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Characterization of Lipopolysaccharide Fractions and Their Interactions with Cells and Model Membranes

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The role of the length of the O-antigen polysaccharide side chain of bacterial lipopolysaccharide (LPS) in biological and model membrane systems was investigated. LPS from *Salmonella typhimurium* ATCC 14028 was chromatographed on a Sephadex G-200 column in the presence of sodium deoxycholate and separated into three fractions on the basis of molecular size. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blot (immunoblot), and chemical analyses indicated that these fractions differed from each other primarily in the number of repeating units in the O-antigen polysaccharide side chain. In a biological system fractions 2 and 3 had the same effects to induce mitogenesis in murine lymphocytes, but fraction 1 was less effective than the other two fractions. In a model membrane system, LPS induced changes in small unilamellar vesicles (SUVs) which were measured by changes in the behavior of a fluorescent probe, 1,6-diphenylhexa-1,3,5-triene (DPH), and interaction of increasing amounts of all LPS fractions with SUVs gradually increased DPH anisotropy. Fractions 2 and 3 had similar effects on the SUVs as detected by changes in DPH anisotropy, while fraction 1 had almost twice as much activity as the other two fractions. These results suggest that the polysaccharide side chain of LPS may modulate the ability of biologically active lipid A to interact with cells and model membranes. In addition, factors other than changes in membrane fluidity may play a role in mediating LPS-induced cell activation.

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria. The structure of LPS, particularly that from members of the family *Enterobacteriaceae*, is composed of three well-defined structural regions: (i) the hydrophobic lipid A which anchors it in the outer leaflet of the outer membrane and has about seven fatty acid chains linked to the diglucosamine backbone, (ii) the core oligosaccharide, and (iii) the O-antigen polysaccharide side chain. The polysaccharide extends into the cell surroundings (see references 5, 12, 15, 22, 23, and 36 for reviews). This structure is generally for a monomer unit in smooth strains of bacteria. However, because of its amphipathic nature, LPS in an aqueous solution exists as aggregates of macromolecular subunits. In addition, LPS from rough mutants is deficient in various amounts of core oligosaccharide.

For bacteria, LPS seems to be important for maintaining the integrity of the microorganisms in an adverse environment and as a barrier against the entry of small molecules into the bacteria (24, 28, 40). On the other hand, LPS is responsible for inducing various pathophysiological effects in hosts, including fever, leukocytosis, hypotension, and toxic shock, which can lead to death (see references 25 and 26 for reviews). However, there is little information on the molecular basis of LPS interactions with cellular membranes.

In the immune system LPS is a potent mitogen for bone marrow-derived lymphocytes (B cells) which are stimulated in vitro (6, 32) and in vivo (38) to DNA synthesis and immunoglobulin synthesis and secretion without the assistance of T-helper cells. This preference of LPS for B-cell activation suggests that these cells have a specific binding site or receptor for LPS on the plasma membrane. Some investigators have also examined the physical properties of the membrane after interaction with LPS. The results have demonstrated that LPS interaction with phospholipid monolayers or bilayers is based on both hydrophobic and hydrophilic forces (2, 30, 37). The sequence of interaction, however, is not discernible from these studies.

Recently, Jacobs (13) studied the binding of LPS at the single-cell level by evaluating binding in the presence of various charged molecules and after different target cell treatments. The results suggested a two-step process for the interaction of LPS with the cell membrane. The first step is an ionic interaction between two amphipathic aggregates to overcome electrostatic and hydration repulsions, equivalent to adherence. The characteristics of this step are temperature independent, susceptible to inhibition by polycations such as polymyxin B, and reversible. The second step is an insertion of the lipid A portion of LPS into the cell membrane, or hydrophobic interaction, equivalent to coalescence. This step is characterized as temperature dependent, not inhibitable by polymyxin B, and not reversible (13, 35).

