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Toxicity and Immunogenicity of *Neisseria meningitidis* Lipopolysaccharide Incorporated into Liposomes

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To obtain nontoxic and highly immunogenic lipopolysaccharide (LPS) for immunization, we incorporated *Neisseria meningitidis* LPS into liposomes. Native LPS and its salts were incorporated by the method of dehydration-rehydration of vesicles or prolonged cosonication. The most complete incorporation of LPS into liposomes and a decrease in toxicity were achieved by the method of dehydration-rehydration of vesicles. Three forms of LPS (H^+ form, Mg^{2+} salt, and triethanolamine salt) showed different solubilities in water, the acidic form of LPS, with the most pronounced hydrophobic properties, being capable of practically complete association with liposomal membranes. An evaluation of the activity of liposomal LPS in vitro (by the *Limulus* amoebocyte test) and in vivo (by monitoring the pyrogenic reaction in rabbits) revealed a decrease in endotoxin activity of up to 1,000-fold. In addition, the pyrogenic activity of liposomal LPS was comparable to that of a meningococcal polysaccharide vaccine. Liposomes had a pronounced adjuvant effect on the immune response to LPS. Thus, the level of anti-LPS plaque-forming cells in the spleens of mice immunized with liposomal LPS was 1 order of magnitude higher and could be observed for a longer time (until day 21, i.e., the term of observation) than in mice immunized with free LPS. The same regularity was revealed in a study done with an enzyme-linked immunosorbent assay. This study also established that antibodies induced by immunization belonged to the immunoglobulin M and G classes, which are capable of prolonged circulation. Moreover, liposomal LPS induced a pronounced immune response in CBA/N mice (defective in B lymphocytes of the LyB-5$^+$ subpopulation). The latter results indicate that the immunogenic action of liposomal LPS occurs at an early age.

The prevention of infection caused by group B meningococci remains a problem as yet unsolved. At present, the main efforts of researchers are directed towards the development of a group B meningococcal vaccine based on polysaccharide and protein antigens (14, 19). Another antigen contained in the cell wall of meningococci is lipopolysaccharide (LPS). Antibodies to LPS are known to be capable of neutralizing meningococcal endotoxin, thus preventing the development of Schwartzmann's phenomenon, both local and generalized. In addition, these antibodies produce a bactericidal effect on meningococci and protect animals in different experimental systems (24). LPS has antigenic determinants common to *Neisseria meningitidis* isolates of different serogroups and serotypes (18, 28), as well as to isolates of other gram-negative bacteria (21). For these reasons, the development of a prophylactic preparation based on LPS may play a decisive role in the prophylaxis of meningococcal diseases and other infections accompanied by endotoxemia. However, the toxicity of lipid A, contained in LPS, makes it impossible to prepare a vaccine based on purified meningococcal LPS, and the removal of this toxic component leads to a decrease in the immunogenic potency of the antigen. Moreover, LPS itself induces a faint, transitory response, limited mainly to immunoglobulin M (IgM) antibodies and not leading to immunological memory (12).

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Liposomes, i.e., double-layer phospholipid membranes enclosed in vesicles, are used as biodegraded and biocompatible carriers for bacterial and viral antigens (for a review, see reference 16). The incorporation of a number of biological preparations, including LPS and lipid A of gram-negative bacteria, into liposomes permits a decrease in their toxicity and prevents the development of allergic reactions (9). The development of immunogens based on lipid A incorporated into liposomes makes it possible to achieve a considerable increase in the immune response to lipid A itself and to protein antigens and also to convert polysaccharide antigens into thymus-independent type 1 antigens (4, 26). These results are of particular importance for the immunization of persons incapable of a response to thymus-independent type 2 antigens (e.g., young children). This study was aimed at obtaining a nontoxic and highly immunogenic form of meningococcal LPS by the incorporation of the native preparation into liposomes.

MATERIALS AND METHODS

Animals. Experiments were done with CBA/Ca *lac* mice obtained from the Stolbovaya animal facility of the Academy of Medical Sciences and CBA/N mice from the animal facility of the Central Research Institute of Tuberculosis, Moscow, Russia.

