THE EFFECT OF LIPID A ON THE FLUIDITY AND PERMEABILITY PROPERTIES OF PHOSPHOLIPID DISPERSIONS

Shlomo ROTTEM

Biomembrane Research Laboratory, Department of Clinical Microbiology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

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1. Introduction

The envelope of Gram-negative bacteria is a complex structure which consists of an outer membrane, an intermediate peptidoglycan layer and a cytoplasmic membrane [1]. The outer membrane differs from the cytoplasmic membrane in a number of biochemical and physiological properties. Both membranes contain the same classes of lipids but only the outer membrane contains lipopolysaccharide (LPS) [2,3]. In addition, the outer membrane of smooth Gram-negative bacteria was found to be impermeable to hydrophobic components capable of penetrating through the cytoplasmic membrane [4,5]. It was postulated [5–7] that the external side of the outer membrane of smooth *Salmonella typhimurium* strains is composed exclusively of LPS and protein molecules. The absence of phospholipids from this part of the membrane does not allow the exogenous hydrophobic components to dissolve in the interior of the outer membrane, a prerequisite for the diffusion process [8]. In deep rough mutants, where a much higher permeability to hydrophobic substances was described [5], phospholipids were present on the external part of the outer membrane also, resulting in the formation of bilayer regions in which hydrophobic components can be dissolved [6,7]. An alternative explanation to the low permeability of the outer membrane of the smooth strains can be based on our findings by EPR studies that the fluidity of the outer membrane is much lower than the fluidity of the cytoplasmic membrane [9]. The association of decreased membrane fluidity with decreased permeability has been established for a variety of membrane systems [10,11]. To determine the reason for the lower fluidity of the outer membrane we recently studied the effects of varying the composition and content of the LPS of the outer membrane on its fluidity [12]. The results indicated that the polysaccharide moiety of the lipopolysaccharide restricted the mobility of lipid hydrocarbon chains in the outer membrane. To further our understanding of the reasons for the lower fluidity of the outer membrane the effect of the lipid A moiety of *Proteus mirabilis* LPS on the mobility of spin-labeled fatty acids in egg lecithin liposomes was studied. Our results suggest that the incorporation of lipid A into the phospholipid liposomes restricted the mobility of the hydrocarbon chains of spin-labeled fatty acids and decreased permeability of the liposomes to non-ionic substances.

2. Materials and methods

The rough *Proteus mirabilis* strain R 45 and the smooth strain S 1959 were kindly provided by Dr K. Kotełko (University of Łodz, Poland). The organisms were grown in 1 liter basal salt medium [13] for 16–20 h at 37°C with vigorous shaking. The cells were harvested by centrifugation at 10 000 × *g* for 10 min at 4°C, and washed once with deionized water. Cell lipids were extracted by three successive extractions with chloroform–methanol (2/1) at 45°C for 1 h. LPS was extracted by the phenol/chloroform/light petroleum procedure [14]. Ribonucleic acid contaminating the LPS preparations was removed by ribo-

nuclease treatment [3]. Lipid A was liberated from LPS by hydrolysis in 0.1 N acetic acid for 1 h at 100°C. The precipitate formed was collected by centrifugation at 5000 × *g* for 10 min, washed twice in deionized water, freeze dried, weighed, and stored as a 5 mg/ml solution in chloroform–methanol (2/1). In some experiments the lipid A preparation (5 mg) was chromatographed on an activated silicic acid column (30 × 1.5 cm) and eluted stepwise with mixtures of 10–60% diethylether in *n*-hexane, followed by acetone and methanol [15].

