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Altered In Vivo Activity of Liposome-Incorporated Lipopolysaccharide and Lipid A

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We compared the abilities of free and liposome-incorporated *Salmonella minnesota* wild-type lipopolysaccharide (LPS) and lipid A to activate peritoneal macrophages and induce lethal toxicity in mice. Incorporation of lipid A into multilamellar vesicles resulted in a 100-fold-decreased potency to prime macrophages for phorbol myristate acetate-triggered release of H_2O_2. In addition, liposome incorporation reduced the lethality of LPS and lipid A at least 10-fold in dactinomycin-sensitized mice. Similar results were obtained with multilamellar liposomes delivered intravenously and when small unilamellar vesicles were employed. The observed difference in toxicity was not dependent on dactinomycin treatment, since a similar decrease was obtained with large doses of liposomal LPS in unsensitized mice. Control liposomes, prepared without LPS and lipid A, did not reduce the activities of the free compounds. The administration of a sublethal amount of liposomal LPS induced within 20 days, but not during the first week, tolerance to a subsequently injected lethal dose of free endotoxin. The latter observation suggests that early-phase tolerance is not the mechanism responsible for the reduced toxicity of liposomal LPS. These data show that liposomal LPS and lipid A have reduced endotoxic activity in vivo and are consistent with our hypothesis that a direct interaction of lipid A with appropriate plasma membrane components is necessary to efficiently trigger biologic responses. This interaction, however, is prevented by the stable insertion of LPS into the liposomal membrane.

Lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, triggers many of the pathological sequelae of gram-negative sepsis, including shock, disseminated intravascular coagulation, tissue injury, and death (21, 37, 43). Although the precise cellular and molecular mechanisms whereby LPS elicits these pathological events have not been elucidated, mononuclear phagocytes (monocytes and macrophages) appear to play a central role (16, 36, 47). LPS induces many responses from macrophages, among which are the development of an enhanced respiratory burst capacity (24, 40) and the secretion of inflammatory mediators, including prostaglandins (46), colony-stimulating factors (52), interleukin-1 (IL-1) (12) and tumor necrosis factor alpha (cachectin) (TNF) (4, 32). Recently, TNF has been shown to reproduce the manifestations of septic shock when administered to experimental animals, indicating that it is a major mediator of endotoxin-induced injury (1, 33, 39, 45, 53).

In previous in vitro studies on the mechanisms of LPS action, we modified the interaction of LPS and lipid A (the active moiety of LPS) with macrophages by incorporating these compounds into the lipid bilayer of liposomes (phospholipid vesicles). By using this approach, it was demonstrated that the potency of LPS and lipid A as inducers of macrophage secretory (IL-1 and TNF), phagocytic, and tumoricidal activities is reduced 100- to 1,000-fold by liposome incorporation (9, 10). Experiments performed with radiolabeled LPS revealed that this decreased potency of liposomal LPS was not a result of reduced cellular uptake (10). These findings suggested that incorporation of LPS into the bilayer of liposomes prevents the interaction of the active lipid A moiety with key plasma membrane structures involved in signal transduction (9, 10). To extend these in vitro studies, we have now investigated whether liposome incorporation alters the macrophage-activating and lethal properties of LPS and lipid A in vivo.

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MATERIALS AND METHODS

Mice. Female 6- to 12-week-old BALB/c mice from the breeding colony of the National Institutes of Health Bethesda, Md., weighing between 15 and 20 g, were used in all experiments. For toxicity experiments, mice were selected that varied in weight by less than 2 g.

LPS and lipid A. Monophosphoryl lipid A and diphosphoryl lipid A, both derived from *Salmonella minnesota* R595 rough (Re) LPS, were obtained from List Biological Laboratories, Campbell, Calif., and Ribi ImmunoChemical Research Inc., Hamilton, Mont., respectively. Clear aqueous stock solutions of lipid A (0.5 mg/ml) were made in 0.5% triethylamine. For the incorporation of lipid A in liposomes, stock solutions (0.5 mg/ml) of monophosphoryl lipid A in chloroform and of diphosphoryl lipid A in chloroform-methanol (4:1) were prepared. *S. minnesota* wild-type (wt) LPS (List) was dissolved (5 mg/ml) in pyrogen-free water by heating at 37°C, vortexing, and brief sonication in a bath-type sonicator (Laboratory Supply Company, Inc., Hicksville, N.Y.).

