FITC-labeled lipopolysaccharide: use as a probe for liposomal membrane incorporation studies

V.S. Trubetskoy[1], N.V. Koshkina[1], V.G. Omel'yanenko[1], V.L. L'vov[2], B.A. Dmitriev[2], A.B. Petrov[3], and V.P. Torchilin[1]

[1] *USSR Cardiology Research Center,* [2] *Gamaleya Institute of Epidemiology and Microbiology,* [3] *Mechnikov Research Institute for Vaccines and Sera, Moscow, USSR*

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FITC-labeled LPS from *Neisseria meningitidis* can be used as a probe to follow the process of LPS incorporation into liposomal membrane and to study its interaction with a bilayer. The incorporation of FITC-LPS into the bilayer was proved by physicochemical methods as well as by liposomal LPS toxicity decrease in actinomycin D-sensitized mice. Fluorescence intensity increase was observed upon the insertion of FITC-LPS into the membrane of dehydration/rehydration vesicles and vesicles obtained by co-sonication of lipid suspension and FITC-LPS. Following FITC-LPS fluorescence polarization it was shown that the substance seems to be clusterized in the liposomal membrane starting from FITC-LPS/lipid molar ratio 1:800.

Lipopolysaccharide; Liposome; Toxicity; Fluorescent probe; Fluorescence polarization

1. INTRODUCTION

The incorporation of LPS into liposomes appeared to be one of the most effective ways to modulate numerous biological activities of LPS. The use of liposomal LPS substantially decreases the LPS-mediated production of interleukin-1 and tumor necrosis factor by cultured macrophages [1–3], macrophage tumoricidal activity [3,4], the toxicity of LPS preparation in mice [5] and *Limulus* amoebocyte gelation potency [1].

The active part of LPS molecule, responsible for the above-mentioned effects appeared to be a lipid A moiety [6]. The value of the parameters, describing different LPS effects, can vary in a range of 3 orders of magnitude depending on whether lipid A is inserted into the membrane bilayer or not. So, the presence of even very small quantities of free LPS in liposomal LPS can substantially change the total effect of the given preparation.

The importance of the exact estimation of LPS association with liposomes is quite evident and, consequently, the reliable methods for monitoring the process of LPS insertion into bilayer are highly desirable. In this paper we describe the use of FITC-labeled LPS (FITC-LPS) as a tool for tracing the incorporation of LPS into liposomes and for the detailed elucidation of LPS-membrane interactions.

Correspondence address: V.P. Torchilin, 121552 USSR Cardiology Research Center, 3rd Cherepkovskaya str., 15a Moscow, USSR

Abbreviations: LPS, lipopolysaccharide; FITC, fluorescein isothiocyanate; DRV, dehydration/rehydration vesicles; DPH, 1,6-diphenylhexatriene; AD, actinomycin D

2. MATERIALS AND METHODS

2.1. *LPS*

LPS was isolated from group B *Neisseria meningitidis* strain B125 using Westphal's method [7] and purified by repeating ultracentrifugation at 105 000 × *g* for 4–6 h, at 4°C, followed by Sephadex G-100 gel-filtration in 0.01 M Tris-HCl, 0.2 M NaCl, 0.25% sodium deoxycholate, 1 mM EDTA, pH 8.0. According to our preliminary studies and previously reported structure of the carbohydrate moiety [8] LPS from *N. meningitidis* group B is a lipooligosaccharide without repeating polymeric saccharide structures with an approximate molecular mass of about 4000 Da. FITC labeling was performed as in [3] with subsequent separation of the mixture on Sephadex G-50 column (29 × 1.6 cm) in 0.01 M Tris-HCl, 0.25% sodium deoxycholate, 1 mM EDTA, pH 8.0, and exhaustive dialysis against water. FITC-LPS contained 0.33 mol FITC per mol LPS as calculated according to [9].