Liposomes were prepared by pipetting 0.5 ml egg lecithin solution (10 mg/ml in chloroform, Makor, Jerusalem) into 20 ml test tubes. Varying volumes (0–1.0 ml) of lipid A solution (5 mg/ml in chloroform–methanol) were then added. The solvent was evaporated under N_2, and 0.3 ml diethylether was added to each tube. The ether was quickly evaporated to dryness using a Vortex mixer and hot air, forming a dry thin film of lipid on the test tube wall. The lipids were then dispersed in 5 ml solution containing 40 mM KCl in 4 mM Tris·HCl (pH 8.0), at 25°C by agitation on a Vortex mixer for 2 min, followed by a brief ultrasonic treatment (30–60 s) using a W-350 Heat Systems sonicator equipped with a microtip. The lipid dispersions were then incubated at 37°C for 1 h with constant shaking.

Liposomes were labeled with *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of 5-ketostearic acid (5-doxylstearate) and 12-ketostearic acid (12-doxylstearate), products of Syva (Palo Alto, CA). For spin-labeling of the liposomes, 2 μl 12.5 mM stock solution of the spin label in ethanol were added to a test tube containing 0.3 ml diethylether. After the solvents were evaporated, forming a thin film of the label on the bottom of the test tube, 1 ml liposome suspension was added and the test tube was incubated at 37°C for 1 h with vigorous shaking. The liposomes were then transferred to a disposable pipette sealed at one end, and EPR spectra were obtained in a Varian E-4 spectrometer equipped with a temperature control unit. The freedom of motion of the spin-labeled fatty acids in the liposome preparations was assessed from the order parameter (S) calculated according to the following equation [16]:

$$S = \frac{T'_{\parallel} - T'_{\perp} - C}{T'_{\parallel} + 2T'_{\perp} + 2C} \times 1.723$$

where $T'_{\parallel}$ and $T'_{\perp}$ (in gauss) are equal to one-half the separation of the outer and inner extrema, respectively, and $C = 1.4\,G - 0.053\,(T'_{\parallel} - T'_{\perp})$, and from the motion parameter (τ_0) calculated according to [17] and expressed in ns. Higher S and τ_0 values are associated with a more restricted mobility of the hydrocarbon chains.

For permeability experiments, the liposome suspension was mixed rapidly with equal volumes of a solution containing 400 mM glycerol or erythritol, 40 mM KCl and 4 mM Tris·HCl (pH 8.0). The initial change in absorbance caused by liposome swelling was monitored at 500 nm. Initial swelling rates were calculated as in [10].

3. Results and discussion

Hydrolysis by 0.1 N acetic acid of LPS from the rough mutant *Proteus mirabilis* R 45 yielded a lipid A preparation that was eluted from a silicic acid column as a single peak with 60% diethylether in *n*-hexane (by vol.). The LPS of the wild strain S 1959 was not hydrolyzed under these mild conditions and boiling for 60 min with 0.1 N HCl was required for hydrolysis. The preparation of lipid A obtained in this case was heterogenous and exhibited 4 separate peaks when chromatographed on the silicic acid column. Liposomes containing egg lecithin and lipid A (1:1 by wt) could be readily obtained. Mg^{2+}, which was found to improve the incorporation of LPS into phospholipid liposomes, apparently by neutralizing repulsive forces on the negatively charged LPS molecules [18], was not required for formation of the lipid A/phospholipid liposomes. The phospholipid:lipid A ratio of 1 is equivalent to or slightly higher than the expected ratio in the native outer membrane, so that attempts were not made to obtain liposomes containing higher proportions of lipid A. The egg lecithin–lipid A liposomes chromatographed on a Sepharose 4B column [19] were eluted in a single peak (at the void volume) with most of the fractions in the peak having a similar lipid A:egg lecithin ratio, suggesting that the preparation is homogenous and consists of liposomes with mixed lipid A–lecithin bilayers rather than separate phospholipid and LPS dispersions.

