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Immunization Against Experimental Murine Salmonellosis with Liposome-Associated O-Antigen

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Partially delipidated *Salmonella typhimurium* (O-1,4,5,12) lipopolysaccharide was incorporated into small multilamellar liposomes composed of either naturally occurring or synthetic phospholipids. Vaccination of mice with the liposome-lipopolysaccharide complexes induced a cellular response specific for O-1,4,5,12 determinants, as determined by the development of a delayed-type hypersensitivity reaction. The liposome-lipopolysaccharide vaccines were significantly more effective, compared with other nonviable vaccines tested, in protecting mice against a lethal intravenous challenge infection with virulent *S. typhimurium*. Protection afforded by the liposome-lipopolysaccharide vaccines was comparable to that conferred by a live *S. typhimurium* vaccine. Results suggest that liposome-induced modulation of the host immune response in favor of cell-mediated immunity may be more efficacious in preventing diseases in which cell-mediated immunity is of prime importance.

The prevention of salmonellosis by vaccination has been the subject of many investigations (for a recent review, see reference 14). Despite this, the mechanism of protective immunity against *Salmonella* infections remains a controversial subject.

Salmonella typhimurium is classified as a facultative, intracellular bacterium by virtue of its ability to survive and multiply within the specialized phagocytic cells of the host reticuloendothelial system. In their sheltered intracellular environment, the salmonellae are provided with a considerable amount of protection from several host defense mechanisms (e.g., specific antibody and complement). Although both cell-mediated and humoral immune responses are evoked in naturally occurring and in experimentally induced salmonellosis (4), the relative degree of protection afforded by each of the two arms of the specific immune response is still unclear. Thus, it has been observed that mice vaccinated with killed *Salmonella* bacterins or with soluble *Salmonella* antigens were not sufficiently protected from a lethal challenge inoculum despite the presence of a substantial amount of specific antibody (5, 9, 26). Others have observed that a significant degree of protection was indeed provided by humoral mechanisms (3, 30).

Undoubtedly, humoral immunity plays a substantial role in preventing bacteremia and in negating some of the noxious effects of endotoxin (17). However, a large body of evidence suggests that intracellular bacterial multiplication is controlled only by the presence of activated macrophages generated as a result of a T-lymphocyte-mediated response (10, 15, 20). This cell-mediated response, induced as a result of natural infection or by the use of live vaccines, is capable of protecting animals against a challenge infection (6, 25, 29). These observations, along with data from adoptive transfer experiments (26), suggest that cellular immunity is the overriding protective factor against *Salmonella* infections.

Several studies have demonstrated that the O-antigenic polysaccharide (O-PS) of the *S. typhimurium* lipopolysaccharide (LPS) molecule is a key component associated with bacterial virulence (reviewed in reference 35). Unfortunately, in terms of vaccine potential, the O-polysaccharide chain itself is not very immunogenic, and there are substantial drawbacks associated with the use of the native LPS molecule as an immunogen. As a result, attempts have been made at synthesizing a vaccine which incorporates *Salmonella* O-antigenic determinants but is devoid of the toxic properties inherent in the lipid A moiety of the LPS. Through the covalent attachment of *Salmonella* O-polysaccharide subunits to carrier molecules, Svenson and co-workers were able to induce a good antibody response to the haptenic oligosaccharide (41, 42). However, the ability of these preparations to induce a state of specific cellular reactivity and macrophage activation was not reported. Although live *Salmonella* vaccines can induce a cellular immunity that is protective in experimental models, the widespread use of such vaccines in humans and animals could pose potential problems. In view of this, the construction of a nonviable vaccine composed of O-antigenic determinants and capable of inducing a specific cell-mediated immune (CMI) response would be highly desirable. This has in the past proved to be relatively difficult (7).

Phospholipid bilayered vesicles (liposomes) have received a considerable amount of attention as carriers for the delivery of a wide variety of biologically active substances to cells and tissues in vitro and in vivo (for reviews, see references 28 and 37). More recently, liposomes have been employed as immunological adjuvants for the enhancement or modulation of immune responses to various antigens. The adjuvant effect of liposomes has been demonstrated with diphtheria toxin (1), albumin (46), adenovirus capsid protein (19), influenza subunit proteins (2), *Plasmodium* antigens (40), hepatitis B surface antigen (27), tumor antigens (21, 39), and various haptens (18). Further, evidence suggests that liposomes can influence the resulting immune response to an associated antigen in favor of the generation of CMI (43).

