversion of LPS into a triethylamine salt form (8). We found that EDTA treatment of eLPS of different chemotypes leads to the further removal of divalent cations (Table 1), which increases the net negative charge of the molecule because of the presence of phosphate groups and acidic Kdo residues. This probably decreases the stability of the LPS aggregates by charge-charge repulsion. In fact, solubilization of EDTA-treated LPS into a subunit form by sodium deoxycholate, a detergent with high critical micelle concentration (4 mM) and low aggregate number ($N = 2$ to 3) at a pH of ≥8.0 (19), and the subsequent removal of excess detergent by dialysis resulted in reassociation of LPS into monodisperse micelles with reduced size in the absence of divalent cations (Fig. 2). Although EDTA treatment might also alter LPS-deoxycholate interactions, it seems that depletion of divalent cations is vital for dispersion of LPS aggregates.

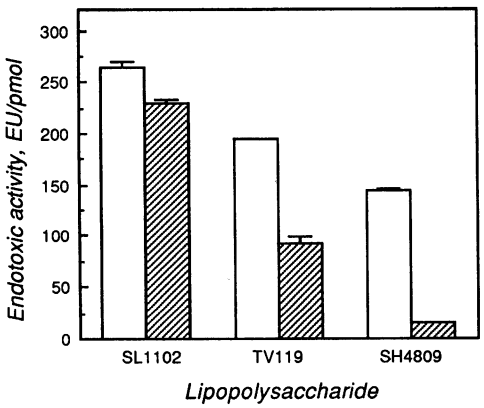

FIG. 6. Comparative evaluation of the endotoxic properties of LPS preparations by using the LAL assay. A standard calibration curve for determination of the endotoxic activity of eLPS (□) and dLPS (▨) was created by using the recommended procedure for the water method and LPS standard provided by the manufacturer. The endotoxic activity was measured in triplicate for each LPS preparation tested. The optimal conditions for the assay were predetermined in pilot experiments. The data represent average values of three separate experiments utilizing three different batches of the eLPS and dLPS preparations.

Comparative chromatographic investigations of eLPS and dLPS from different *Salmonella* strains also indicated that EDTA-deoxycholate treatment reduced polydispersity and size of the LPS macromolecular aggregates (Fig. 3). In addition, the treatment improved LPS dispersion in aqueous solutions as assessed by filtration via a 0.45-μm-pore-size filter (Table 2). We also found that the method is efficacious irrespective of the chemotype of LPS subjected to the disaggregation. Besides *S. typhimurium* SL1102 LPS (Re), TV119 LPS (Ra), and SH4809 LPS (smooth), a list of successfully treated LPSs includes those isolated from *S. typhimurium* SL1181 (Rd2), SL1032 (Rd1), SL805 (Rc), TV148 (Rb3), TV161 (Rb2), and SL733 (Rb1; data not shown). Moreover, lyophilized dLPS preparations can be easily and quickly reconstituted in aqueous solutions. The reduced size of dLPS micelles remained unchanged at least for the smooth dLPS from *S. typhimurium* SH4809 as estimated by gel filtration chromatography (data not shown).

The dLPSs were found to differ from the parent eLPS preparations only in the physical state of their macromolecular aggregates. No chemical and/or conformational modifications were demonstrated by using well-characterized MAbs against different LPS epitopes as probes. None of the dLPSs showed a reduced reactivity with MAbs in direct immunoassays. However, the results of a direct ELISA are difficult to interpret since the amounts of eLPS and dLPS coated onto the plates could be different. Adsorption to a microtiter plate may also induce conformational reorganization of the molecule or mask specific epitopes on LPS, which then affect the binding capacities of MAbs to LPS. Therefore, soluble-antigen inhibitory assays were carried out and considered primarily in the present study. By using an inhibitory ELISA technique, we were able to demonstrate the difference between eLPS and dLPS from *S. typhimurium* SH4809 in reactivity with MAbs. We found that specific epitopes in the O-polysaccharide chain and the core of dLPS are more readily accessible for binding with MAST-107 and MATy-1 MAbs, respectively (Fig. 4). Similarly, the higher binding of MATy-1 and 6B8 MAbs to dLPS versus eLPS of Ra and Re chemotypes was also demonstrated on a soluble-antigen ELISA (data not shown). These results would reflect intrinsic differences in the availability of the innermost epitopes between polydisperse eLPS and monodisperse dLPS macromolecular aggregates of reduced size. It is likely that the high density of polysaccharide present in large macromolecular aggregates of eLPS sterically hinders the inner epitopes of LPS. In contrast, a relative decrease of polysaccharide density within small LPS micelles should be

expected and may favor the availability of some specific structures.