Bacteria and cultivation. *N. meningitidis* 125, group B, serotype 2b, immunotype L6, from the collection of the Tarasevich State Research Institute for the Standardization

and Control of Medical Biological Preparations, was used. The first passage was made on 20% Hottinger serum agar at 37°C for 18 h. After the second passage, the culture was diluted in saline and transferred to a fermentor (model AHKYM 2M). For the cultivation of meningococci in the fermentor, a semisynthetic culture medium was prepared by the method of Cohen and Wheeler and contained strongly hydrolyzed casein, various salts, and yeast dialysate (6). The inoculum was placed into the fermentor containing medium with a dissolved oxygen content reaching 20 to 25% of complete saturation at 37°C and a pH of 7.4. The exponential phase usually lasted 4 h. During this period of cultivation, the maximum growth rate and the generation time were calculated, permitting continuous synchronous cultivation of *N. meningitidis*. The culture thus grown was concentrated in a Pellicon cassette system (Millipore) with a molecular weight cutoff of 10,000. The cells thus obtained were washed with acetone and dried in air.

LPS extraction. Dried cells (ca. 10 g) were suspended in 250 ml of water, the suspension was heated to 70°C, and an equal volume of a 90% aqueous phenol solution at the same temperature was added. This mixture was stirred for 30 min at 70°C, cooled, and dialyzed against distilled water for 1 week. The dialysate thus obtained was made free of denatured material, concentrated in a rotary evaporator, and freeze-dried. The dry residue was dissolved in a minimal amount of distilled water and centrifuged at 105,000 × *g* three times for 4 h each time. The gel-like sediment was suspended in distilled water and freeze-dried.

Acid form and salts of LPS. The Na^+ salt of LPS, sufficiently soluble in water, was obtained after chromatography on a column packed with Sephadex G-200 in a buffer solution of EDTA and sodium deoxycholate (27). At this stage, LPS was separated from the high-molecular-weight components. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of LPS, loaded at up to 1 μg on the gel as described previously (28), revealed only one band corresponding to approximately 4,000 Da. Electrodialysis of the solution of the Na^+ salt of LPS until a constant current strength was achieved and the pH was 2.8 to 3.0 led to the formation of the scarcely soluble acidic form of LPS (H^+ LPS). During the treatment of H^+ LPS with triethanolamine (Et_3N) until the pH was 7.5 to 8.0, the complete dissolution of precipitated LPS and the formation of the Et_3N salt of LPS were observed. To obtain the Mg^{2+} salt of LPS, moderately soluble in water, we added saturated magnesium hydroxide solution to the H^+ LPS suspension until the pH was 8.0. The surplus Et_3N and $Mg(OH)_2$ were removed by dialysis against distilled water, and the LPS preparation was freeze-dried.

LPS incorporation into liposomes. Liposomes were prepared from egg phosphatidylcholine and cholesterol (Sigma) in the molar ratio 7:3. The basic LPS/lipid ratio was 1:10. In some experiments, this ratio varied from 1:0.1 to 1:1,000. The incorporation of LPS into liposomes was performed by the method of dehydration-rehydration of vesicles (DRV) and the method of prolonged cosonication (25, 27). In the DRV method, 150 μl of pyrogen-free deionized water was added to a lipid film, well dried from the traces of organic solvents, and the lipid was resuspended by vigorous vortexing. One hundred micrograms of fluorescein isothiocyanate (FITC)-LPS or LPS suspension (1-mg/ml stock in pyrogen-free deionized water) was added to the lipid suspension. The final mixture was sonicated three times for 1 min each time at 0°C under an argon flow) and freeze-dried. The dried residue was reconstituted with 1 ml of pyrogen-free saline. In the prolonged cosonication method, the lipid was resuspended in 150 μl of saline, and 100 μl of FITC-LPS or LPS (the above-mentioned stock was used) was added to the formulated liposomes. After being vortexed, the mixture was sonicated for up to 35 to 40 min under an argon flow (0°C; 40 W; probe-type Labsonic system from Lab Line Instruments). Free LPS was separated from liposomal LPS by Ficoll density gradient centrifugation with FITS-LPS as a probe (27). For removal of noncoupled FITC after labeling, the FITC-LPS sample was separated on a Sephadex G-50 column (29 by 1.6 cm) containing 0.01 M Tris-HCl–0.25% sodium deoxycholate–1 mM EDTA (pH 8.0) and exhaustively dialyzed against deionized water.

