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Physicochemical and immunological properties of albumin-associated dialkyl-ether phosphatidylcholine liposomes

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Multilamellar and unilamellar liposomes were prepared from *sn*-3-dihexadecylphosphatidylcholine/cholesterol/dicetylphosphate (7:2:1) in the presence of bovine serum albumin. Liposome-associated bovine serum albumin was separated from free bovine serum albumin by Blue Sepharose CL-6B affinity column chromatography. The chromatographed fractions were analyzed for their protein and liposomal phosphorus contents. The recovered albumin-containing liposomes were characterized morphologically by electron microscopy on negatively stained preparations. These preparations showed vesicular organizations of multilamellar or unilamellar phospholipid bilayers depending on the method of preparation used in each case. An analysis of the particle size distribution indicated that the mean radius was 280 ± 50 nm for the multilamellar bovine serum albumin-liposomes and 150 ± 50 nm for the unilamellar preparations. The efficacy of unilamellar and multilamellar dialkyl-ether phosphatidylcholine liposomes in eliciting antibody formation was examined. Mice were injected with liposome-entrapped bovine serum albumin and the albumin-specific plaque-forming cell response was evaluated. The unilamellar vesicles were found to be more effective than their multilamellar counterparts in promoting the elicitation of the anti-bovine serum albumin plaque-forming cell response. Within each category of lamellar structure, i.e., unilamellar or multilamellar bilayers, liposomes composed of dialkyl-ether phosphatidylcholines are less efficient than those of diacyl-ester phosphatidylcholines in potentiating the humoral immune response. These results demonstrate that liposome-mediated enhancement of the antibody response is determined, at least in part, by the lamellar arrangement of the vesicles and by the characteristic chemical structures of the phospholipids used.

Introduction

Phospholipid vesicles (liposomes) have been shown to be useful for the delivery of drugs [1,2], enzymes [3] and antigens [4]. In the last decade, numerous studies with liposomes were conducted to elucidate the lipid-protein interactions in different membrane structures; liposomes were also used in the reconstitution of enzymatic activities, and in the potentiation of the immune response [5–8]. In most of the immunological studies, multilamellar phospholipid vesicles were used. More recently, we demonstrated that large unilamellar liposomes can also serve as an effective immunopotentiating carrier for weak protein antigens [9]. The phospholi-

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pids used in all previous studies were invariably diacyl-ester phosphatidylcholines. In addition to the naturally occurring ester-bond-containing lipids widely spread among many organisms, the ether-bond-containing lipids are now recognized to be present also in nature [10,11]. Kates and his co-workers, for example, reported that lipids of the cell walls of extremely halophilic bacteria are predominantly ether-bond lipids [12,13]. Ether phosphatidylcholines, like dioleyl-ether phosphatidylcholine, are more resistant to hydrolysis and less susceptible to enzymatic digestions [14]. In view of the apparently more stable nature of ether-phosphatidylcholine liposomes, the possible application of this class of phospholipids for the delivery of antigens and other biologicals seems attractive.

In this study, we examined the immunogenicity of albumin entrapped in dialkyl-ether-phosphatidylcholine liposomes. The procedures for the preparation of unilamellar and multilamellar ether-phosphatidylcholine liposomes were described. The liposome preparations were also characterized for lamellar structure and size distribution by electron microscopy.

Materials and Methods

Animals. Female A/J mice, 6 weeks old, were purchased from Jackson Laboratories, Bar Harbor, ME. They were kept on standard mouse diet and water ad libitum.

Chemicals. Bovine serum albumin (essentially free of fatty acids) and dicetyl phosphate were purchased from Sigma, St. Louis, MO; cholesterol was from P-L Biochemicals, Milwaukee, WI; *sn*-3-dihexadecylglycerophosphorylcholine was from Serdary Research Labs., London, Ont.; and *sn*-3-dimyristoylphosphorylcholine was from Fluka, Buchs, Switzerland. Octyl β-D-glucopyranoside was purchased from Calbiochem, La Jolla, CA, and Blue Sepharose CL-6B was from Pharmacia, Uppsala, Sweden. (*Methyl*-^{14}C)-labeled bovine serum albumin, [4-^{14}C]cholesterol and octyl β-D-[U-^{14}C]glucopyranoside were purchased from New England Nuclear, Boston, MA. All solvents were of the highest purity commercially available from Fisher Scientific Co., Toronto, Ont.