Liposomes. All phospholipids were obtained from Avanti Polar Lipids, Inc., Birmingham, Ala., and cholesterol (Chol) (C-8253) was obtained from Sigma Chemical Co., St. Louis, Mo. Lipid A was incorporated into multilamellar vesicles (MLV) or small unilamellar vesicles (SUV) consisting of egg phosphatidylcholine (PC), bovine brain phosphatidylserine, or egg phosphatidylglycerol (PG) and Chol in a molar ratio of 4:1:4 by the chloroform method, as described previously (10). Control liposomes were prepared with sterile, pyrogen-free phosphate-buffered saline. To incorporate up to 20 μg of wt LPS per μmol of total liposomal lipid, our previously

described dry method was used to make both MLV and SUV (10, 11). To incorporate up to 100 μg of wt LPS per μmol of total liposomal lipid and to prepare relatively large amounts of phospholipid vesicles, the dry method was slightly modified as follows. The liposomal lipid mixture in chloroform (100 μmol of total lipid) was dried in borosilicate glass culture tubes (16-mm diameter) by rotary evaporation and high vacuum. Next, the lipids were suspended in 2 ml of water containing the desired amounts of wt LPS (2.5 to 10 mg). After being vortexed and heated at 40 to 45°C, the lipid suspension was sonicated for two 12-min periods with intermittent vortexing. The resulting opalescent solution was lyophilized overnight and suspended in 1.5 ml of phosphate-buffered saline. MLV and SUV were then prepared by vortexing (MLV) and sonication (SUV) (10, 11). To avoid potential contamination of the liposome preparations with environmental endotoxins, no attempts were made to separate unincorporated lipid A or wt LPS from the liposomes. More than 99% of the added lipid A or wt LPS was incorporated into the phospholipid vesicles used in this study, as indicated by at least 100-fold-reduced clotting activity in the *Limulus* amoebocyte lysate assay (10, 11).

Peritoneal macrophage H_2O_2 release. Groups of four to six mice were injected intraperitoneally (i.p.) with 0.5 ml of free monophosphoryl lipid A (diluted in saline, 0.01% triethylamine final concentration) or liposomal lipid A suspensions in phosphate-buffered saline. Four days after injection (24), mice were sacrificed and peritoneal exudate cells were harvested by lavage with Hanks balanced salt solution. Peritoneal exudate cells were pelleted at 200 × *g* for 10 min (4°C) and suspended in Dulbecco modified Eagle medium (Microbiological Associates, Walkersville, Md.) supplemented with 2 mM glutamine, 25 μg of gentamicin per ml, 25 μg of streptomycin per ml, 25 IU of penicillin per ml, and 10% heat-inactivated (56°C, 30 min) fetal calf serum (Hyclone Laboratories, Logan, Utah). Peritoneal exudate cell viability (trypan blue exclusion) was greater than 95% in all experimental groups. One million peritoneal exudate cells were seeded into 11-mm-diameter plastic tissue culture wells (Costar, Cambridge, Mass.) and incubated for 90 min at 37°C in 5% CO_2 to permit macrophage adherence. Nonadherent cells were removed by washing each well twice with warm (37°C) Hanks balanced salt solution immediately before the assay. Less than 3% of the adherent cells from all experimental groups were polymorphonuclear leukocytes, as determined in Wright-stained monolayers. Macrophage H_2O_2 release was measured by the horseradish peroxidase-catalyzed oxidation of phenol red, as described by Pick and Keisari (41). A 1-ml volume of warm (37°C) phenol red solution (140 mM NaCl, 10 mM K_2HPO_4, 5.5 mM dextrose, 0.28 mM phenol red, 50 μg of horseradish peroxidase per ml [Sigma]), pH 7.4, was added to each well with 50 ng of phorbol 12-myristate 13-acetate per ml (PMA; Sigma) as the triggering stimulus. After 60 min, 0.6 ml of the solution was removed and adjusted to pH 12.5 with 0.01 ml of 1.0 N NaOH, and the A_{610} was read. A standard curve was prepared with each assay, using dilutions of a stock 10 mM H_2O_2 solution. The concentration of the stock was verified for each assay by using an extinction coefficient for H_2O_2 of 81 M/cm at 230 nm (41). For most experiments, a duplicate set of macrophages was prepared in parallel for determination of cell protein (31).