We report here that vaccination of mice with liposomes containing *S. typhimurium* LPS elicits a specific cell-medi-

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ated response directed toward O-PS determinants. In addition, the liposome-LPS complex is more efficacious in comparison with other vaccines examined in protecting mice against a lethal challenge dose of virulent *S. typhimurium*.

MATERIALS AND METHODS

Reagents. Egg phosphatidylcholine (PC) and cholesterol were obtained from Sigma Chemical Co., St. Louis, Mo. Dipolmitoylphosphatidylcholine (DPPC) was obtained from Calbiochem, LaJolla, Calif. All other reagents were of analytical grade.

Bacteria storage and culture. *S. choleraesuis* serovar *typhimurium* (*S. typhimurium*) used throughout this study was isolated from a calf which had died of acute enteric salmonellosis. This strain was strongly agglutinated by O:1,4,5,12 and H:i,1,2 antisera. Maximum virulence of the bacteria was attained by sequential passage in ICR mice. The intravenous 50% lethal dose in ICR mice was determined to be 5.0×10^3 by the method of Reed and Muench (33). The passaged bacteria were inoculated into brain heart infusion broth, harvested during the early-logarithmic-growth phase, and stored at −80°C in small samples of 10^9 bacteria per ml.

Preparation of vaccines. (i) Killed bacterins. Samples of the frozen stock culture of *S. typhimurium* were used for the preparation of bacterins. The culture was thawed, inoculated into brain heart infusion broth, and harvested during the early-logarithmic-growth phase. For the preparation of Formalin-killed bacterins, 1 volume of bacterial suspension was mixed with 1 volume of 0.6% Formalin in saline and held at 4°C for 24 h. A second bacterial suspension was heated at 70°C for 1 h for the production of the heat-killed bacterin. Both preparations were washed extensively in phosphate-buffered saline (PBS) before their use.

(ii) LPS. *S. typhimurium* LPS was extracted and purified from a Formalin-killed preparation of bacteria by the Westphal hot phenol-water procedure as described by Lindberg and Holme (22). The phenol-water extract was exhaustively dialyzed against distilled water and then lyophilized. A 3% solution (wt/vol) of the crude LPS in distilled water was centrifuged at 100,000 × *g* for 4 h. The LPS pellet was suspended in distilled water and lyophilized for storage. LPS was partially delipidated by subjecting it to alkaline hydrolysis in 0.2 N NaOH for 4 h at 56°C. The preparation was then adjusted to a pH of 3.5 by the addition of 1 N HCl and extracted with chloroform to remove free fatty acids. The aqueous phase was removed and readjusted to a pH of 7.0 and exhaustively dialyzed against distilled water. The delipidated LPS was further purified by gel filtration on a Sephacryl S-1000 column. Free O-PS was prepared from the purified LPS by acid hydrolysis in 1% acetic acid at 100°C for 4 h. The O-PS was separated from the precipitated lipid A by centrifugation and then dialyzed against distilled water. The total hexose content of the LPS and O-PS was assayed by the phenol-sulfuric acid method (13). The final LPS and O-PS preparations contained no detectable protein as measured by the Lowry assay (23).

(iii) Liposome-associated antigens. *S. typhimurium* LPS was incorporated into small, multilamellar liposomes composed of either DPPC-cholesterol (7:3 molar ratio) or PC-cholesterol (7:3) as follows. The dried lipids (50 μmol total) were hydrated for 30 min in 3.0 ml of an aqueous solution of purified *S. typhimurium* LPS at a concentration of 2.5 mg/ml. The dispersions were agitated at high speed with the aid of a mechanical vortex mixer for 3 min (37°C for PC liposomes; 50°C for DPPC liposomes) and then filtered through 1.0- and 0.4-μm polycarbonate membranes. Liposome-associated LPS was separated from nonassociated LPS by gel filtration on a Sephacryl S-1000 column. All liposome preparations were assayed for associated *Salmonella* LPS by the phenol-sulfuric acid procedure and corrected for the appropriate blanks. The sterility of all vaccine preparations was checked before use.