Almost all studies which have examined the biological activity of LPS or the binding characteristics of LPS and which have used material from smooth (wild-type) enteric bacteria have not taken into consideration that this is a mixture of LPS molecules with different amounts of carbohydrate. Jessop and Lambert (16) used two strains of *Serratia marcescens* to investigate the role of surface polysaccharide in determining the sensitivity to serum killing. They found that the resistant strain has about 33% more phenol-extractable polysaccharide material than the sensitive strain, inferring that the resistant strain contains a longer O-antigen polysaccharide side chain than the sensitive strain. This was

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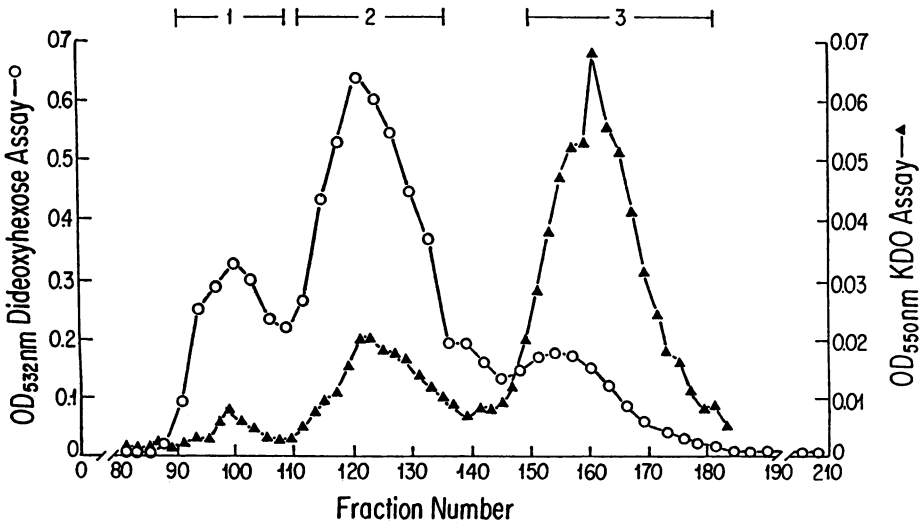

FIG. 1. Profile of LPS fractionation from *S. typhimurium* ATCC 14028 on a Sephadex G-200 column. Each fraction was analyzed for KDO and 3,6-dideoxyhexose. The horizontal axis indicates fraction numbers in the order of elution; the vertical axes indicate optical densities (OD) of KDO and 3,6-dideoxyhexose. Fractions were pooled according to the peaks, denoted by 1, 2, and 3, and were named fractions 1, 2, and 3, respectively. Recovery of material prepared in four separate fractionations averaged 88%, ranging from 80 to 91% by weight. The average material recovery of each fraction was 17, 36, and 35% by weight for fractions 1, 2, and 3, respectively. In one experiment, LPS fraction preparations were incubated with tracer radioactive ^{14}C-sodium deoxycholate (DOC) and dialyzed against the eluting buffer without DOC to determine the residual DOC in the fractions. Result shows that >99.9% of DOC was removed from the fractions by equilibrium dialysis by the end of 12 changes of buffer.

confirmed by Grossman et al. (9), who used LPS mutants of *Salmonella montevideo* to study the role of LPS size in serum resistance. They demonstrated that the survival of the organisms during serum treatment depends on the length of the O-antigen polysaccharide side chain associated with LPS. The longer the O-antigen polysaccharide side chain is, the more resistant the organisms are to serum killing.

Similar results can be obtained in an isolated in vitro system. Porat et al. (34) fractionated LPS from both sensitive and resistant strains of *Escherichia coli* on a Sephadex column and tested the role of the length of the O-antigen polysaccharide side chain in serum killing in a liposomal model. As expected, liposomes containing higher-molecular-weight LPS fractions from sensitive or resistant strains are not lysed by normal human serum, while liposomes containing lower-molecular-weight LPS fractions from sensitive or resistant strains are lysed by normal human serum. In this paper, we have fractionated LPS from *Salmonella typhimurium* into three populations on the basis of amounts of O-antigen polysaccharide side chain and have evaluated and compared mitogenic properties of LPS fractions and their interaction with model membranes.