Preparation of multilamellar liposomes. The method described by Bangham et al. [15] was adapted as follows: phosphatidylcholine (42.7 μmol), cholesterol (12.2 μmol) and dicetyl phosphate (6.1 μmol) giving a molar ratio of 7 : 2 : 1 were dissolved in 25 ml of chloroform. The solution was evaporated to dryness at 37°C under reduced pressure by rotary evaporation to form a thin film around the wall of a round-bottom flask. To this film, 1 μmol bovine serum albumin (previously purified by Blue Sepharose CL-6B column chromatography) in 5 ml of 0.075 M sodium phosphate buffer (pH 7.4) was added and the phosphatidylcholine was hydrated at a temperature above the transition temperature (55°C for dihexadecylphosphatidylcholine and 35°C for dimyristoylphosphatidylcholine). The lipid film was dispersed by vortexing. The suspension was kept at the hydration temperature for 12 h, filtered through a 0.65 μm membrane (Millipore), and applied to a Blue Sepharose CL-6B column for chromatography as described below.

Preparation of unilamellar liposomes. The procedure used for the preparation of unilamellar liposomes was essentially the same as that described by Mimms et al. [16]. A lipid film having the same composition as described above for multilamellar vesicles was obtained and hydrated, at a temperature above the transition temperature of the phosphatidylcholine, with 5 ml of purified bovine serum albumin (1 μmol) in 0.075 M sodium phosphate buffer (pH 7.4) containing 0.13 M octyl β-D-glucoside. The molar ratio of glucoside to phosphatidylcholine was 15 : 1. The solution was kept at the hydration temperature for 2 h before dialyzed at the same temperature against 0.15 M phosphate-buffered saline (pH 7.4) for 36 h. In separate experiments using octyl β-D-[^{14}C]glucoside, only 0.04% of the glucoside was detectable after such a dialysis. A turbid liposome suspension was formed after dialysis and was filtered through a 0.65 μm membrane (Millipore) before its application to a Blue Sepharose CL-6B column for chromatography.

For the preparation of liposomes not containing albumin, the lipid film was hydrated using buffer solution only. In order to facilitate the identification of liposomal peaks in column chromatography and the estimation of protein association with liposomes, 2–4 μg [^{14}C]cholesterol

(specific activity; 0.14 μCi/μg) or 5 μg ^{14}C-labeled albumin (specific activity: 0.02 μCi/μg) were used, respectively.

Column chromatography of liposomes and bovine serum albumin. In order to separate liposome-associated albumin from free albumin, multilamellar and unilamellar liposomes containing this antigen were prepared as described in previous sections and subjected to column chromatography as follows. In control experiments, free albumin, multilamellar and unilamellar liposomes (both without albumin), were subjected also to the same chromatographic procedure.

A Blue Sepharose CL-6B column (2.6 × 18.5 cm) was preequilibrated with either 0.01 M Tris-HCl buffer (pH 7.4) containing 0.17 M NaCl or 0.15 M phosphate-buffered saline (pH 7.4). The binding capacity of the column for bovine serum albumin was 150 mg at 4°C. Liposome-associated albumin was separated from free albumin by passing the mixture through the column at a flow-rate of approx. 45 ml/h. Fractions of 4 ml each were collected. Albumin retained by the column was eluted with 1.5 M NaCl in Tris-HCl buffer (0.01 M, pH 7.4). The elution was monitored at 280 nm and aliquots were taken from each fraction for the determination of phosphorus, protein and radioactivity, if applicable. All operations were performed at room temperature (20–23°C) when liposomes were chromatographed. Chromatography of albumin was done at 4°C.

Electron microscopy. Liposomes were negatively stained with a solution of 2% sodium phosphotungstate (pH 7.4) on carbon-coated copper grids (400 mesh) and examined in a Philips EM-300 electron microscope operated at 60 kV. The size distribution of liposomal particles was determined at a magnification of 20000–30000 ×, and the magnifications used to examine the lamellar structures are specified in the legend of Fig. 3.

Radioactive and other measurements. Radioactivity was measured with a Packard Tri-Carb Model 3003 scintillation spectrometer. Buhler's scintillation fluid [17] was used and the efficiency for ^{14}C counting was about 45%.

Phosphorus was measured by the method of Bartlett [18] and protein determination was performed in the presence of 1% sodium dodecyl sulfate as described by Markwell et al. [19].