Lethal toxicity assay. Groups of four preweighed mice were used for each experimental condition. Free diphosphoryl lipid A diluted in sterile, pyrogen-free saline (0.01% triethylamine final concentration) and free wt LPS diluted in saline were administered i.p. (0.5 ml) or intravenously (i.v.) (0.2 to 0.3 ml) in the tail. The liposomal lipid A and LPS preparations diluted in phosphate-buffered saline were injected i.p. or i.v. in volumes similar to those of the free compounds. In most experiments, the animals were sensitized with 550 μg of dactinomycin per kg of body weight (from *Streptomyces* species; Sigma) (2, 50). This dose of dactinomycin was used because it was not lethal for control mice in our system. Dactinomycin was dissolved in ethanol (1 mg/ml) and was diluted in saline prior to use. The final concentration was checked by measuring the A_{430} (50) and adjusted as necessary. Dactinomycin (0.5 ml) was injected i.p. within 20 min after the mice received the LPS or lipid A preparations. Deaths were recorded daily for 7 to 10 days. The cumulative mortality after 4 days was summated (deaths after 4 days rarely occurred).

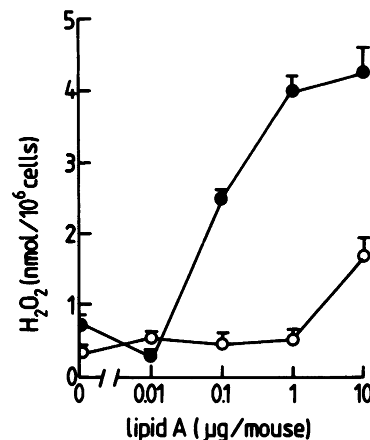

FIG. 1. Stimulation of peritoneal macrophage H_2O_2-releasing capacity by free (●) or MLV-incorporated (○) monophosphoryl lipid A. The MLV consisted of PC, phosphatidylserine, and Chol in a molar ratio of 4:1:4. Groups of four to six mice were injected i.p. with the indicated amounts of free lipid A or liposomal lipid A (2.5 μmol of total lipid per mouse). After 4 days, peritoneal macrophages were harvested, and PMA-triggered H_2O_2 release was determined. The data shown are the mean values of quadruplicate wells expressed as nanomoles of H_2O_2 released in 1 h per 10^6 cells (each bar represents 1 standard deviation).

RESULTS

Augmentation of peritoneal macrophage H_2O_2-releasing capacity by free and liposomal lipid A. In initial experiments, we compared the abilities of free and liposome-incorporated monophosphoryl lipid A to augment peritoneal macrophage H_2O_2-releasing capacity when injected i.p. into mice. Four days after injection, peritoneal macrophages were isolated, and PMA-triggered H_2O_2 release was measured. H_2O_2 release was studied as the parameter of macrophage activation because it can be determined within 90 min of cell harvest, thus reducing the influence of in vitro culture on cellular function. Free lipid A was a potent activator of macrophage H_2O_2-releasing capacity (Fig. 1). The dose of free lipid A required to enhance H_2O_2 release was 0.1 μg per mouse. In contrast, MLV-incorporated lipid A was approximately 100-fold less potent, the minimum active dose being 10 μg per mouse. The amount of H_2O_2 released after administration of control MLV was not significantly different from the values obtained with control saline (Fig. 1). The MLV used in this experiment contained phosphatidylserine, which has been

TABLE 1. Effect of control liposomes on the macrophage-activating potency of free monophosphoryl lipid A[a]

Treatment	Macrophage H_2O_2 release (nmol/mg of protein)[b] at lipid A dose of (μg/mouse):			
	10	1	0.1	0
Free lipid A	472 ± 6	404 ± 16	166 ± 6	55 ± 3
Free lipid A plus control MLV	471 ± 14	341 ± 7	208 ± 7	95 ± 5

[a] Experimental conditions were as described in the legend to Fig. 1.
[b] Mean values (± standard deviation) of quadruplicate wells.

shown to block gamma interferon-induced microbicidal activity of peritoneal macrophages (19). We therefore performed control experiments in which free lipid A was coinjected with control MLV (containing phosphatidylserine). The data in Table 1 clearly demonstrate that coinjected control MLV did not reduce the activating potency of free lipid A. Thus, the reduced potency of MLV-lipid A is not explained by a direct effect of MLV on the responsiveness of macrophages to lipid A.