LPS fractionation by Sephadex chromatography. Several reports (7, 10, 11, 27, 31, 33) have demonstrated that the heterogeneity of the O-antigen polysaccharide side chain of LPS can be separated into subunits of different sizes in the presence of sodium deoxycholate. LPS from *S. typhimurium* (ATCC 14028; List Biological Laboratories, Inc., Campbell, Calif.) was fractionated in the presence of 0.25% sodium deoxycholate by chromatography on a Sephadex G-200 column according to the procedure described by Peterson and McGroarty (33). Because 2-keto-3-deoxyoctulosonic acid (KDO) and 3,6-dideoxyhexose are located in the core oligosaccharide and O-antigen polysaccharide side chain of LPS, respectively, each fraction was assayed for KDO (8) and 3,6-dideoxyhexose (3, 43). The elution profile for LPS samples could be sorted into three peaks by these sugar

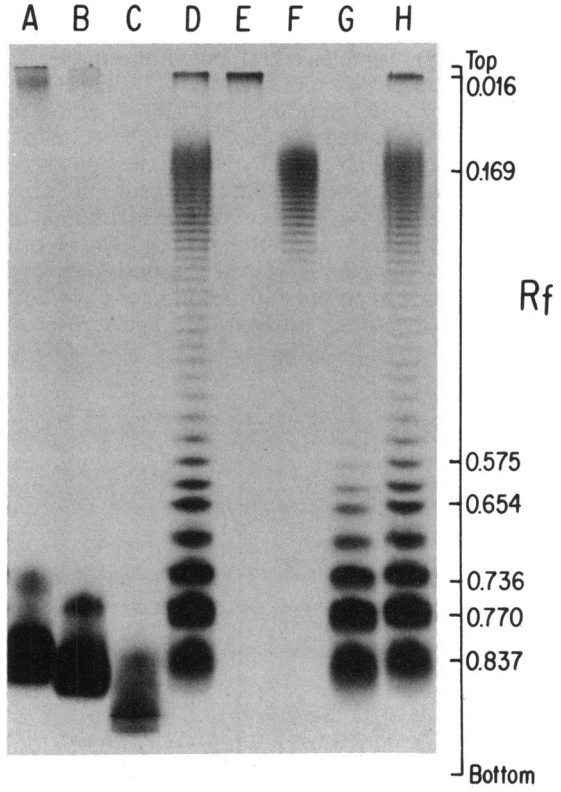

FIG. 2. Analysis of LPS fractions by SDS-PAGE: silver stain. A 15% polyacrylamide gel was prepared and run according to the method of Laemmli (20). The gel was fixed and stained as described by Tsai and Frasch (41). Samples were treated with equal volumes of a sample buffer containing 2% SDS and were heated to 100°C for 5 min. Lanes: A, *Salmonella minnesota* Ra (5 μg); B, *S. minnesota* Rb (5 μg); C, *S. minnesota* Re (5 μg); D and H, *S. typhimurium* unfractionated LPS (6.25 μg); E, *S. typhimurium* fraction 1 (6.25 μg); F, *S. typhimurium* fraction 2 (6.25 μg); G, *S. typhimurium* fraction 3 (6.25 μg). R_f values are shown on the right.