LPS activity in the LAL test. In vitro endotoxin activity was tested by the *Limulus* amoebocyte (LAL) test (Atlas Bioscan). The samples were diluted with pyrogen-free saline prepared with water supplied by the manufacturer, and the NaCl was incinerated at 250°C for 3 h. LAL reagent in a volume of 0.1 ml was added to 0.1 ml of various dilutions of the samples containing LPS and incubated at 37°C for 1 h. The formation of a dense opaque clot that did not move when the test tube was turned 180° was considered a positive reaction.

Pyrogenic properties of LPS. Testing of the pyrogenic properties of LPS was done in accordance with the State Pharmacopoeia of Russia by use of chinchilla rabbits weighing 1.5 to 2.5 kg. The animals were kept at a constant temperature for 5 days prior to the test and weighed, and their rectal temperature was taken. For this test, animals with an initial temperature of 38.5 to 39.5°C were selected. The dilution of the preparation being tested was considered pyrogen free if, during 3 h of observation, no increase in temperature exceeding 0.6°C in comparison with the initial temperature occurred in any of the rabbits or if the sum of temperature increases in three rabbits did not exceed 1.4°C.

Immunization of mice. General-purpose CBA/Ca *lac* mice, 6 weeks old, 6 to 10 animals per group, were injected intraperitoneally (i.p.) with 10 μg of liposomal or free LPS dissolved in 0.5 ml of saline. For the dose-response experiment, mice were injected with 0.1, 1.0, 10.0, or 50.0 μg of liposomal or free LPS. As controls, mice injected with saline were used. For obtaining hyperimmune sera, a separate group of mice was injected with 10^6, 10^7, 10^8, 10^9, and 10^9 *N. meningitidis* cells i.p. at 1-week intervals and bled on day 7 after the last injection.

Anti-LPS PFC. The numbers of anti-LPS plaque-forming cells (PFC) were determined in the system proposed by Cunningham (7) by use of a chamber made up of two glass slides and into which spleen cells from immunized mice, sheep erythrocytes (SRBC) sensitized with LPS, and guinea pig complement were introduced. SRBC were sensitized with LPS treated with 0.3 N NaOH at 37°C for 18 h and neutralized with 0.3 N HCl. Treated LPS (1 ml) at a concentration of 50 μg/ml was added to 0.1 ml of the dense sediment of washed SRBC. After 2 h of incubation at 37°C, SRBC were washed with saline three times and used in the test. A 7% suspension of sensitized SRBC (0.05 ml) and guinea pig complement (0.05 ml) were added to 0.1 ml of spleen cell suspension (about 2×10^6 spleen cells in 1 ml of medium 199). After 1 h of incubation at 37°C, the number of hemolysis zones was calculated by visual examination.

Antibody assay. Anti-LPS antibodies were assayed in an enzyme-linked immunosorbent assay (ELISA). Polystyrene microplates (Nunc, Roskilde, Denmark) were treated with poly-L-lysine with a molecular weight of 50,000 (Sigma), dissolved in 0.1 M phosphate buffer (pH 7.2) at a concentration of 10 μg/ml, and placed into each well in a volume of 100

TABLE 1. Effectiveness of incorporation of different forms of *N. meningitidis* LPS into liposomes

Form of LPS	% Incorporation of LPS into liposomes obtained by the following method[a]:	
	DRV	Prolonged cosonication
H^+	99.9	94.2
Mg^{2+}	93.0	68.2
Et_3N LPS	32.0	ND

[a] For determination of the percent incorporation, FITC-LPS was used. Free FITS-LPS was determined by centrifugation in Ficoll discontinuous gradients. ND, not determined.

μl. The microplates were allowed to stand at 8°C for 18 h. After the microplates were washed, each well was filled with 100 μl of LPS at a concentration 10 μg/ml in the same buffer. Serial twofold dilutions of serum, starting at 1/100, and affinity-purified antibodies, used as standards, both having an initial protein concentration of 500 μg/ml, were prepared in duplicate in phosphate-buffered saline (PBS) containing 0.05% Tween 20. Affinity-purified antibodies from the sera of mice that had received multiple injections of *N. meningitidis* cells were prepared as described previously (13). In brief, 0.3 g of freeze-dried CNBr-activated Sepharose 4B (Pharmacia) was coupled to 4 mg of LPS. Hyperimmune serum (0.3 ml), diluted to 2 ml with borate buffer solution (0.1 M, pH 8.0) containing 0.5 M NaCl, was applied to a column (4 by 1 cm) with a sorbent. Antibodies to LPS were eluted with 3 M KCNS. The antibodies thus obtained were dialyzed against PBS (pH 7.4) containing 0.02% NaN_3, concentrated with polyethylene glycol 40,000 to 1 ml, and stored at −70°C. The protein content in the samples, detected by the Lowry method, was 0.62 mg/ml. The assay was done with peroxidase conjugated to various classes of mouse immunoglobulins; as a substrate, H_2O_2–*o*-phenylenediamine was used. A standard curve was constructed; 1 U of activity corresponded to the activity of 1 μg of affinity-purified anti-LPS antibodies in a reaction with peroxidase-conjugated anti-mouse polyvalent IgM, IgG, and IgA (Sigma).