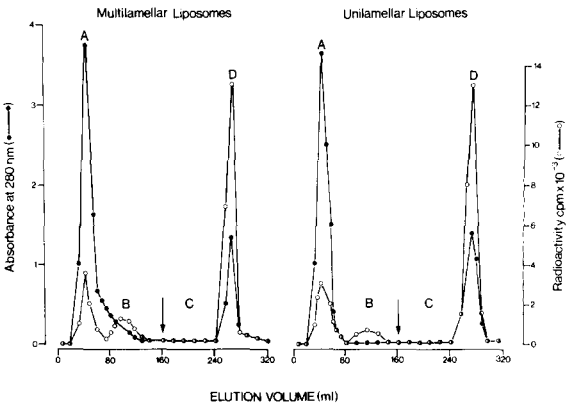

Fig. 1. Blue Sepharose CL-6B chromatography of liposome preparations. Dialkyl-ether-phosphatidylcholine liposomes, in multilamellar or unilamellar form, were prepared in the presence of bovine serum albumin and 0.1 μCi of ^{14}C-methylated bovine serum albumin was added as a tracer. Each liposomal mixture was subjected to affinity chromatography as described in detail under Materials and Methods. The inverted arrow indicates the initiation of high-salt elution using 1.5 M NaCl.

All spectrophotometric measurements were obtained using a C. Zeiss PMQ-II spectrophotometer equipped with a digital photometer indicator PI-2.

Hemolytic plaque assay. Procedures used for the

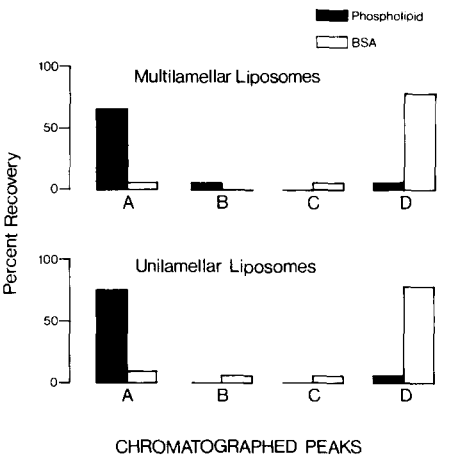

Fig. 2. Recovery of lipids and albumin after Blue Sepharose CL-6B chromatographic fractionation. Aliquots obtained for different fractions of the chromatographed peaks of Fig. 1 were analyzed for phosphatidylcholine contents using phosphorus determination and albumin contents using radioactivity (^{14}C) measurements and protein determination, all described under Materials and Methods. Each bar represents the recovery of albumin or lipid, expressed as a percentage of the original input to the column, from all the fractions of each peak. BSA, bovine serum albumin.

preparation of spleen cells and for the determination of bovine serum albumin-specific plague-forming cell response were performed as previously described in detail by Shek and Sabiston [20].

Results

Chromatographic separation of liposome-entrapped and untrapped bovine serum albumin