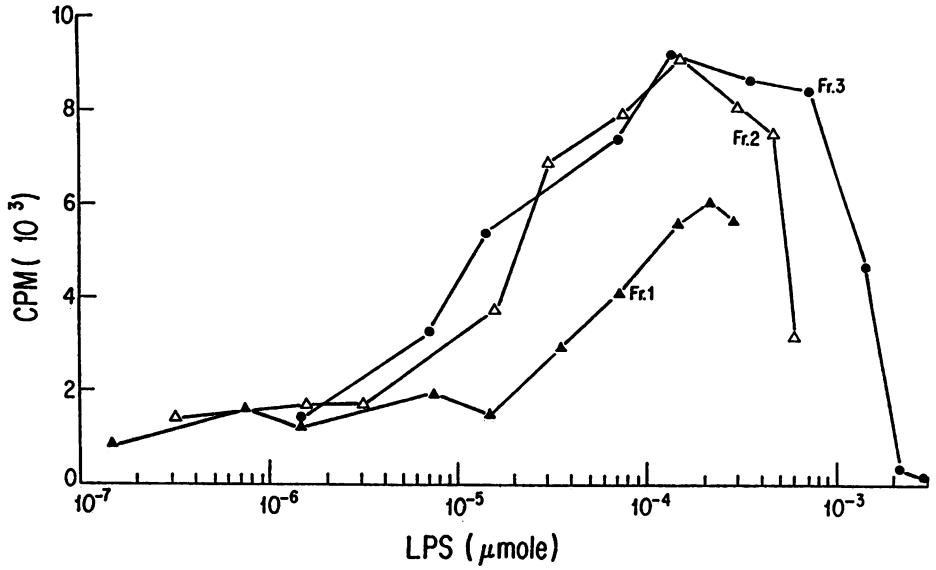

FIG. 3. Splenic mitogenesis induced by LPS fractions. Cultures containing 2.5 × 10^6 cells per ml from C3H/St mouse spleens (from at least three mice per experiment) were incubated in 0.2 ml of medium with various amounts of LPS fractions (Fr.) as indicated. After a 48-h incubation, the cultures were pulsed with 0.5 μCi of ^{125}I-iododeoxyuridine per well for another 18 h. The vertical axis indicates the radioactivity of iododeoxyuridine incorporated into DNA. The optimal concentration for unfractionated LPS to induce splenic mitogenesis was 5 μg/ml. Each point indicates the average of six determinations.

analyses (Fig. 1). Samples were pooled into three fractions according to the peaks, indicated by 1, 2, and 3 in Fig. 1 (which were named fractions 1, 2, and 3, respectively); dialyzed; and lyophilized.

Our results are in agreement with the previous report by Peterson and McGroarty (33) that three peaks are separable from *S. typhimurium* LPS. However, the distribution of each fraction (data not shown) is different from that reported by Peterson and McGroarty (33). On the basis of phosphate content, they have reported a distribution of 6, 44, and 50% of total LPS recovered for fractions 1, 2, and 3, respectively. The recovery reported here has been relatively high in fraction 1 (17%). The difference in results may be due to LPS preparations from different commercial sources which could have been grown under different conditions.

Analysis of LPS fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot. Samples of each fraction were subjected to electrophoresis in 15% polyacrylamide gels in a Laemmli system (20). After electrophoresis the gels were stained with silver nitrate (41) or underwent electroblotting onto Zeta-probe membranes (Bio-Rad, Richmond, Calif.) followed by detection by radio-