Statistical analysis. The results are presented as the geometric mean ± the standard error (SE) (antilogarithm of the standard error of the mean of the logarithms). Before statistical analysis, antibody concentrations or the numbers of PFC were log converted, a procedure that normalized their distribution. Findings were considered statistically significant only when P was ≤0.05.

RESULTS

Incorporation of LPS into liposomes. As demonstrated earlier, a decrease in LPS toxicity depends on the completeness of LPS incorporation into liposomes (27). LPS salts with different solubilities in water probably show different degrees of incorporation into phospholipid bilayers. In these experiments, three kinds of FITC-LPS with different solubilities in water were used: a practically insoluble acidic form of LPS, moderately soluble Mg^{2+} LPS, and readily soluble Et_3N LPS. Liposomes were obtained by two methods: DRV and prolonged cosonication (for up to 30 min). Table 1 shows that the highest percentage of incorporation into vesicles (exceeding 99%) was established for H^+ LPS, scarcely soluble in water and prepared by the DRV method. For this reason, we used the DRV method for the incorporation of H^+ LPS into liposomes in the remaining experiments.

LPS activity in the LAL test. To determine the minimum amount of phospholipids sufficient for decreasing the toxic properties of LPS, we obtained liposomal LPS preparations with different LPS/lipid ratios (1:0.1 to 1:1,000 by weight) by the DRV method and determined the LAL gelation potencies of these preparations. As indicated in Table 2, a decrease in the activity of LPS occurred even at an LPS/lipid ratio of 1:1 (by weight) and reached its lowest level at an LPS/lipid ratio of 1:100. In further experiments evaluating immunobiological properties, the preparations were obtained by the DRV method at an LPS/lipid ratio of 1:10.

Pyrogenic properties of liposomal LPS. The main test of the control of endotoxin toxicity in preparations intended for parenteral administration is the pyrogen test in rabbits. In accordance with World Health Organization requirements for polysaccharide vaccines (29), a 10^{-4} μg dose of a polysaccharide vaccine (one human dose is about 100 μg) must not induce a pyrogenic reaction in a rabbit. As shown in Table 3, liposomal LPS was pyrogen free at a dose of 0.1 μg, and the pyrogenic properties of native LPS decreased 1,000 times. The level of detoxication achieved in these experiments was comparable to the pyrogenic properties of group A meningococcal polysaccharide vaccines in Russia. Thus, the pyrogenic properties of the liposomal form of *N. meningitidis* LPS corresponded to the requirements of the State Pharmacopoeia of Russia and the World Health Organization requirements for meningococcal polysaccharide vaccines.

Induction of anti-LPS PFC in mice. Table 4 shows the dose dependence of the induction of PFC after immunization of mice with free and liposomal LPS. All doses of liposomal LPS studied in our investigation (0.1 to 50 μg) induced the extensive accumulation of PFC. The level of PFC induced by liposomal LPS was 10-fold higher than the level induced by free LPS.

A study of the dynamics of PFC accumulation (Fig. 1) revealed three important facts: in comparison with free LPS, liposomal LPS induced a more prolonged accumulation of

TABLE 2. Activity of *N. meningitidis* LPS incorporated into liposomes in the LAL test

Prepn	Gelation of LAL with LPS at the following concn (μg/ml):				
	4×10^{-8}	4×10^{-7}	4×10^{-6}	4×10^{-5}	4×10^{-4}
Free LPS	−	+	+	+	+
LPS-liposome mixture	−	+	+	+	+
Liposomal LPS at an LPS/lipid ratio of:					
1:0.1	−	+	+	+	+
1:1	−	−	+	+	+
1:10	−	−	−	+	+
1:100	−	−	−	−	+
1:1,000	−	−	−	−	+