The eLPS and dLPS from R-chemotype and smooth LPS were used to assess whether a relationship exists between immunomodulatory potency and the physical state of their macromolecular aggregates in aqueous solution. We found that dLPSs of all three chemotypes at very low concentrations were more potent activators of the proliferative response of splenocytes from C3H/HeN mice than eLPS, although only the difference in the activity between smooth eLPS and dLPS was statistically significant (Fig. 5). These results are consistent with recently obtained data that BSA-ReLPS complexes with an apparent mass of about 70 kDa were more stimulatory at low concentrations than ReLPS on the LPS-responsive murine pre-B cell line 70Z/3 (31). Perhaps the mitogenic activation of splenocytes is a receptor-mediated process which occurs via a recently described 73-kDa LPS binding protein on murine lymphocytes and macrophages (12).

Our experiments carried out to compare the endotoxicity of the dLPS and eLPS showed that dLPS had a reduced activity as estimated both in the in vivo lethal test on C57BL/6 mice (Table 3) and in vitro by using the LAL assay (Fig. 6). The most noticeable differences in biological activity were, however, found in the differential profiles of eLPS and dLPS from smooth bacteria. On the other hand, our data indicate that the most drastic change in the size of macromolecular aggregates after EDTA-deoxycholate treatment was obtained with smooth LPS as well (Fig. 2). It is also intrinsically obvious that the dispersed smooth LPS micelles may be considerably more stable than those of R chemotypes while exposed to blood or LAL cations because of the presence of the hydrophilic O-polysaccharide chain setting up the repulsive forces and preventing formation of large colloidal aggregates. Whatever the precise mechanism underlying the differential profiles of eLPS and dLPS activity, we conclude that the differences between the quaternary configuration of dLPS and eLPS macromolecular aggregates define the endotoxic properties of the LPS preparations.

It is generally accepted that macrophages are the cells mediating the lethal activity of LPS via production of a variety of mediators of endotoxic shock and, in particular, tumor necrosis factor (5). Although the biochemical pathway by which macrophages recognize and become activated by LPS is still poorly understood, two distinct mechanisms for this process have been proposed, namely, specific and nonspecific. The specific interaction relates to a receptor-mediated interaction. However, a large body of experimental evidence suggests that most endotoxic effects are a result of nonspecific interactions of large hydrophobic LPS aggregates with target systems (reviewed in reference 16). On the other hand, it is well known that the hydrophobicity, charge, and chemical composition of the particle surface play important roles in the determination of the nature of cell-particle interaction in phagocytosis. A proposed general physical model for phagocytosis states that a phagocyte engulfs particles whose surface hydrophobicity is greater than its own (35). It is also clear that divalent cation deprivation increases the net negative charge of dLPS and alters the balance between hydrophobic and hydrophilic forces in the molecule. These changes may influence dLPS interaction with macrophages and thereby diminish its endotoxic properties.

Alternatively, several lines of evidence provide support for the concept of serum-mediated inactivation of LPS because of its disaggregation by interaction and binding to serum proteins as an obligatory step in detoxification (24, 32). Among other serum proteins, HDLs are believed to be important factors which bind and neutralize LPS (33, 34). In addition, it has been demonstrated that incubation of isolated HDL with LPS does not result in complexing and neutralizing of the endotoxic properties. However, pretreatment of LPS by sodium deoxycholate, which disperses LPS aggregates, enhanced its subsequent binding to HDL (18). It is likely that the disaggregation of LPS micelles accomplished by combined EDTA-deoxycholate treatment might facilitate the LPS binding to HDL and neutralization of its endotoxic properties.

Finally, although the molecular mechanisms involved in LPS interaction with target cells are still incompletely understood, we believe that LPS must be in a highly aggregated state to be endotoxic. Our findings that detoxification of LPS can result from a reversible change of its physical state coincide with this concept.

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