The application of Blue Sepharose CL-6B col-

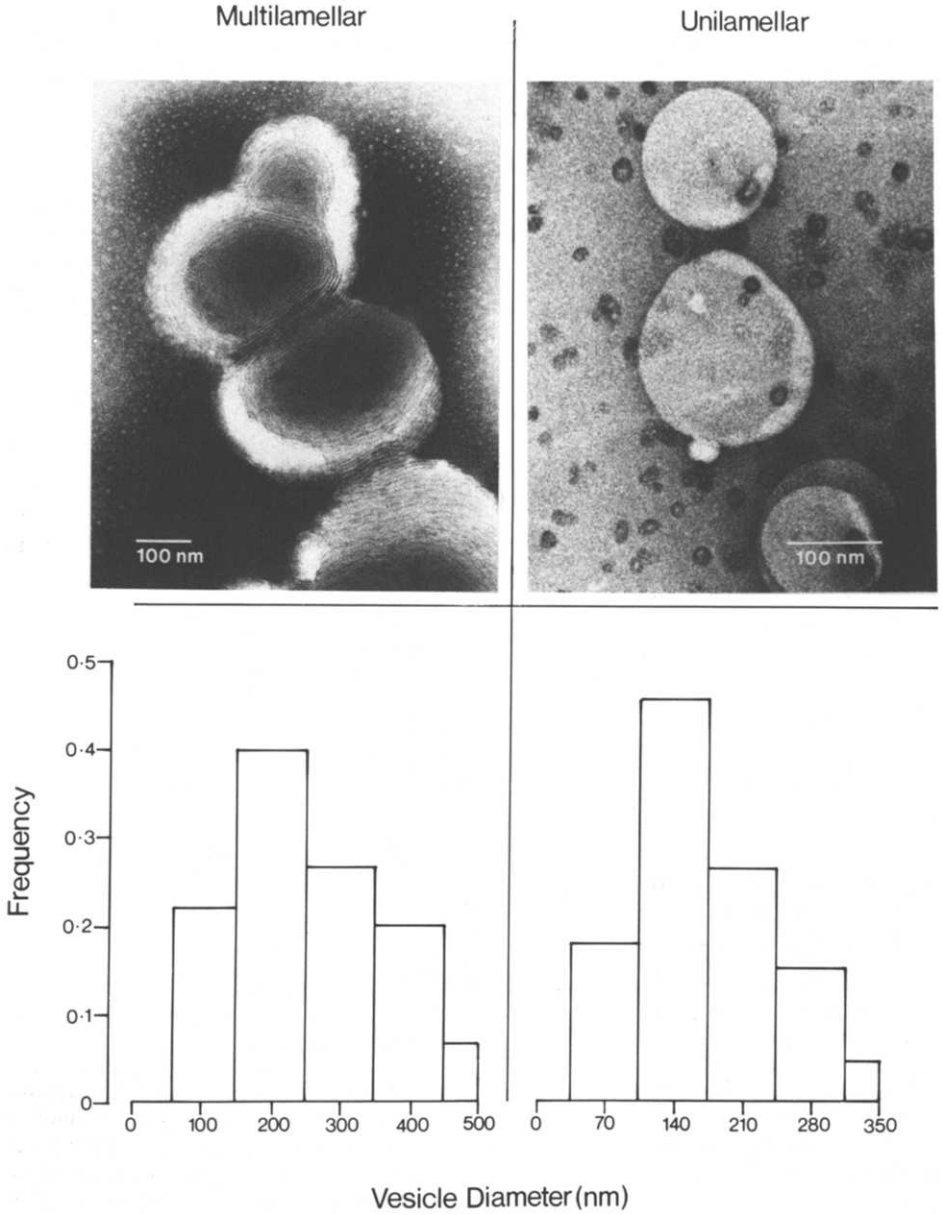

Fig. 3. Electron micrographs and size distribution histograms of albumin-associated multilamellar and unilamellar dialkyl-ether-phosphatidylcholine liposomes. The liposomal preparations were negatively stained with sodium phosphotungstate and the vesicles were examined for lamellar structure at a magnification of ×130000 and ×200000 for multilamellar and unilamellar liposomes, respectively. Diameters of 150 randomly chosen vesicles were measured for each liposome preparation.

umn chromatography was found useful for the separation of untrapped bovine serum albumin from liposome-entrapped protein. It can be seen from Fig. 1 that liposome-associated albumin was eluted in peak A, and free albumin was recovered in peak D. The recovery of phosphatidylcholine applied to the column was about 70–75% in peak A and only 3–4% in peak D (Fig. 2). In terms of protein recovery, most (80–90%) of the protein applied was eluted with peak D, and about 6% of albumin was recovered in peak A (Fig. 2). In addition to peaks A and D, two minor and rather broad peaks (B and C) were also observed (Fig. 1). The amount of phosphatidylcholine and protein in these fractions was low or negligible (Fig. 2).

Electron microscopy

The lamellar appearance and size distribution of bovine serum albumin-containing liposomes were examined by electron microscopy. Vesicular structures were evident from the electron micrographs obtained (Fig. 3); the formation of unilamellar or multilamellar arrangement is related to the presence or absence of octylglucoside in the preparative procedures as described in Materials and Methods. An analysis of the particle size distribution of albumin-associated ether-phosphatidylcholine liposomes was performed and the results are shown in Fig. 3 (lower panel). The mean vesicle size was found to be 280 ± 50 nm for multilamellar liposomes and 150 ± 60 nm for unilamellar liposomes. The range of vesicle diameters for each liposome preparation is indicated by the particle size distribution histograms.