Immunization of mice. Groups of mice were vaccinated with one of the following test vaccines: (i) heat-killed *S. typhimurium* (5×10^8 cells in 0.1 ml), (ii) Formalin-killed *S. typhimurium* (5×10^8 cells in 0.1 ml), (iii) PC-cholesterol liposomes containing *S. typhimurium* (O-1,4,5,12) LPS (10 μg of LPS in 0.1 ml), (iv) DPPC-cholesterol liposomes containing *S. typhimurium* LPS (10 μg of LPS in 0.1 ml), (v) DPPC-cholesterol liposomes containing PBS, (vi) *S. typhimurium* LPS in complete Freund adjuvant (10 μg of LPS in 0.1 ml), or (vii) live *S. typhimurium* (5×10^2 cells in 0.1 ml). All vaccines were administered intravenously with the exception of LPS in Freund adjuvant, which was given intraperitoneally. Each group received two vaccine doses (1 week apart) with the exception of the group that received a single inoculation of live *S. typhimurium*.

Challenge of vaccinated mice. At 2 weeks after the second vaccination, mice were challenged intravenously with 5×10^6 (1,000 50% lethal doses) viable *S. typhimurium* in a 0.1-ml saline suspension. The number of organisms was determined before infection by direct enumeration with a Petrof-Hauser counting chamber and confirmed by triplicate plate counts. The relative degree of protection afforded by each vaccine was assessed by the number of mice surviving 30 days after infection.

Measurement of DTH. In a separate set of experiments, similarly vaccinated mice were examined for delayed-type hypersensitivity (DTH) response to *S. typhimurium* O-antigenic determinants with liposome-encapsulated O-1,4,5,12 LPS as the test inoculum. At 2 weeks after the second vaccination, each mouse received 0.025 ml of the liposome-LPS complex intradermally in the left hind footpad and the same volume of PBS in the right hind footpad. Footpad thickness was measured 48 h later with dial calipers accurate to 0.01 mm. Representative mice from each group were killed, and the distal segments of their hind limbs were submitted to the Department of Pathology, New York State College of Veterinary Medicine, for histological evaluation of the footpads.

Antibody response. At 2 weeks after the second vaccination, the specific antibody titer to *S. typhimurium* LPS was measured by indirect hemagglutination of LPS-coated sheep erythrocytes.

RESULTS

Association of bacterial LPS with liposomes. DPPC-cholesterol liposomes prepared in the presence of *S. typhimurium* LPS (2.5 mg/ml) entrap ca. 6.0 to 10.0 μg of LPS per μmol of liposomal lipid, corresponding to a capture efficiency of 1.5 to 2.8%. The amount of LPS which associates with PC-cholesterol liposomes was slightly less. The volume of liposome vaccines was adjusted to correspond to 10 μg of liposome-associated LPS in a volume of 0.1 ml. Although all liposome vaccines were used within several days of their production, we have found that these preparations are stable at 4°C for several months.

DTH to various test preparations. To accurately identify cellular reactivity in vaccinated mice, we examined the

TABLE 1. DTH reaction to various test preparations injected intradermally in mice immunized with live *S. typhimurium* and in nonimmunized mice[a]

Group no. (n = 16)	Footpad inoculum	Mean footpad thickness (mm) ± SE		Significance
		Preinoculation	48 h	
1	Heat-killed *S. typhimurium*	1.716 ± 0.197	2.287 ± 0.443	$P < 0.0001$
2	O-1,4,5,12 polysaccharide	1.797 ± 0.171	1.799 ± 0.126	NS
3	O-1,4,5,12 LPS	1.753 ± 0.172	2.069 ± 0.219	$P < 0.0001$
4	DPPC-C liposomes–O-1,4,5,25 LPS	1.756 ± 0.148	2.222 ± 0.229	$P < 0.0001$
5	DPPC-C liposomes–PBS	1.781 ± 0.149	1.763 ± 0.119	NS
6	PBS	1.709 ± 0.143	1.772 ± 0.117	NS
7	Heat-killed *S. typhimurium*	1.815 ± 0.156	1.968 ± 0.155	$P < 0.05$
8	O-1,4,5,12 LPS	1.712 ± 0.150	1.950 ± 0.148	$P < 0.001$
9	DPPC-C liposomes–O-1,4,5,12 LPS	1.737 ± 0.158	1.784 ± 0.170	NS
10	DPPC-C liposomes–PBS	1.772 ± 0.089	1.809 ± 0.139	NS

[a]Abbreviations: C, cholesterol; NS, not significant. Mice in groups 1 to 6 were immunized with 0.1 50% lethal dose of live *S. typhimurium* 2 weeks before testing. Mice in groups 7 to 10 were not immunized.

