Oil-in-Water Liposomal Emulsions: Characterization and Potential Use in Vaccine Delivery

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Received January 12, 1999. Final revised manuscript received June 25, 1999.
Accepted for publication September 7, 1999.

Abstract □ Emulsification of mineral oil by phospholipids donated by liposomes composed of dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, cholesterol, and lipid A by extrusion resulted in the formation of oil-in-water liposomal emulsions containing a substantial number of intact liposomes. Increasing the proportion of liposomes from 25 mM to 150 mM phospholipid and increasing the oil content from 2.5% (v/v) to 42.5% (v/v) changed the flow characteristics of the emulsions from fluid liquidlike to viscous. Likewise, the degree of stability of the emulsions was liposomal phospholipid concentration-dependent, ranging from partial emulsification in the range 25–100 mM to complete stabilization in the range 125–150 mM. Despite some loss of liposome integrity, as evidenced by the release of liposomal trapped glucose, emulsification of liposomes containing encapsulated prostate-specific antigen (PSA) exhibited antigen-specific immunostimulation in mice. These results suggest that liposomes containing encapsulated antigen can serve as constituents for the formulation of oil-in-water vaccines.

Introduction

For many years, liposomes have been utilized as carriers of vaccines for inducing high titers of antibodies and for induction of cytotoxic T-lymphocytes.[1−4] The purpose of the present study was to examine the emulsifying characteristics of liposomes as constituents of oil-in-water emulsion adjuvants for vaccines. We and others have also previously reported that liposomes could be emulsified directly within incomplete Freund's adjuvant and other water-in-oil emulsions and that depending on the proportion of liposomes, such emulsification resulted in the formation of stable emulsions containing a large fraction of intact liposomes.[5,6] The liposomes that were destroyed by the emulsification process donated phospholipid molecules to assist the stabilizing properties of Span 80 and Arlacel A, respectively.[5,6] Thus, if liposomes containing encapsulated antigen were emulsified within Freund's adjuvant or any other water-in oil emulsion, at least some of the antigen would be released free into the emulsion and might thereby lose the beneficial immunostimulatory effects derived from encapsulation in liposomes. These observations raised the question whether the emulsification of oil by nondegraded liposomes in oil-in-water emulsions for vaccine design could be more difficult to achieve than with water-in-oil emulsions such as Freund's adjuvant because of more efficient donation of phospholipid molecules to effect droplet dispersion.

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The fact that liposomes can be emulsified directly by an oil emulsion such as incomplete Freund's adjuvant or any oil without being either completely degraded themselves or without degrading the emulsion itself is very appealing in adjuvant technology. The immunogenic power of emulsions is well-known, as they are extremely powerful for inducing antibodies to emulsified antigens.[7−10] However, although emulsions are not noted for inducing cytotoxic T-lymphocyte (CTL) responses,[7−10] liposomes are among the most potent carriers for inducing CTLs.[3,4] Therefore, if the properties of each were additive, formulations of liposomes with water-in-oil or oil-in-water emulsions could have potential for being versatile adjuvants.

The data reported herein demonstrate that emulsification of mineral oil by phospholipids donated by liposomes results in the formation of oil-in-water emulsions that still contain substantial amounts of intact liposomes. Furthermore, it is also shown that formulations of liposome-encapsulated antigen in the oil-in-water emulsions exhibited strong immunostimulating activity for induction of antibodies and an antigen-specific lymphoproliferative response in mice.

Materials and Methods

Lipids—Dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), cholesterol (CHOL), and lipid A (LA) were purchased from Avanti Polar Lipids (Alabaster, AL). The fluorescent phospholipid *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (N-NBD-PE) was purchased from Molecular Probes (Eugene, OR). LA was stored at −20 °C and dissolved in chloroform containing 10% methanol (v/v) before use. Solutions of phospholipids and CHOL were prepared in chloroform containing 0.75% ethanol (v/v) and stored at −20 °C until use. The phospholipid concentration in stock solutions, liposomes, and liposomal emulsions was determined by assaying aliquots for organic phosphorus by using the procedure of Bartlett[11] as modified by Gerlach and Deuticke.[12] Cholesterol content of stock solutions, liposomes, and liposomal emulsions was determined as described elsewhere.[13]

Emulsion Constituents—Materials used in emulsion preparations included light mineral oil (Sigma Chemical Co., St. Louis, MO) and liposomes containing or lacking PSA. Occasionally, trace amounts of N-NBD-PE-labeled liposomes were used in the formulation of emulsions to determine the presence of liposomal spherules.

Other Reagents—Chloroform was redistilled before use. After distillation, ethanol was added to chloroform as a preservative at 0.75% (v/v). Sodium deoxycholate from Sigma was purified by crystallization from hot acetone−water. All other reagents were reagent grade and were used without further purification.