We next determined whether altering the phospholipid composition (PG versus phosphatidylserine) of the liposomes influenced the potency of MLV-incorporated lipid A. When lipid A was incorporated into MLV containing PG, a similar 100-fold reduction in potency was observed (Fig. 2). This effect was again not due to a liposome-mediated alteration in macrophage responsiveness to lipid A, since coinjection of control MLV with free lipid A did not alter the dose-response curve of free lipid A (Fig. 2).

Lethal toxicity of free and liposome-incorporated lipid A in dactinomycin sensitized mice. We next sought to determine whether the lethal toxicity of lipid A was modified by liposomal delivery. Since monophosphoryl lipid A is a relatively detoxified preparation of lipid A (23), we used diphosphoryl lipid A in the toxicity experiments. However, free diphosphoryl lipid A administered i.p. in doses of up to 200 μg failed to induce a lethal effect in normal mice. This finding is in agreement with previous studies which showed 50% lethal dose (LD_{50}) values of more than 1 mg for *S. minnesota*-derived lipid A (17, 18). Such large quantities, however, are impractical to incorporate into phospholipid vesicles. Consequently, dactinomycin-sensitized mice were

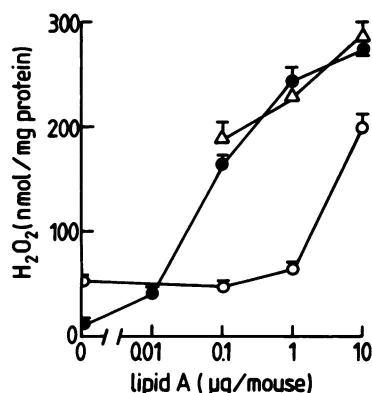

FIG. 2. Stimulation of peritoneal macrophage H_2O_2-releasing capacity by free monophosphoryl lipid A (●), MLV-incorporated lipid A (○), or free lipid A plus control MLV (△). The MLV consisted of PC, PG, and Chol in a molar ratio of 4:1:4. The other experimental conditions were as described in the legend to Fig. 1.

TABLE 2. Lethal toxicity of free and MLV-incorporated diphosphoryl lipid A in dactinomycin-sensitized mice

Dose (μmol of lipid/mouse) and treatment[a]	No. of mice killed[b] at lipid A dose of (μg/mouse):				
	10	1	10^{-1}	10^{-2}	0
0.5					
Free lipid A	4	4	1	0	0
Free lipid A plus control MLV	4	4	1	ND[c]	0
MLV-lipid A	4	1	0	ND	0
2.5					
Free lipid A	4	4	2	0	0
Free lipid A plus control MLV	4	4	0	ND	0
MLV-lipid A	4	0	0	ND	0

[a] MLV were PC-PG-Chol (4:1:4).
[b] Total deaths 4 days after lipid A administration. Four mice were used per experimental condition. Lipid A was administered i.p.
[c] ND, Not determined.

used to conduct the toxicity studies with liposomal lipid A. This model has been widely employed to study the toxic properties of endotoxins (2, 50, 56). In our experiments, dactinomycin sensitization reduced the lethal i.p. dose of lipid A from greater than 200 μg to 0.1 to 1.0 μg per mouse.