TABLE 3. Pyrogenic reactions in rabbits after the intravenous injection of free and liposomal *N. meningitidis* LPS

Prepn	Dose (μg)	Total increase in temp in three rabbits after the following no. of h[a]:				Pyrogenic effect
		1	2	3	4	
Free LPS	10^{-4}	0.4	1.0	1.1	0.5	–
	10^{-3}	0.5	1.5	1.5	1.3	+
	10^{-2}	2.4	3.2	3.5	0.6	+
	10^{-1}	3.2	3.6	5.1	3.2	+
Liposomal LPS	10^{-3}	0.2	0.2	0.5	0.1	–
	10^{-2}	0.7	1.0	0.7	0.5	–
	10^{-1}	0.2	1.2	1.1	0.3	–
	10^{0}	0.6	1.7	2.1	1.5	+
Group A polysaccharide vaccine	10^{-1}	0.8	0.7	0.6	0.3	–

[a] Sum of the changes in temperature in degrees Celsius.

PFC; the peak of the response with liposomal LPS was observed later, on day 6 after immunization; and both preparations induced cyclic changes in the PFC level at 1 to 3 days.

We believe that the liposomal form of the vaccine will be used for subcutaneous immunization. The influence of the route of administration of liposomal LPS was studied with mice immunized with doses of 1 and 10 μg (Table 5). Administration of the preparation by subcutaneous injection was found to be less effective than administration by i.p. and intravenous injections. However, the adjuvant action of the subcutaneous injection of liposomal LPS was retained, and a sixfold increase in the PFC level with a dose of LPS of 10 μg was observed. It should be pointed out that free LPS at a dose of 10 μg, introduced intravenously, produced a toxic effect in mice that probably accounted for the decrease in the immune response with an increase in the dose from 1 to 10 μg.

The serious drawback of currently used meningococcal polysaccharide vaccines is the absence of an immunogenic effect at an early age. This phenomenon is believed to be due to the delayed appearance of the subpopulation of B lymphocytes responding to polysaccharide antigens (T-cell-independent antigens of type 2) in ontogenesis. In mice, these B lymphocytes have marker LyB-5. CBA/N mice have no lymphocytes of this phenotype because of genetic defect *xid* (1). Table 6 indicates that liposomal LPS effectively induced the accumulation of PFC in CBA/Ca *lac* mice and CBA/N *xid* mice. Therefore, liposomal forms of LPS may be immunogenic at an early age.

TABLE 4. Levels of PFC induced by LPS after immunization of CBA/Ca *lac* mice with different doses of free and liposomal LPS

Prepn[a]	Dose (μg)	No. of PFC/spleen[b]
Liposomal LPS	0.1	3.32 ± 0.19 (2,120)
	1.0	3.93 ± 0.14 (8,510)
	10.0	4.48 ± 0.28 (30,130)
	50.0	4.79 ± 0.21 (62,040)
Free LPS	0.1	2.44 ± 0.25 (280)[c]
	1.0	3.27 ± 0.18 (1,871)
	10.0	3.48 ± 0.26 (3,063)
	50.0	3.73 ± 0.22 (5,400)
Saline		2.20 ± 0.05 (160)

[a] Mice were immunized i.p., and the number of PFC in their spleens was determined on day 5.
[b] Log_{10} PFC per spleen ± SE for groups of 10 mice; geometric means (antilogarithms) are shown in parentheses.
[c] Not significantly different from the control (saline-treated mice).

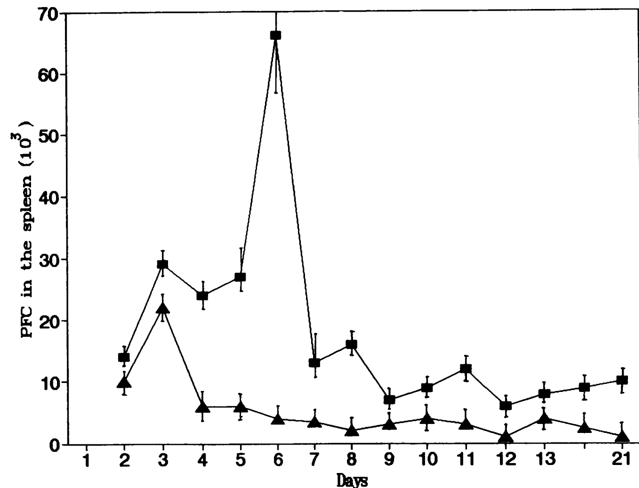

FIG. 1. Dynamics of the accumulation of PFC in mice immunized with free (▲) and liposomal (■) LPS (LPS content, 10 μg). Vertical bars represent the SE for six animals. An absence of bars indicates that the results do not differ significantly from the control results (saline-treated mice).