2.2. *Preparation of liposomes*

Liposomes were prepared from egg phosphatidylcholine and cholesterol (Sigma) in molar ratio of 7:3. 1 mg of the lipid mixture was dried in argon flow in pyrogen-free test tubes. FITC-LPS was incorporated into liposomes (1 μg/10 μg of total lipids) using two alternative methods.

2.2.1. DRV method

150 μl of pyrogen-free deionized water were added into the test tube and lipids were resuspended by vortexing. 100 μg of FITC-LPS suspension (1 mg/ml in pyrogen-free water) were then added. The final mixture was sonicated (3 × 1 min, 0°C, argon atmosphere, 40 W, probe-type Labsonic system, Lab Line Instruments, USA) and freeze-dried. The dry preparation was reconstituted with 1 ml of pyrogen-free saline.

2.2.2. Prolonged sonication method

100 μl of FITC-LPS (1 mg/ml in pyrogen-free saline) were added to 1 mg of lipids resuspended in 150 μl of the same solution. After vortexing the mixture was co-sonicated for up to 35 min at the conditions indicated above. During the process aliquots were taken for fluorescence measurements and toxicity determinations.

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The separation of unincorporated FITC-LPS from liposome mixture was performed by Ficoll gradient centrifugation as described in [10]. All fluorescence measurements were performed using Hitachi F-4010 fluorescence spectrophotometer (λ_{ex} = 490 nm, λ_{em} = 520 nm). Liposomes for fluorescence polarization measurements were prepared by prolonged sonication method. To incorporate the DPH probe into liposomes aliquots of 1 mM DPH solution in tetrahydrofuran were added to the liposome suspension in saline to obtain 1:200 DPH/lipid molar ratio. After 1 h of incubation, fluorescence polarization was measured using polarization assembly of the spectrofluorimeter.

2.3. *Toxicity assay in mice*

Inbreed CBA/Ca lac Sto mice weighing from 18–20 g were used in toxicity tests. AD (Sigma) was dissolved in DMSO (Serva; 1 mg/ml) and then was diluted in pyrogen-free saline. Subtoxic dose of AD was determined before the experiments. The maximal dose of this agent in a range of 15–35 μg per animal at which all animals in the group have remained alive during 3 days was used for the sensitization. Mice simultaneously received i.p. AD solution and the dilutions of LPS preparations. Deaths were observed during 72 h after injection. Each group contained 5 animals. Each test was repeated at least 3 times.

3. RESULTS

Free LPS in water solutions forms large aggregates (micelles) with an apparent molecular weight of several million daltons [11]. The fluorescence of FITC-LPS occurring in micelles is partially quenched and becomes dequenched upon aggregate destruction (e.g. after the addition of detergent). The fluorescence intensity in this case increases by several dozen-fold (data not shown). The similar phenomenon is also observed when FITC-LPS is incorporated into liposomal membrane. Comparing curve no. 2 and the initial point of curve no. 1 (Fig. 1) one can notice FITC fluorescence increase upon FITC-LPS incorporation into DRV as well as during its prolonged co-sonication with lipids in saline (curve no. 1). The prolonged sonication of preformed

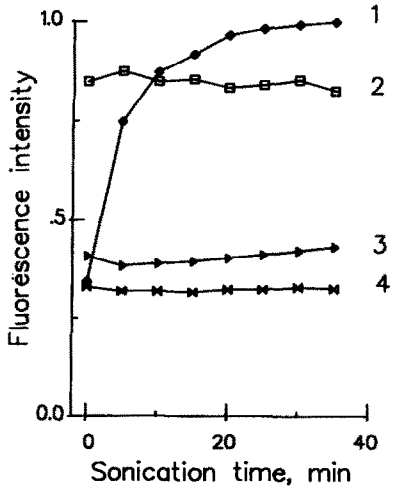

Fig. 1. FITC-LPS fluorescence intensity changes during sonication (1 = mixture of lipids and FITC-LPS; 2 = preformed DRV with FITC-LPS; 3 = FITC-LPS alone; 4 = mixture of lipids and FITC-LPS without sonication).