Various amounts of free lipid A or lipid A incorporated into MLV were injected into mice (0.5 μmol of total lipid), followed within 20 min by the administration of dactinomycin. Liposomal lipid A was 10-fold less toxic than either free lipid A or free lipid A simultaneously injected with control MLV (Table 2). Analysis of the LD_{50}s revealed values of 0.18 μg for free lipid A and 2.4 μg for liposomal lipid A (44). In addition, the incorporation of lipid A into SUV (0.5 μmol of lipid per mouse) also resulted in a 10-fold reduction in lethal toxicity (results not shown). The reduced toxicity of liposomal lipid A did not appear to be dependent on a particular lipid dose, since a similar reduction in potency was observed when MLV-incorporated lipid A was administered in a lipid dose of 2.5 μmol per animal (Table 2).

Lethal toxicity of free and liposomal wt LPS in sensitized and normal mice. Smooth or wt LPS is a potential contaminant of liposomes prepared for pharmaceutical use. For this reason, we also studied the toxic properties of *S. minnesota* wt LPS incorporated into liposomes or mixed with preformed vesicle preparations. MLV-incorporated wt LPS injected i.p. into dactinomycin-sensitized mice was more than 10-fold less toxic than free wt LPS (Table 3). Analysis of the LD_{50}s revealed values of 0.05 μg for free LPS and 3.2 μg for liposomal LPS (44). When the route of administration was changed from i.p. to i.v., a similar reduction in toxicity was observed (Table 3). This result was also obtained when SUV were used as carriers for i.v. LPS. Conversely, free LPS mixed with control MLV or SUV and injected either i.p. or i.v. was essentially as toxic as the free compound alone (Table 3).

To investigate whether the observed difference in toxicity of liposome-associated versus free LPS was related to the dactinomycin treatment, we conducted experiments to assess the LD_{100} of liposomal versus free LPS in unsensitized mice. The LD_{100} for wt LPS was found to be 100 to 150 μg for unsensitized animals. Next, various amounts of LPS were incorporated into MLV (up to 100 μg/μmol of total lipid), and the vesicles were injected i.v. To administer large doses of liposomal LPS, it was necessary to use a relatively large amount of total liposomal lipid (20 μmol per mouse). In unsensitized mice, liposome-incorporated wt LPS was 10-

TABLE 3. Lethal toxicity of free and liposomal wt LPS in dactinomycin-sensitized mice

Type of injection[a]	No. of mice killed[b] at LPS dose of (μg/mouse):				
	10	1	10^{-1}	10^{-2}	0
i.p.					
Free LPS	4	4	3	0	0
Free LPS plus control MLV	4	4	0	ND[c]	0
MLV-LPS	4	0	0	ND	0
i.v.					
Free LPS	4	4	1	0	0
Free LPS plus control MLV	4	4	1	ND	0
MLV-LPS	4	1	0	ND	0
i.v.					
Free LPS	4	4	4	0	0
Free LPS plus control SUV	4	4	4	ND	0
SUV-LPS	4	2	0	ND	0

[a] The MLV and SUV dose was 0.5 μmol of total liposomal lipid per mouse. Liposomes were composed of PC-PG-Chol (4:1:4).
[b] Total deaths 4 days after LPS administration. Four mice were used per experimental condition.
[c] ND, Not determined.

fold less toxic (LD_{100}, 1,000 μg) than free LPS or free LPS mixed with control vesicles (LD_{100}, 100 μg) (Table 4).

Induction of lethal tolerance by liposomal wt LPS. Single or multiple daily injections of a nontoxic dose of LPS can induce resistance or tolerance to the lethal effects of readministered endotoxin (25). Two phases of tolerance can be distinguished. In mice, early-phase tolerance develops within 24 h and starts waning in the next few days. In contrast, late-phase tolerance, which is LPS type specific, requires several days to develop after a single injection of LPS (25). Induction of early-phase tolerance could account for the reduced toxicity we observed with liposomal LPS. To test the possibility that gradual release of LPS from the liposomes induced early-phase tolerance, the following experiment was performed. Mice were injected with a sublethal dose of MLV-incorporated wt LPS and after different time periods reinjected with a lethal dose of free LPS. The administration of 200 μg of free LPS 1 to 7 days after the injection of 200 μg of MLV-LPS resulted in death of all the animals (Table 5). In fact, these mice appeared more sensitive since death occurred within 24 h, whereas the controls died between 24 and 48 h. In contrast, when free LPS was administered 20 days after the liposomal form, complete tolerance was observed (Table 5). These mice also survived the administration of a second lethal dose (200 μg) of free