Level of anti-LPS antibodies. To determine the level of antibodies in an ELISA, we made a standard preparation of affinity-purified antibodies from the sera of mice after multiple immunizations with *N. meningitidis* cells. Titration curves for anti-LPS IgG and IgM antibodies in the sera of intact mice and those immunized with meningococci, free LPS, or LPS incorporated into liposomes are presented in Fig. 2. The content of antibodies in the sera of intact mice was 0.086 U/ml and increased 230-fold after multiple immunizations with *N. meningitidis* cells. All groups of mice were found to have approximately the same ratio of IgG antibodies to IgM antibodies in their blood sera. Among IgG antibodies, those belonging to subclass IgG3 prevailed (data not shown).

Experiments testing the dose dependence of the immune response (Table 7) indicated that liposomes had a pronounced adjuvant effect on the concentration of serum antibodies. In contrast to free LPS, LPS incorporated into liposomes (5 μg) permitted the induction of 3.73 U/ml on day 7 after injection. By day 21, a high level of antibodies to liposomal LPS (0.815 U/ml) was retained; this level exceeded the level induced by free LPS fourfold. No changes in the class of induced antibodies in mice immunized with the liposomal LPS preparations were observed.

DISCUSSION

The results presented in this paper indicate that the incorporation of *N. meningitidis* LPS into liposomes led, as shown in experiments with mice, to a considerable decrease in the activity of endotoxin and to an increased capacity of endotoxin to induce humoral responses. In previous work, we established that after the association of *N. meningitidis*

TABLE 5. Influence of different routes of administration on the level of PFC after immunization of CBA/Ca *lac* mice with *N. meningitidis* LPS incorporated into liposomes

Prepn[a]	Dose (μg)	No. of PFC/spleen after injection by the following route[b]:		
		Subcutaneous	i.p.	Intravenous
Free LPS	1.0	2.41 ± 0.15[c] (259)	2.84 ± 0.19 (691)	3.31 ± 0.30 (2,035)
	10.0	2.54 ± 0.13 (350)	3.65 ± 0.26 (4,456)	3.28 ± 0.33 (1,889)
Liposomal LPS	1.0	2.50 ± 0.20[c] (319)	4.36 ± 0.23 (22,976)	4.40 ± 0.27 (25,235)
	10.0	3.30 ± 0.14 (2,010)	4.54 ± 0.17 (34,422)	4.67 ± 0.18 (46,766)
Saline		2.28 ± 0.09 (189)		

[a] The number of PFC was determined on day 5 after immunization.
[b] Log_{10} PFC per spleen ± SE for groups of 10 mice; geometric means (antilogarithms) are shown in parentheses.
[c] Not significantly different from the control (saline-treated mice).

LPS with phospholipid bilayers, the toxicity of LPS for mice treated with actinomycin D decreased up to 1,000 times, the completeness of the incorporation of LPS reflecting the degree of its detoxication (27). The possibilities of practically complete incorporation of LPS into liposomes and alteration of some of its immunobiological properties, in particular, a decrease in its toxicity and a change in its macrophage-stimulating activity, were experimentally shown by a number of researchers using preparations of lipooligosaccharides and LPSs (R-type and S-type LPSs) from gram-negative bacteria (3, 9, 10). One of the methods necessary for ensuring the effective incorporation of LPS into phospholipid bilayers is cosonication (11). As noted in these investigations, R-type LPS was incorporated into liposomes in different ways because LPS obtained from R mutants was less hydrophilic because of its short carbohydrate chains. In some experiments, the presence of Mg^{2+} ions was necessary for the preparation of the mixed LPS-phospholipid bilayers, while in other experiments, this condition was not essential (11, 20). As shown in the present study, the solubility of LPS in water was also of importance and changed with the conversion of one form of LPS into another (H^+ LPS and Mg^{2+} and Et_3N salts). The acidic form of LPS, least soluble in water, was almost completely (≥99.9%) incorporated into liposomes, while with the increase in solubility from Mg^{2+} LPS to Et_3N LPS, a larger amount of LPS remained free, i.e., not incorporated into liposomes (Table 1). The main difference in the physical structures of liposomal LPS formulations produced by the prolonged cosonication method and DRV consisted of the location of the LPS. Liposomes obtained by the prolonged cosonication method are formed as small, unilamellar vesicles. As LPS was added to the preformed liposomes, it was primarily located on the outer surface of the vesicles. By the DRV method multilamellar liposomes were produced. Because of this multilamellar structure, LPS was incorporated into the outer and inner membranes of these liposomes. A high degree of incorporation of LPS into liposomes was indirectly confirmed by a decrease in its activity in the LAL test. In experiments with *Salmonella minnesota* LPS, like *N. meningitidis* LPS in its chemical structure, 100-fold suppression of LAL coagulation