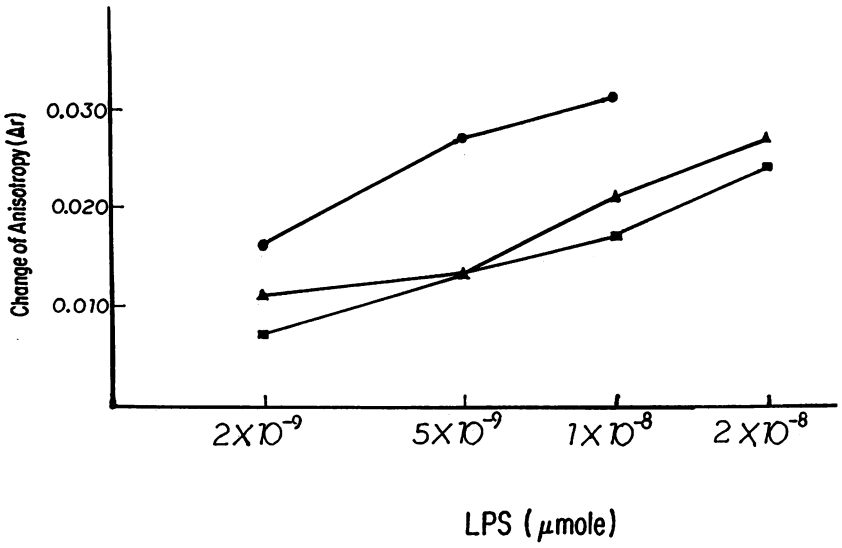

FIG. 4. Dose responses to changes in DPH anisotropy (Δr) induced by LPS fractions interacting with SUVs. The reaction mixture volume was 2 ml containing 20 μM lipid SUVs. Measurements were at 37°C. Anisotropy values are the averages from three separate determinations. The anisotropic change induced by 50 μg of unfractionated LPS was 0.046. ●, fraction 1; ▲, fraction 2; ■, fraction 3.

immunoassay using a monoclonal anti-O4 antibody (4) as a probe according to the method described by Johnson et al. (17). The ladderlike banding patterns of rough, smooth, and fractionated LPS are observed in silver-stained gels (Fig. 2) and immunoblot membranes (data not shown), indicating that LPS aggregates are heterogeneous with three predominant subpopulations. These results of LPS fraction banding patterns are consistent with the findings of Munford et al. (27) and Peterson and McGroarty (33). However, Goldman and Leive (7), Palva and Makela (31), and Hitchcock and Brown (11) reported that doublet banding patterns occur in lower-molecular-weight LPS from both *S. typhimurium* and *E. coli*. Such doublets have not been observed in the experiments reported here. The results from those studies indicate that further heterogeneity may exist in the lipid A-core oligosaccharide region, including heterogeneity in the number or distribution of phosphate groups. The exact explanation for double banding patterns is not clear.

However, comparison of the immunoblot pattern with the electrophoretic pattern, on the basis of R_f values, shows that the two fastest migrating bands (R_f = 0.837 and 0.770) in both unfractionated and fraction 3 LPS were missing. One band (R_f = 0.837) was not expected to contain any O-antigen polysaccharide side chain. The other band (R_f = 0.770), however, was assumed to contain one repeating unit of O-antigen polysaccharide side chain but did not appear on the immunoblot. This is consistent with evidence that the O4 epitope consists of one abequose and an adjacent galactose which would be part of a second repeating unit and would be detectable only with a minimum of two repeating units (18). Fraction 1 was barely detected by the monoclonal anti-O4 antibody in the immunoblot analysis. Fraction 1 was not detected by a monoclonal antibody directed against a KDO-containing epitope in the core glycolipid in a dot blot assay (data not shown).

Chemical analysis. As mentioned before, because 3,6-dideoxyhexose is present in the O-antigen polysaccharide side chain whereas phosphate groups and KDO are present only in the lipid A-core oligosaccharide, each fraction was assayed for KDO, total phosphate, and 3,6-dideoxyhexose. The 3,6-dideoxyhexose content of LPS fractions indicated a decrease in the chain length of the O-antigen polysaccharide side chain from fraction 1 to fraction 3; the KDO content increased as the chain length decreased (Fig. 1). In addition, the 3,6-dideoxyhexose-to-phosphate ratios increased from fraction 3 to fraction 1, and the amount of phosphate per milligram of LPS increased from fraction 1 to fraction 3 (data not shown).

Up to this point, analyses of fractions 2 and 3 of LPS from *S. typhimurium* have been comparable to results from other laboratories (7, 27, 31, 33). The average numbers of repeating units of the O-antigen polysaccharide side chain calculated from chemical analyses were 31.5 and 1.5 for fractions 2 and 3, respectively. Their corresponding molecular weights were 33,638 and 6,614, respectively. However, several tests for fraction 1 revealed some similarities to and some differences from the other LPS fractions. Because fraction 1 contained 3-OH $C_{14:0}$ (data not shown), 3,6-dideoxyhexose, KDO, and phosphate, it was concluded that this fraction was LPS. The number of repeating units estimated from the ratio of 3,6-dideoxyhexose to phosphate was 72, corresponding to a molecular weight of 70,121. The presence of this long-side-chain LPS population in *S. typhimurium* has been reported by Munford et al. (27).