TABLE 4. Lethal toxicity of free and MLV-incorporated wt LPS in normal (unsensitized) mice

Treatment[a]	No. of mice killed[b] at LPS dose of (mg/mouse):								
	2	1.5	1	0.5	0.2	0.15	0.1	0.05	0
Free LPS	ND[c]	ND	ND	4	4	4	4	0	0
Free LPS plus control MLV	ND	ND	ND	4	4	4	4	0	0
MLV-LPS	4	4	4	0	0	ND	ND	ND	0

[a] Administered i.v. The MLV dose was 20 μmol of total liposomal lipid per mouse. MLV were composed of PC-PG-Chol (4:1:4).
[b] Total deaths 4 days after LPS administration. Four mice were used per experimental condition.
[c] ND, Not determined.

TABLE 5. Induction of lethal tolerance by liposomal wt LPS

Pretreatment	No. of mice killed after (days between administration of liposomal LPS and free LPS)[a]:					
	1	3	5	7	20	20 and 34
MLV-LPS	4	4	4	4	0	0
Control	4	4	4	4	4	4

[a] Total deaths 4 days after free-LPS administration. Four mice per experimental condition. Mice were injected i.v. with MLV (PC-PG-Chol)-incorporated LPS (200 μg of LPS in 20 μmol of total lipid), followed by i.v. challenge with 200 μg of free LPS on the day indicated.

LPS, given 14 days after the initial challenge dose (Table 5). No survival was observed in the simultaneously injected control groups which had not been exposed to liposomal LPS. These results suggest that a sublethal dose of liposomal LPS is able to induce late-phase but not early-phase tolerance.

DISCUSSION

The data presented in this in vivo study indicate that the macrophage-activating potency and lethal toxicity of liposome-incorporated LPS or lipid A are reduced 100- and 10-fold, respectively. The liposome dose, vesicle type, and route of administration did not appear to influence this effect. The decreased activities of liposomal LPS and lipid A were also not due to an effect of the phospholipid vesicles themselves, since control liposomes administered together with the free compounds did not significantly alter the potency of the free compounds. In addition, we have previously demonstrated that the procedure used to incorporate LPS into liposomes does not result in inactivation of this bacterial component; LPS with normal biological activity can be recovered from these liposomal LPS preparations (11).

Previously, we investigated the effect of liposome incorporation on the ability of LPS and lipid A to activate macrophages in vitro (9, 10). These studies demonstrated that compared with the free compounds, vesicle-incorporated LPS was at least 100-fold less potent in inducing the tumoricidal state or the secretion of IL-1 and TNF or in stimulating Fc-receptor-mediated phagocytosis. Both free and liposomal LPS, however, became associated with the macrophages in comparable amounts and appeared to be internalized by the endocytic pathway. From these observations, we concluded that the direct interaction of the hydrophobic part of the lipid A moiety of LPS with plasma membrane components is needed to optimally activate these cells in vitro. Thus, due to the stable insertion of the hydrophobic lipid A moiety in the liposomal membrane, this direct interaction is prevented during the association of LPS- or lipid A-containing vesicles with macrophages. Possibly, LPS initially interacts with specific lipid A-binding proteins, which have been recently demonstrated in the plasma membranes of macrophages (20) and splenocyte subpopulations (29).

It is possible that the decreased in vivo potency of liposomal LPS observed in the present study is the result of abrogation of a necessary mode of interaction of lipid A with the macrophage plasma membrane. Although macrophages play a major role as effector cells for the LPS-induced (patho)physiological effects (16, 36, 47), the in vivo interaction of liposomal LPS-containing liposomes with other LPS-responsive cells, such as B lymphocytes (35) or vascular

endothelial cells (30, 34), may similarly result in reduced stimulation.