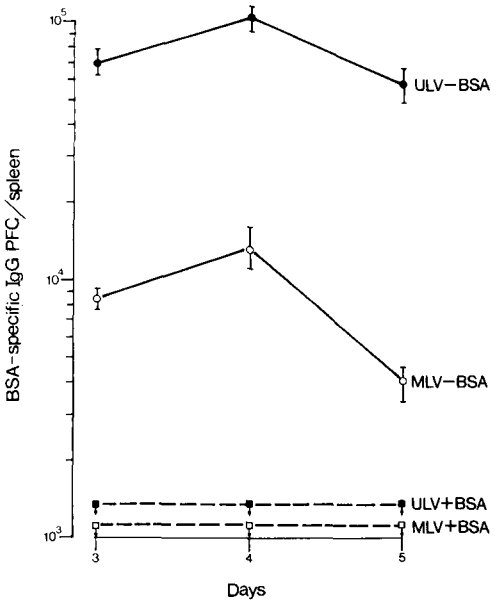

Fig. 4. Kinetics of the bovine serum albumin-specific plaque-forming cell response elicited by albumin-associated ether-phosphatidylcholine liposomes. A/J mice were immunized intraperitoneally twice, 3 weeks apart, with 30 μg of bovine serum albumin entrapped in either multilamellar vesicles (MLV-BSA) or unilamellar vesicles (ULV-BSA). Control animals were given separate but simultaneous injections of the same dose of free albumin and lipid vesicles (MLV + BSA; ULV + BSA). The splenic albumin-specific IgG plaque-forming cell response of all immunized animals was examined 3–5 days after the antigenic challenge. Each point represents the mean response ± S.E. for ten animals.

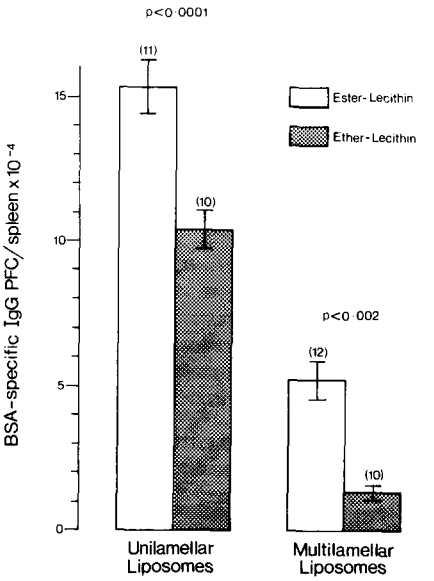

Fig. 5. Comparison of the effectiveness of ester-phosphatidylcholine and ether-phosphatidylcholine liposomes in eliciting the antibody response to entrapped bovine serum albumin (BSA). Different groups of A/J mice were immunized with albumin (30 μg) entrapped in unilamellar or multilamellar liposomes prepared from ester and ether phosphatidylcholines. The peak splenic anti-albumin plaque-forming cell (PFC) response was evaluated 4 days after antigenic challenge. Each vertical bar represents the mean response ± S.E. of the number of animals indicated in each bracket. The *P* value (Student's *t*-test) compares the significance of the difference in the plaque-forming cell response between the two groups of animals immunized with albumin-containing ester-phosphatidylcholine and ether-phosphatidylcholine liposomes.

Liposome-mediated potentiation of the antibody response. Dialkyl-ether phosphatidylcholine liposomes

Mice were immunized with two intraperitoneal injections, 3 weeks apart, of liposome-entrapped bovine serum albumin (30 μg). Animals in the control groups werer similarly immunized with the same dose of free albumin in combination with a simultaneous injection of albumin-free liposomes. The splenic anti-albumin plaque-forming cell response of all immunized animals was assayed 3–5 days after the antigenic challenge and the results are shown in Fig. 4. The entrapment of albumin in unilamellar ether-phosphatidylcholine liposomes was found very useful for engendering a pronounced enhancement of the albumin-specific plaque-forming cell response. The multilamellar vesicles were only marginally effective in potentiating the same antibody response. Control animals immunized with free albumin together with empty liposomes failed to elicit a significant response.

Comparison of ester-phosphatidylcholine and ether-phosphatidylcholine liposomes in immune promotion

In order to examine whether the chemical structure of the phosphatidylcholine, differing by an ether- versus an ester-bond, may alter the extent of liposome-mediated immunopotentiation, the plaque-forming cell response of appropriately immunized animals was compared. It can be seen from Fig. 5 that dialkyl-ether-phosphatidylcholine liposomes are less effective than diacyl-ester-phosphatidylcholine liposomes in promoting the immunogenicity of the entrapped albumin antigen.