ability of various test preparations to elicit a DTH reaction in mice immunized with live *S. typhimurium*. Live vaccines have previously been shown to induce cellular immunity which correlates well with the development of a DTH response (24). Results (Table 1) show that mice so immunized develop a significant increase in footpad thickness at 48 h after intradermal inoculation with heat-killed cells, *S. typhimurium* LPS, and liposome-associated LPS (groups 1, 3, and 4). However, significant footpad swelling was also observed when heat-killed *Salmonella* organisms and free LPS were inoculated into the footpads of nonimmunized mice (groups 7 and 8). Upon histological examination, it was determined that the inflammation induced in nonimmunized mice was due to an acute reaction characterized by the infiltration of neutrophils and by local edema. This reaction peaked early but was still detectable at 48 h. On the contrary, the local response to liposome-associated LPS in immunized mice was predominantly the result of a mononuclear infiltrate. There was no significant inflammation associated with DPPC-cholesterol liposomes containing either PBS or LPS in nonimmunized mice. No reaction was observed when free O-1,4,5,12 polysaccharide was injected into the footpads of immunized mice. This is presumably due to the inability of the free polysaccharide to remain at the site of injection for a sufficient period of time (36). The specificity of the DTH response to O-1,4,5,12 antigenic determinants is exemplified by the data in Table 2. A positive DTH to *S. typhimurium* can be detected with liposome-associated O-1,4,5,12 LPS but not with liposome-associated O-3,10 LPS derived from *S. anatum*. Although these two salmonellae possess similar determinants in the core oligosaccharides and in lipid A, the absence of cellular cross-reactivity may be due to inaccessibility to shared determinants which are in close association with or intercalated into the liposome bilayer. Alternately, this may be due to the relatively low epitope density of the core and lipid A region in comparison with the numerous repeating units of the O-polysaccharide chains. Insertion of the LPS molecule into liposomes minimizes nonspecific inflammation evoked by the lipid A moiety of the molecule (34). These results suggest that, through the association of *S. typhimurium* LPS with DPPC liposomes, a specific DTH response can be elicited to O-antigenic determinants, and nonspecific inflammation associated with free LPS can be minimized. As a result, DPPC liposomes containing *S. typhimurium* O-1,4,5,12 were used for all subsequent determinations of DTH reactivity.

Development of immunity to *S. typhimurium* in vaccinated mice. Table 3 compares the various vaccine groups with respect to the development of a DTH and formation of specific antibody to *S. typhimurium*. The highest antibody response was elicited by *S. typhimurium* LPS in complete Freund adjuvant and by live *S. typhimurium*. Comparable titers were achieved by killed cells and liposome-LPS vaccines. Only liposome-LPS vaccines and live *S. typhimurium* were capable of inducing DTH responses to the test preparation.

Survival of challenged mice. Figure 1 shows the percent survival of the various vaccine groups over a period of 30 days after an intravenous challenge of 1,000 50% lethal doses of virulent *S. typhimurium*. At this challenge level, the degree of protection conferred by the liposome-LPS vaccines was comparable to that provided by a sublethal dose of live *S. typhimurium*. Vaccination with DPPC-LPS liposomes protected more than 40% of the challenged mice. A 30% survival rate was observed for mice receiving the PC liposomes-LPS vaccine. All mice vaccinated with heat-killed cells or with LPS in complete Freund adjuvant died within 2 weeks after challenge. Similarly, there was no protection afforded by liposomes containing PBS.

TABLE 2. Comparison of DTH reactions to liposome-associated and free LPS derived from *S. typhimurium* (O-1,4,5,12) and *S. anatum* (O-3,10) in mice immunized with live *S. typhimurium*[a]

Group no. (n = 16)	Footpad inoculum	Mean footpad thickness (mm) ± SE		Significance
		Preinoculation	48 h	
1	O-1,4,5,12 LPS	1.805 ± 0.163	2.010 ± 0.177	$P < 0.001$
2	DPPC-C liposomes–O-1,4,5,12 LPS	1.793 ± 0.151	2.235 ± 0.218	$P < 0.0001$
3	O-3,10 LPS	1.782 ± 0.109	2.205 ± 0.162	$P < 0.001$
4	DPPC-C liposomes–O-3,10 LPS	1.741 ± 0.125	1.800 ± 0.140	NS
5	DPPC-C liposomes–PBS	1.766 ± 0.136	1.783 ± 0.132	NS

[a] See Table 1, footnote *a* for an explanation of abbreviations.