The increased capacity of LPS-elicited peritoneal macrophages to produce reduced oxygen metabolites after being triggered by PMA or other secondary stimuli was initially observed by Johnston and co-workers (24). In vitro, LPS can prime peritoneal macrophages directly for the increased production of oxygen metabolites (40), but the mechanism by which LPS activates macrophages in vivo is probably more complex, involving both direct and indirect effects. Mediators induced by LPS likely contribute to the induction of the primed state. For instance, both TNF and gamma interferon are able to activate peritoneal macrophages for PMA-triggered secretion of H_2O_2 (13, 38). Moreover, these cytokines have been shown to be produced in vivo after LPS administration (6, 26, 33). In vitro, the direct interaction of LPS with mononuclear phagocytes stimulates TNF secretion (4, 32). Macrophages are necessary as accessory cells for the LPS-induced production of gamma interferon by T lymphocytes (27). The reduced potency of vesicle-incorporated lipid A to prime peritoneal macrophages in vivo which was observed in this study is probably the result of the ineffective interaction of liposomal lipid A with macrophages and possibly other responsive cells.

Cytokines released from macrophages certainly play an important role in the pathogenesis of endotoxic shock. Although mortality from endotoxemia can be considered the integral effect of the large variety of LPS-induced responses (21, 37, 43), macrophage-derived TNF appears to be a central mediator. The administration of recombinant TNF mimics LPS in the induction of different pathophysiological effects (1, 33, 39, 45, 53), and passive immunization with anti-TNF antibodies protects mice and rabbits from endotoxin-induced death (5, 33). Other LPS-induced mediators are probably also required. For example, Kiener and coworkers demonstrated that treatment of mice with detoxified monophosphoryl and toxic diphosphoryl lipid A resulted in comparable TNF levels in the circulation, but only the diphosphoryl component was lethal (26). In addition, both LPS and IL-1 synergize with TNF in the induction of lethal shock (48, 54, 55). Other evidence also indicates the involvement of TNF and IL-1 in endotoxemia. Treatments that sensitize mice for LPS-induced lethality also render these animals hyperresponsive to the toxic effects of both TNF and IL-1 (3, 28, 56). Given the important role of TNF and IL-1 in endotoxic shock and our previous observation that the secretion of these cytokines from macrophages in vitro was considerably reduced upon incorporation of LPS in liposomes, we conclude that impaired secretion of these mediators may contribute to the decreased in vivo toxicity of the liposomal form.

The finding that liposomal LPS did not induce early-phase tolerance indicates that this phenomenon probably does not play a significant role in the decreased toxicity of liposomal LPS. Recent reports suggest that TNF and IL-1 are also involved in the induction of early-phase endotoxin tolerance (15, 54, 56). This suggests that the levels of TNF and IL-1 induced by liposomal LPS are probably not sufficient to induce early-phase tolerance. It is known that the protection observed during late-phase tolerance correlates with the presence of antibodies directed against the carbohydrate chain (O antigen) of LPS (25). Since liposome-incorporated LPS has been shown to induce humoral immunity (8), the late tolerance observed in our study was probably also the result of the generation of specific antibodies, LPS-antibody complexes are thought to be less toxic due to neutralization and/or increased clearance by the host (42).

In vivo, free LPS is possibly detoxified by both intravascular mechanisms and the phagocytic cells comprising the reticuloendothelial system (51). Ultimately, most of the LPS seems to be processed by the liver, initially by the liver macrophages or Kupffer cells. Intravenously administered liposomes, irrespective of the presence of LPS as a constituent, are taken up mainly by the macrophages of the liver and the spleen (22, 49). Within a few hours, i.p. injected liposomes also reach the blood circulation via the abdominal lymphatics and are subsequently cleared by the same tissues as i.v. administered vesicles (14, 22, 49). Thus, liposomal LPS is probably also detoxified by cells of the reticuloendothelial system (7).

It is possible that factors such as the rate of clearance of free and liposomal LPS or the stability of phospholipid vesicles in whole blood and lymphatic fluids also influence the relative potency of these LPS forms in vivo. Enhanced clearance and processing may decrease the activity of liposomal LPS, whereas destabilization in body fluids may increase its activity. Mononuclear phagocytes appear to be the main effector cells for LPS-induced responses and, in addition, play an important role in the clearance and processing of the LPS molecule. Thus, it is probable that the reduced in vivo activities of liposomal LPS observed in this study are the result of ineffective interaction of the hydrophobic portion of the lipid A moiety with critical receptors on these cells.

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