TABLE 6. Level of PFC in the spleens of CBA/Ca *lac* and CBA/N mice after immunization with liposomal LPS

Prepn[a]	No. of PFC/spleen in the following mice[b]:	
	CBA/Ca *lac*	CBA/N
Free LPS	2.80 ± 0.25 (630)[c]	3.18 ± 0.36 (1,501)
Liposomal LPS	4.11 ± 0.24 (12,748)	3.94 ± 0.21 (8,678)
Saline	2.16 ± 0.32 (144)	2.40 ± 0.28 (250)

[a] The response was registered on day 6 after i.p. immunization (the dose of LPS was 10 μg).
[b] Log_{10} PFC per spleen ± SE for groups of eight mice; geometric means (antilogs) are shown in parentheses.
[c] Not significantly different from the control (saline-treated mice).

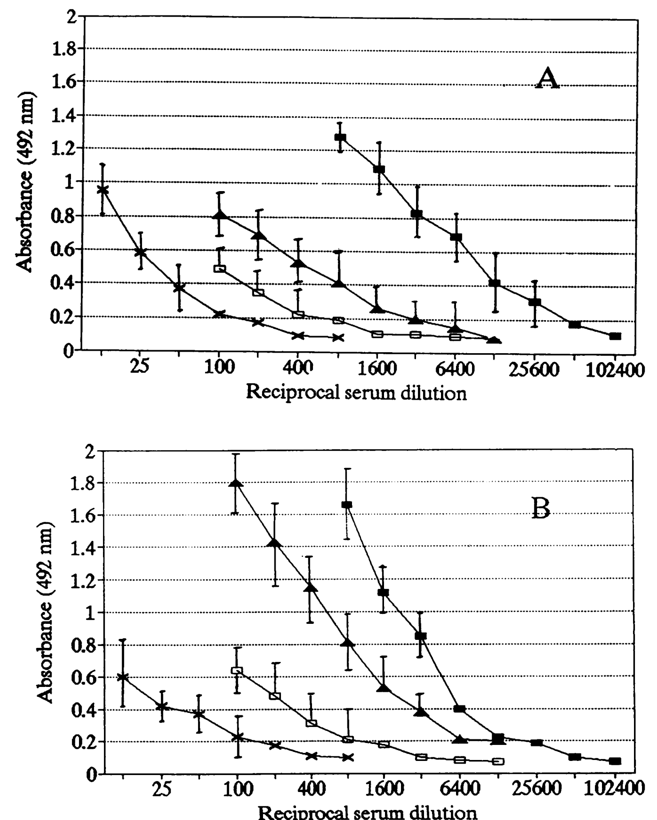

FIG. 2. ELISA analysis of IgG (A) and IgM (B) anti-LPS antibodies in mice. Sera were collected from intact mice (×) and from mice on day 14 after a single injection of liposomal LPS (▲) or free LPS (□) and on day 7 after multiple immunizations with *N. meningitidis* cells (■). Six animals were used per group. Results are expressed as the mean ± SE (bars) for three independent experiments. An absence of bars indicates that the results do not differ significantly from the results for the nearest curve or the blank control.