TABLE 3. Development of immune response to *S. typhimurium* (O-1,4,5,12) LPS in mice vaccinated with various preparations[a]

Group no. (n = 16)	Vaccine	Route	Mean anti-LPS titer (log_2)[b]	Mean footpad thickness (mm) ± SE		Significance
				Preinoculation	48 h	
1	Heat-killed *S. typhimurium*	i.v.	5.7	1.588 ± 0.103	1.655 ± 0.116	NS
2	Formalin-killed *S. typhimurium*	i.v.	5.2	1.681 ± 0.075	1.760 ± 0.107	NS
3	DPPC-C liposomes–O-1,4,5,12 LPS	i.v.	5.4	1.750 ± 0.096	1.981 ± 0.153	$P < 0.005$
4	PC-C liposomes–O-1,4,5,12 LPS	i.v.	5.7	1.808 ± 0.144	1.921 ± 0.186	$P < 0.05$
5	O-1,4,5,12 LPS in CFA	i.p.	7.2	1.700 ± 0.071	1.817 ± 0.082	NS
6	DPPC-C liposomes–PBS	i.v.	<1.0	1.681 ± 0.075	1.715 ± 0.084	NS
7	Live *S. typhimurium*	i.v.	7.0	1.716 ± 0.192	2.287 ± 0.443	$P < 0.0001$

[a] Abbreviations: i.v., intravenous; i.p., intraperitoneal; CFA, complete Freund adjuvant. See footnote *a* of Table 1 for additional explanations.
[b] By indirect hemagglutination.

DISCUSSION

The importance of cellular immunity in host defense against facultative intracellular bacteria is well documented (8, 20). The macrophage, as a key antigen-presenting cell, plays a central role in the induction of CMI through processing and presentation of antigens, in an immunogenic form, to antigen-specific T cell subpopulations (44). Further, the nature of the interaction between antigens and macrophages is largely a function of the physical characteristics of the antigen. In turn, the outcome of the interaction between antigens, macrophages, and lymphocytes may have a profound effect on the resulting immune response (31). Thus, it has been observed that lipid-modified antigens preferentially induce cell-mediated responses as opposed to humoral responses (12, 16, 32). The ability of chemically modified antigens to induce CMI was correlated with their hydrophobicity and to the affinity of the modified molecules for macrophages and immunocompetent cells. Additionally, lipid-modified antigens displayed a longer retention time in lymphatic tissue than did the unaltered antigen (11). Others have shown that antigens bound to macrophages are strong