Discussion

Dialkyl-ether phosphatidylcholines, which were at one time considered to be phospholipids of unusual biological properties, are now recognized to be rather common phospholipids of many organisms [10,11]. Since we described the chemical synthesis of major representatives of dialkyl-ether phosphatidylcholines some time ago [21], these lipids have been readily available. In this study, the saturated derivatives of phosphatidylcholines were used. Both multilamellar and unilamellar liposomes were studied because of the difference in their structural organization and the possible influence of this difference on their association with albumin. It is relevant to note that in most published studies (for review, see Ref. 5), almost as a rule, multilamellar liposomes prepared from diester phosphatidylcholines were used. Furthermore, as far as we are aware, this is the first study in which the efficacy of multilamellar and unilamellar dialkyl-ether-phosphatidylcholine liposomes in immunopotentiation was examined.

The liposomes used in this study were negatively charged with dicetylphosphate and stabilized with cholesterol, because this liposomal formulation has been shown to be very efficient for immune enhancement [22]. We have not, however, examined whether the molar ratio of phosphatidylcholine/cholesterol/dicetylphosphate (7 : 2 : 1) used in this study and frequently reported in the literature, was indeed, the optimal for promoting antibody formation.

The application of agarose column chromatography, originally described by Huang [23], can be used for the separation of sonicated liposomes on the basis of size differences. We have nevertheless, opted for the affinity chromatography method using Blue Sepharose CL-6B for the separation of liposome-associated albumin from free albumin, because this variety of Sepharose has a very high affinity for albumin and was found useful for the preparation of albumin-free serum [24]. By using this method, free albumin was retained in the column while liposome-associated albumin was eluted in the void volume (Fig. 1). We are not in a position to exclude the presence of, if at all, a small amount of vesicles not associated with albumin, since 'empty' liposomes would be also eluted in the fractions of peak A.

It is conceivable that protein molecules can associate with liposomes by localizing in the lipid bilayers and/or within the entrapped aqueous volume. On the basis of our previous studies with phospholipid vesicles containing cytochrome *c* oxidase [25,26] and apolipoprotein [27,28], one might expect that some albumin molecules are embedded in the lipid bilayers of our liposome preparations. However, since bovine serum albumin is a rather hydrophilic protein, the predominant localization of the protein within the aqueous compartments (internal and external to the lipid bilayer) of the multilamellar and unilamellar liposomes remains a distinct possibility.

The precise distribution of dialkylphosphatidylcholine liposome-associated albumin molecules can only be determined with further experimentation.

In this study, we examined the efficacy of ether-phosphatidylcholine liposomes in promoting the immunogenicity of entrapped bovine serum albumin antigens. Unilamellar vesicles were found to be more superior than multilamellar vesicles in the potentiation of the anti-albumin plaque-forming cell response (Fig. 4). This observation is consistent with our previous results using ester-phosphatidylcholine liposomes for antigen entrapment [9]. Thus, the lamellar arrangement appears important in determining the potentiating capacity of the liposomal carrier. The lack of response by animals, receiving a simultaneous injection of free antigen and empty liposomes, demonstrates the need for direct physical association between the antigen and the carrier. Another determining factor seems to be the chemical structure of the phospholipid used. It is clear that liposomes composed of ester phosphatidylcholines are more effective than those of ether phosphatidylcholines in engendering an antigen-specific antibody response (Fig. 5). It has been reported that phospholipids containing the diether bond are less susceptible to enzymatic degradation in the body and the rate of metabolism of the ether lipid is slower [14]. We have previously demonstrated that macrophages play an obligatory role in processing liposome-entrapped antigens for the elicitation of liposome-mediated immune enhancement [29]. Thus, depending on the relative efficiency of macrophages in breaking down the diether phospholipids, the more resistant nature of the ether bond to enzymatic digestion might slow down the processing of the entrapped antigen, and therefore, could affect the outcome of the immune response.

In conclusion, our results appear to demonstrate that liposome-mediated potentiation of the antibody response to the entrapped antigen is determined by at least two factors, namely the lamellar arrangement of the liposomal membrane and the chemical structure of the phospholipid used in the liposome preparation.

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