Mitogen assay. Andersson et al. (1) demonstrated that lipid A is the active component for LPS mitogenicity of murine lymphocytes. From these results, we have made an assumption that the same molar amounts of lipid A of each LPS fraction should have the same effect on splenocyte mitogenesis. Mitogen assay was carried out as previously described (14). The result is shown in Fig. 3. On a molar basis fractions 2 and 3 showed the same effect on splenocyte proliferation. Fraction 1 had less effect than the other two fractions over all of the dose ranges studied. The optimal concentration of each fraction for splenocyte mitogenesis was about 1 μM (see Fig. 5). Since each fraction is assumed to contain one lipid A moiety per molecule but differ in side chain length, this result suggested that a very long O-antigen polysaccharide side chain may modulate lipid A-mediated lymphocyte mitogenesis. Although Vukajlovich and Morrison (42) reported that a 3,6-dideoxyhexose-rich fraction from *E. coli* gives the least effect on mitogenesis, their fraction may consist of polysaccharide and not LPS (33), while our fraction 1 contains LPS.

TABLE 1. DPH anisotropic changes (Δr) in SUVs induced by LPS fractions

Fraction no.	Δr with LPS fraction concn of:			
	1.0 μM	2.5 μM	5.0 μM	10.0 μM
1	0.016[a]	0.027	0.031	ND[b]
2	0.011	0.013	0.021	0.027
3	0.007	0.013	0.017	0.024

[a] For unfractionated LPS (50 μg), Δr = 0.046.
[b] ND, not done.

Steady-state fluorescence anisotropy study. It has been suggested that the interaction of LPS with the plasma membrane, which subsequently initiates all activation, is a two-step process (13, 35) and can be modeled by using small unilamellar vesicles (SUVs). The effect of the length of the O-antigen polysaccharide side chain on the fluidity of SUVs was investigated. SUVs were prepared from egg phosphatidylcholine (Avanti Polar Lipids, Inc., Birmingham, Ala.) as described elsewhere (35). All fluorescence assays were carried out with 20 μM lipid. The steady-state fluorescence anisotropy of LPS-SUVs containing 1,6-diphenylhexa-1,3,5-triene (DPH) (Molecular Probes, Eugene, Oreg.) as the fluorescence probe was measured as described previously (35). The results are shown in Fig. 4 and Table 1. In all three LPS fractions, interaction of increasing amounts of LPS with SUVs gradually increased DPH anisotropy. The data were then analyzed by the SPSS-PC+ statistics program on an IBM personal computer (Table 2). The statistical results show that all dose responses to changes in DPH anisotropy induced by LPS fractions interacting with SUVs were linear. In addition, further statistical analyses show that both frac-

TABLE 2. Statistical analyses of dose responses to DPH anisotropic changes by LPS fractions[a]

Fraction no.	Correlation coefficient (r)	Intercept (SE)	Slope (SE)
1	0.7792	0.01509 (0.00333)	0.00334 (0.00102)
2	0.8866	0.00998 (0.00166)	0.00175 (0.00029)
3	0.8937	0.00692 (0.00164)	0.00180 (0.00029)

[a] With the 95%-confident t tests of intercepts and slopes, the regression line of fraction 1 was found to be significantly different from the other two regression lines, but there was no statistically significant difference between the regression line of fraction 2 and the regression line of fraction 3.