Table 1 shows that at 37°C the large hyperfine splitting ($2T_{\parallel}$) and the order (S) and motion (τ_0) param-

Table 1
The freedom of motion of 5-doxylstearate and 12-doxylstearate in mixed lipid A–egg lecithin liposomes

Liposome preparation	5-Doxylstearate		12-Doxylstearate		
	$2T_{\parallel}$ (G)	S	$2T_{\parallel}$ (G)	S	τ_0 (ns)
Egg lecithin (1.5 mg/ml)	47.7	0.54	36.5	0.26	1.9
Egg lecithin (1.5 mg/ml) + lipid A (0.75 mg/ml)	49.7	0.56	38.5	0.30	2.6
Egg lecithin (1.5 mg/ml) + lipid A (1.5 mg/ml)	51.7	0.61	39.5	0.32	3.0

eters were higher in the lipid A-containing liposomes, suggesting a restricted mobility of the hydrocarbon chains induced by the lipid A moiety. The motion parameter (τ_0) was calculated only from the 12-doxylstearate spectrum since only this probe moved in a nearly isotropic fashion, warrenting the use of the formula in [20] for determining rotational correlation times (τ_c). Nevertheless, the values obtained here are too slow for the one-line shape theory to apply ($\tau_c > 10^{-9}$), thus, for comparative purposes, the empirical motion parameter (τ_0) was used [17]. Throughout the temperature range tested (10–45°C), there was no indication of heterogeneity in the spectra, and the percent change in the observed spectral parameters resulting from the incorporation of lipid A into the egg lecithin liposomes was the same. These data suggest that our preparations contain lipid A–egg lecithin mixed bilayers rather than separate LPS and phospholipid domains. As is also apparent from table 1, the restricted mobility induced by the lipid A moiety was equally pronounced near the methyl end group (12 position) and the carboxyl end group (5 position) of the probe.

Figure 1 shows the effect of the lipid A moiety on the permeability of non-ionic substances into the liposomes. The figure shows the much lower permeability of erythritol and glycerol into the lipid A-containing liposomes, a finding in accord with the restricted mobility of spin-labeled fatty acids in such liposomes, and in agreement with the studies linking lower permeability of glycerol and erythritol to tighter packing of hydrocarbon chains in the lipid bilayer [10].

Our results showing the effect of lipid A moiety on fluidity and permeability properties of phospholipid bilayers are in line with our previous observations showing that decreasing the LPS content of outer membrane preparations increases the mobility of spin-labeled probes in the membrane [10]. It thus appears that both the polysaccharide and lipid A moieties of the LPS are responsible for the low fluidity of the outer membrane, which may restrict the

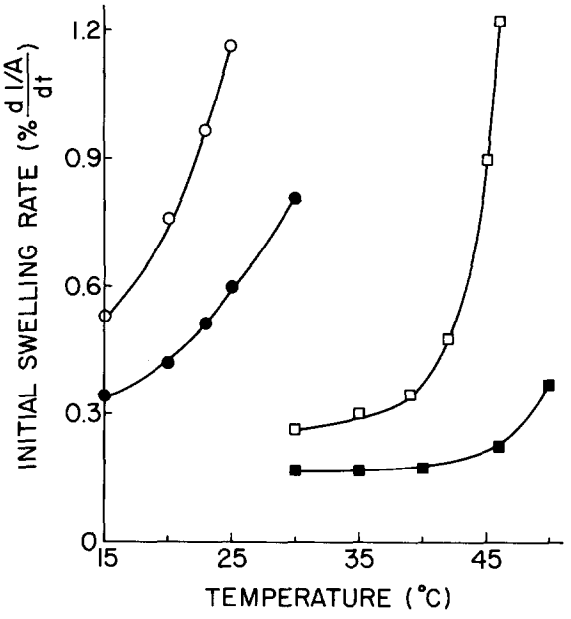

Fig.1. Initial swelling rates of lecithin liposomes (open symbols) and mixed lipid A–lecithin (1:1 by wt) liposomes (closed symbols) in isotonic glycerol (○) or erythritol (□) solutions at various temperatures. The results represent the analyses of at least 3 separate experiments using different preparations of lipid A.

permeation of hydrophobic compounds through the outer membrane.

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