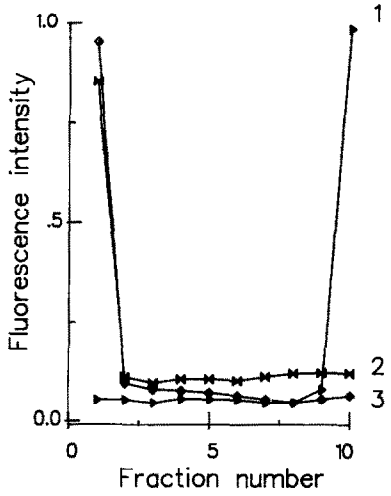

Fig. 2. Separation of liposomes from unincorporated FITC-LPS using Ficoll gradient centrifugation (1 = FITC-LPS alone; 2 = lipids co-sonicated for 35 min with FITC-LPS; 3 = FITC-LPS containing DRVs).

FITC-LPS containing DRVs as well as the sonication of free FITC-LPS does not lead to the substantial fluorescence intensity increase. To demonstrate that the incorporation of FITC-LPS into the bilayer was complete, DRVs and FITC-LPS/lipid mixture were subjected to Ficoll discontinuous gradient centrifugation after 35 min of co-sonication. In both cases more than 97% of the fluorescent label was associated with liposomes (Fig. 2).

The fluorescence polarization studies revealed strong FITC fluorescence depolarization below 1:800 FITC-LPS/lipid molar ratio (Fig. 3). The effect can be attributed to the following reasons: (i) changes in membrane fluidity upon the increase in LPS content, (ii) changes in optical properties of liposome suspension, (iii) FITC-LPS cluster formation in the bilayer. Two first possibilities can be excluded. To minimize light

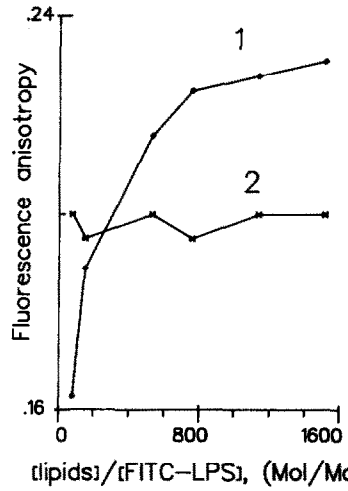

Fig. 3. Fluorescence anisotropy of FITC-LPS (1) and DPH (2) incorporated into liposomes with different lipid/FITC-LPS ratios.

scattering we used liposome concentrations at which absorbance at 490 nm was less than 0.1 and remained constant for all samples. To exclude possible membrane fluidity changes we have shown that fluorescence anisotropy of DPH probe located in the hydrophobic area of the membrane remained unchanged during the increase in LPS content (Fig. 3).

To study the possible changes in the biological activity of LPS after its incorporation into liposomes we have performed the comparative studies of free LPS and liposomal LPS toxicity in mice. As has been recently shown by Dijkstra et al. [5] the incorporation of *Salmonella minnesota* LPS into small unilamellar liposomes or into large multilamellar vesicles results in at least 10-fold decrease in the lethality of AD-sensitized mice. In our experiments liposomal LPS (DRV) reduces its toxicity at least 200-fold (Table I). The control mixture of free FITC-LPS and sonicated lipids possessed the same toxicity as free LPS. Further sonication of FITC-LPS containing DRV resulted in initial decrease of toxicity (approximately by 10-fold) followed by slight increase in toxicity at the end of the sonication period used. It is relevant to note that initially less toxic LPS containing DRVs becomes more toxic after 35 min of sonication.