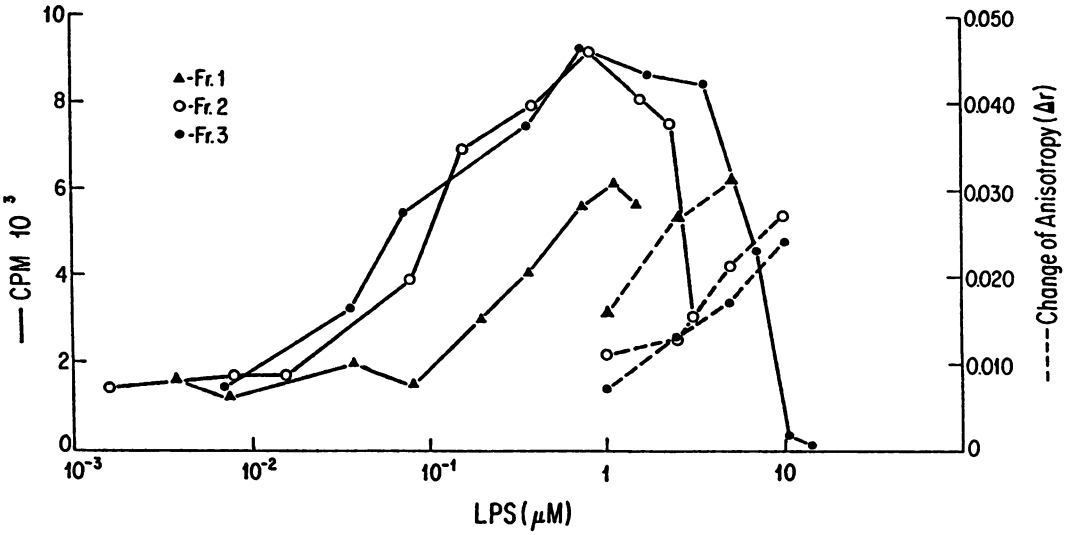

FIG. 5. Comparison between effects of LPS fractions (Fr.) on cellular mitogenesis and changes of DPH anisotropy in model membranes.

tions 2 and 3 had similar effects on the SUVs, detected by a change of DPH anisotropy, while fraction 1 had almost twice as much activity as the other two fractions had at 95% confidence limits. These results indicate that the lipid bilayers of SUVs were more ordered after interacting with LPS fractions and that a very long O-antigen polysaccharide side chain had more effect than LPS with a shorter O-antigen polysaccharide side chain. Thus, the polysaccharide portion of LPS might play an important role in changing the properties of the membrane with which it interacts. The degree of difference between fraction 1 and fractions 2 and 3 has been observed several times and with different fraction preparations. The likely interpretation of these results is that the LPS preparation with a very long O-antigen polysaccharide side chain could exist in a coiled arrangement (19) in which polysaccharide side chains could interact and form a microdomain. Nikaido et al. (29, 39) inferred from the results of nuclear magnetic resonance and electron spin resonance studies that LPS can form microdomains in LPS-phospholipid vesicles and that these microdomains have an effect on the overall order of the lipid bilayer.

Finally, we compared the LPS interactions with cells and model membranes (Fig. 5). The minimal concentration for a measurable change of DPH anisotropy in the model membrane system was about 1 μM. However, the optimal dosage for stimulation of splenocyte mitogenesis was at this concentration. Increasing the LPS fraction concentrations progressively decreased the fluidity of SUVs detected by increasing DPH anisotropy. In addition, the activity of splenocyte mitogenic stimulation by LPS fractions also decreased as the concentration of these fractions increased over 1 μM. The differences observed are not due to using a different ratio of LPS to membrane phospholipid, since the SUV system was designed to achieve the same LPS-to-lipid ratio as is present in the mitogenic assay. These results suggested that other factors in addition to changes in membrane fluidity may play a role in mediating LPS interactions for cell activation. Serum lipoproteins and other bacterial outer membrane components are found to associate with LPS, and they may be important in modulating LPS activities. Cell membrane receptors or specific binding sites on the cell surface (21, 44) may also facilitate the localization of low concentrations of LPS on selected cell populations.

The use of LPS fractions with various lengths of the O-antigen polysaccharide side chain will be useful for further examination of structural control of LPS-membrane interactions and cell activation.

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