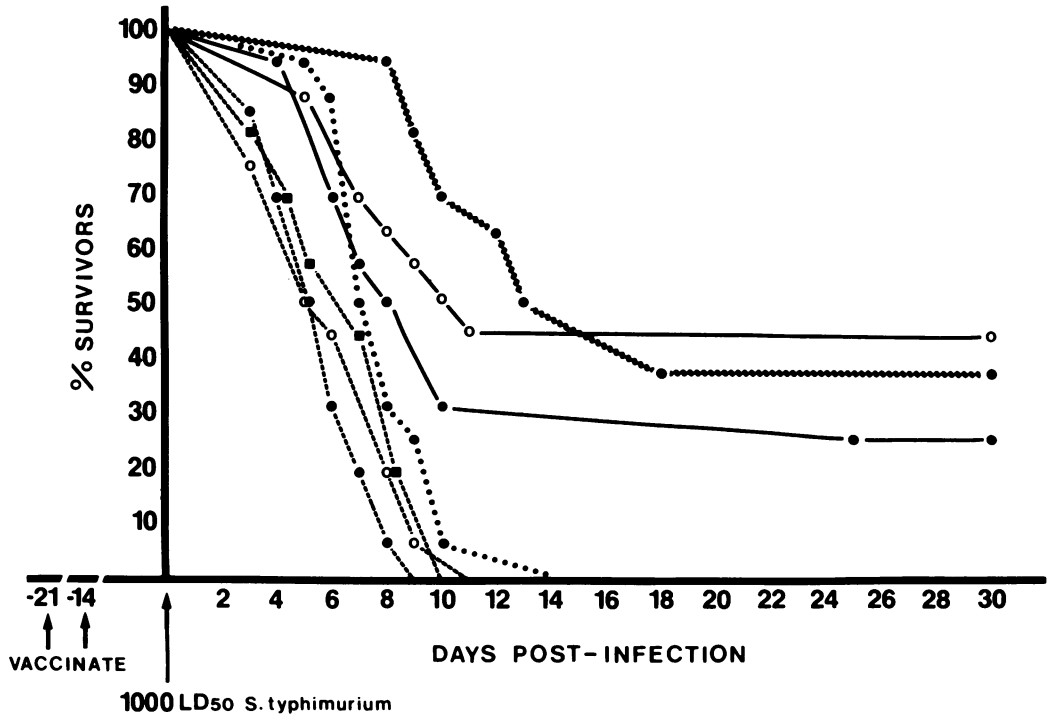

FIG. 1. Comparison of various vaccine preparations in protecting mice against an intravenous challenge of 1,000 50% lethal doses (LD_{50}) of *S. typhimurium*. Symbols: (●~~●) live vaccine, (● . . . ●) LPS-complete Freund adjuvant, (●——●) PC-cholesterol liposomes–LPS, (○——○) DPPC-cholesterol liposomes–LPS, (■- - -■) heat-killed vaccines, (○- - -○) DPPC-cholesterol liposomes–PBS, and (●- - -●) not vaccinated.

inducers of cell-mediated responses (45). In general, poorly soluble antigens which persist in the host for an extended period of time, especially in association with cell membranes, are good inducers of cellular immunity (47).

In view of the facts that immunity to *Salmonella* infections is, for the most part, provided by cellular mechanisms and immunological responses are largely directed toward O-antigenic determinants, it follows that a *Salmonella* vaccine composed of O-antigenic determinants and capable of inducing a CMI response would be protective.

Indeed, results demonstrate that partially delipidated *S. typhimurium* LPS administered in association with either PC liposomes or DPPC liposomes protected a significant number of mice from a high challenge dose of virulent *S. typhimurium*. A comparison of survival experiments with the ability of the vaccines examined to induce a DTH response suggests that protection is correlated with the presence of a DTH and not with specific antibody titer. *S. typhimurium* LPS administered in complete Freund adjuvant induced a relatively high antibody titer and within the first week after challenge provided substantial protection (Fig. 1). However, this was shortly thereafter followed by a sharp decline in survival and resulted in 100% mortality by 2 weeks after infection.

The adjuvant action of liposomes in the stimulation of cellular immune responses has not yet been conclusively established. However, they possess many of the characteristics associated with inducers of cell-mediated responses. It is well established that the majority of liposomes, especially when administered intravenously, are taken up by phagocytic cells of the lymphoreticular system (38). Thus, the association of antigens with liposomes provides a means of targeted delivery of the associated antigens directly to antigen-processing cells of the reticuloendothelial system. Further, the incorporation of LPS into liposomes renders the soluble LPS molecule particulate and much more hydrophobic. Although there have been no studies which compare the relative in vivo toxicity of free and liposome-associated LPS, others have shown that incorporation of LPS into phospholipid bilayers markedly reduced the adverse biological effects of lipid A (34). Additionally, since liposomes are rapidly removed from the circulation by the reticuloendothelial system, the half-life in the blood of liposome-associated LPS is very short. Therefore, it is likely that the liposome-LPS complex is considerably less toxic than the equivalent amount of free LPS, although this remains to be proven.

After endocytosis of the liposome-LPS complex, it is assumed that disruption of the liposome bilayer must occur within the phagolysosomes to expose free LPS molecules for "processing." This, along with the fact that nearly the entire antigenic mass is concentrated in the macrophage population of the reticuloendothelial system, may result in a longer retention time of the LPS and therefore prolonged antigenic stimulation.

The observation that DPPC liposomes provided better protection than did PC liposomes is an interesting one. Liposomes composed of the synthetic DPPC have a critical-phase transition temperature of 41°C. This means that at a normal body temperature of 37°C, DPPC liposomes are "solid" whereas PC liposomes are "fluid" vesicles. It is possible that DPPC liposomes are more slowly degraded than PC liposomes. This may provide macrophage-associated O-antigenic determinants over a longer period of time and thus further favor the induction of cellular responses.

It is difficult to rigorously compare the results presented here with those from other laboratories. Differences in the strains of mice, the species and strains of *Salmonella*, the vaccination regimen, and the mode of challenge may all contribute to dissimilar results. However, under the controlled condition of the experiments described here, vaccination of mice with liposome-associated *S. typhimurium* LPS was comparable to live cells and provided a greater degree of protection against a lethal challenge.

Studies are currently under way to determine the length of immunity afforded by liposome vaccines, the optimum vaccination schedule, the challenge dose against which 100% protection can be achieved, and vaccine efficacy in susceptible mouse strains.

In addition, liposome manipulation of the immune response may prove to be advantageous in the immunization against other diseases in which CMI is of prime importance (e.g., brucellosis, listeriosis, mycobacterial diseases, and many viral infections).

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