TABLE 7. Level of antibodies to meningococcal LPS after immunization of CBA/Ca *lac* mice with free and liposomal LPS

Prepn[a]	Dose (μg)	Antibody level in serum at the following day after immunization[b]:			
		4	7	14	21
Liposomal LPS	1	0.31 (1.48)	0.36 (1.25)	0.29 (1.27)	ND
	5	1.24 (1.25)	3.73 (1.07)	2.45 (1.17)	0.82 (1.10)
	25	0.79 (1.25)	2.42 (1.13)	2.41 (1.08)	ND
Free LPS	1	0.10 (1.20)[c]	0.18 (1.44)[c]	0.13 (1.31)[c]	ND
	5	0.38 (1.18)	0.81 (1.20)	0.42 (1.26)	0.42 (1.36)
	25	0.61 (1.10)	0.89 (1.24)	0.41 (1.78)	ND
Saline		0.09 (1.44)			

[a] In each group, five mice received a single i.p. injection.
[b] Geometric mean of anti-LPS antibodies, in units per milliliter (SE). ND, not determined.
[c] Not significantly different from the control (saline-treated mice).

was achieved (10). In our experiments, this level of suppression was somewhat higher, probably because of the use of the acidic form of LPS.

The pathophysiological effect of LPS is based on the action of the cell-mediated mechanism involving the cells of the reticuloendothelial system. The chain of events is probably as follows: the hydrophobic part of LPS (lipid A) is recognized by special protein receptors on the macrophage membrane, and the mechanism of intensive synthesis and release of monokines, particularly tumor necrosis factor, one of the major mediators of septic shock, is triggered (15, 22). LPS is therefore detoxified by methods ensuring the elimination of lipid A, partial detoxication resulting from deacylation or dephosphorylation of lipid A, as well as screening with hydrophobic substances, e.g., polymyxin B or phospholipids (5, 23). In the latter case, LPS penetrates the phagocyte, remaining unrecognized, so the pathophysiological mechanism of endotoxic shock is not triggered.

The incorporation of *N. meningitidis* LPS into liposomes is accompanied by changes in its antigenic activity. Thus, in the case of inhibition of passive hemagglutination with LPS-sensitized SRBC in the presence of anti-LPS antibodies, liposomal LPS was four- to eightfold more active than native LPS (data not shown). The association of LPS with liposomes leads, seemingly, to changes in the state of micelles and to an increase in the number of antigenic determinants exposed on the surface of vesicles.

The development of vaccines based on LPSs obtained from *N. meningitidis* and other gram-negative bacteria is now attracting special attention. The main approach to solving the problems associated with these attempts consists of the elimination of lipid A and the conjugation of the carbohydrate component of LPS with a protein carrier (2, 17). However, lipid A has, in addition to its toxic action, an adjuvant effect. For this reason, retaining and simultaneously detoxifying lipid A are of special interest for the development of new vaccines.

Attempts aimed at enhancing the immunogenic properties of LPS and lipid A were also made earlier (4, 8). Thus, *Salmonella typhimurium* LPS, incorporated into liposomes, effectively induced delayed-type hypersensitivity. However, these studies were not continued further because of the low percentage of LPS incorporation into liposomes (1.5 to 2.8%). Our experiments demonstrated that after the incorporation of LPS into liposomes, humoral responses appreciably increased. Thus, on day 5 after immunization, almost every cell of 2,000 nucleated cells in the mouse spleen synthesized complement-fixing antibodies. The phenomenon of fluctuations in the number of PFC, observed for several weeks after immunization with free and liposomal LPS, still remains unexplained. This phenomenon was described after immunization with LPS obtained from *Escherichia coli* 055 (12). However, the study of serum antibodies revealed no regularity in the fluctuations in the number of PFC.

An essential drawback of the currently used polysaccharide vaccines is their very low effectiveness in the immunization of young children and their inability to produce immunological memory. As shown by experimental results (Table 6), LPS incorporated into liposomes retained its capacity to induce immune responses in CBA/N mice, deficient in LyB-5$^+$ B lymphocytes; i.e., LPS induced immune responses as a thymus-independent type 1 antigen. Liposomes were found essentially to prolong the time of the accumulation of PFC and the circulation of antibodies. For this reason, one injection of liposomal LPS may be sufficient for the development of prolonged immunity.

The problems of whether antibodies induced by liposomal LPS have bactericidal action, protect from infection, and cross-react with meningococci of other immunotypes (since the retained lipid A carries common determinants of the species) remain unsolved. To solve all these problems, further studies are necessary, and we are conducting these at present.

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