4. DISCUSSION

Previous publications have shown that the incorporation of LPS into liposomal membrane can drastically change its biological properties. Data presented in this paper clearly demonstrate that FITC-LPS can serve as a useful tool for monitoring the endotoxin incorporation into the bilayer. The incorporation of FITC-LPS into DRVs or cosonication of FITC-LPS with empty liposomes resulted in FITC fluorescence increase which seems to be determined by the destruction of free FITC-LPS micelles and the distribution of the fluorophore-labeled molecules over the surface of the liposomal membrane. Similar phenomena connected with dequenching of hydrophobic fluorescent probes upon their association with biological and artificial membranes are, for example, described in [12].

As has been recently shown, the prolonged sonication of multilamellar vesicles with *S. minnesota* LPS results in almost quantitative incorporation of its molecules into liposomes [13]. In the present paper we have demonstrated that using FITC-LPS it is possible to trace the kinetics of this process and to determine the extent of incorporation.

It is well-known that one can observe fluorescence depolarization due to the excitation energy migration when the distance between fluorophores is less than 50 Å. Thus, if the FITC-LPS cluster is forming in the system where upon initial random distribution the distances between individual FITC-LPS molecules have to be greater than 50Å, this may lead to the decrease of fluorescence polarization. It is relevant to note that the energy migration may also cause the decrease in the quantum yield and the latter parameter may be also employed in experiments of similar kind. Nevertheless from our point of view the fluorescence polarization is a more convenient one because it does not require to control the exact probe concentration.

Recently some reports on the basic principles of Gram-negative bacteria outer membrane assembly have appeared [14] where the clusterization of lipid A or lipid A-linked 2-keto-3-deoxyoctonate precursors in the special sites of cytoplasmic membrane was postulated. Such domains facilitate the transfer of LPS precursors to the outer membrane where the biosynthesis of endotoxin molecule is accomplished. These data encouraged us to use FITC-LPS for the elucidation whether LPS molecules within liposomal membrane form clusters. FITC fluorescence anisotropy changes show that LPS molecules starting from LPS/lipid molar ratio of about 1:800 seem to be clusterized. The depolarization in this case is not connected with membrane fluidity since the fluorescence polarization of hydrophobic membrane-associated probe (DPH) is not altered upon increasing LPS/lipid ratio.

Toxicity studies in AD-sensitized mice have also indirectly confirmed the fact of FITC-LPS incorporation into liposomes. It has been assumed that the decrease in LPS toxicity upon the incorporation is taking place due to the enhanced clearance and processing of liposomal LPS injected into the animal [4]. It has been

Table I

Toxicity of liposomal and free LPS preparations in actinomycin D-sensitized mice

Preparation[a]	No. of mice died at LPS dose of (μg/mouse)				LD_{50} (μg)
	10^{-3}	10^{-2}	10^{-1}	1	
Mixture of LPS and lipids, '0 time'	1	3	5	5	$.5\times10^{-3}$
The same after 5 min co-sonication	0	0	2	4	2×10^{-1}
The same after 10 min co-sonication	0	0	2	3	3.2×10^{-1}
The same after 35 min co-sonication	0	0	2	5	1.2×10^{-1}
LPS containing DRV	0	0	0	2	2.0
LPS containing DRV after 35 min sonication	0	0	1	5	2.0×10^{-1}
Free LPS	0	3	5	5	5.0×10^{-3}

[a] Mice received simultaneously i.p. 20 μg actinomycin D

shown many times that liposomes with a large size are cleared from the circulation [15] and processed by phagocytes [16] more rapidly than the small ones. These assumptions can also explain why LPS containing DRVs possess lower toxicity than liposomal LPS obtained by co-sonication. Total change of toxicity during co-sonication appeared to reflect the summation of two processes: initial toxicity decrease due to FITC-LPS incorporation into the membranes of large DRVs followed some increase in the toxicity due to the diminishing of vesicle size under sonication conditions, which, in turn, lowers the rate of LPS processing by phagocytes.

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