Antigen—Protein-specific antigen (PSA) from donor human seminal fluid (M_r 30 000), which had been purified to a single gel band by extensive precipitation and column chromatography steps, including inhibition of protease activity, was purchased from Cortex Biochem, Inc. (San Leandro, CA). The antigen was exten-

10.1021/js990011u CCC: $18.00
Published on Web 10/21/1999

sively dialyzed against isotonic Tris-glycine (20 mM)/NaCl (150 mM) buffer, pH 7.4, and stored at 4 °C until use. Its purity was checked by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using 15% acrylamide gels and silver stain.[14] The results showed that the sample had one predominant band corresponding to the enzyme and two to three minor bands indicating degradation products.

The relative concentration of PSA was determined by the method of Lowry et al.[15] using bovine serum albumin as a standard. The amount of antigen encapsulated in liposomes was determined by using a modification of the Lowry protein assay.[16]

Animals—BALB/c mice were from Jackson Laboratory (Bar Harbor, ME). The animals were handled and housed according to a protocol approved by the Laboratory Animal Care and Use Committee of the Walter Reed Army Institute of Research. They were given standard laboratory food and water ad libitum.

Preparation of Liposomes—Liposomes (multilamellar vesicles) and liposome-encapsulated glucose or PSA were prepared essentially as described elsewhere.[6,16,17] Multilamellar liposomes were made from a mixture of DMPC/DMPG/CHOL in molar ratio of 9:1:7.5. LA was incorporated at a dose of 2.5 μg/μol of phospholipid. Fluorescent liposomes contained a phospholipid, N-NBD-PE, at 2 mol % with respect to the phospholipid concentration.

Preparation of Oil-in-water Liposomal Emulsions— Oil-in-water (o/w) liposomal emulsions were prepared by the stepwise addition method, as described elsewhere,[18] using two 3- or 5-mL B−D syringes connected by a three-way stopcock with rotating male Luer-lock adapter and a switch-key. The Luer-lock adapter (bore diameter = 0.10 cm) supported the 18 gauge needle for sample drawing. Liposomes containing or lacking PSA were added in a stepwise fashion and extruded backward and forward (2 passes/s) with light mineral oil under shear forces for 5 min. Liposomal emulsions (1−3 mL total volume) were also occasionally prepared by vortex-mixing at 2500 rpm for 5 min in 1.5 cm × 10 cm glass tubes. The amounts of liposomes and oil used were such that the final liposomal phospholipid concentration in the emulsions was in the range 25−150 mM and the oil concentration was varied from 2.5 to 42.5% (v/v). The quality of the emulsion was tested by placing a few drops of the sample on the surface of cold (iced) distilled water, as described by Freund.[19] A satisfactory o/w emulsion was produced if the drops lost their integrity by spreading on the surface of the water.

Measurement of Emulsion Viscosity—The viscosity of the emulsions was measured immediately after manufacture using the Cannon model LV 2000 rotational viscometer. The measurement temperature was controlled at 25 °C with a circulating water bath. The Cannon rotational viscometer operates on the principle of a spindle rotating while immersed in the material being tested and measuring the torque necessary to overcome the viscous resistance to rotation. From the spindle speed, torque measurements, and spindle characteristics, the viscometer calculates and displays the viscosity in centipoise (cP) or milliPascal sec (mPa·s). Using TL5 and TL7 spindles for less and more viscous emulsions, respectively, the shear rates were varied in the range 0.084−79.2 s^{-1}.

Emulsion Stability—Emulsion (1−3 mL) was poured from the syringe into 1.0 cm × 10 cm screw-cap glass tubes and incubated at 37 °C. This temperature was chosen for its physiological relevance to antigen stability in immunization studies. The stability of the emulsion was assessed by detection of phase separation with time. The height of the oil phase above the emulsion was measured using a ruler, and the results were expressed in % oil separated using the following formula:

$$\frac{\text{height of oil (upper phase)}}{\text{total height of emulsion sample} \times F}$$

where the factor F stands for the initial fraction of oil in the emulsion. The value of F was in the range 0.025−0.425. The smallest amount of separated oil that could be measured was ≤5%.

The rates of separation of the emulsions were expressed in % oil per minute as reported previously.[20,21] These rates were calculated from the slopes of the time of the percent of oil separated using the initial velocity data.

Liposome Stability—Liposome stability in the o/w emulsions was assayed spectrophotometrically by enzymatic assay of trapped glucose released, as previously described,[16] with the following modification. Normal saline (2 mL) was added to the emulsion (1 mL), and the mixture was vortexed for 2 min at 2000 rpm. Following centrifugation at 27 000 × g for 15 min at 25 °C, the oil phase was removed and the pellet was resuspended in the aqueous supernatant and glucose. As this procedure completely separated the oil from the water and lipids, all of the released glucose was detected in the liposomes resulting from resuspension of the pellet in the aqueous phase. This was confirmed in control experiments by demonstrating that the total amount of trapped glucose detected in emulsified liposomes was identical to the amount detected in the absence of an emulsion after disruption of the liposomes with chloroform and 10% Triton X-100.[16] Glucose release values of emulsified liposomes above 5% compared to baseline were judged to be positive.

The presence of liposomal spherules in the emulsion was determined by fluorescence microscopy using trace amounts of N-NBD-PE-labeled liposomes in the emulsion. A few drops of the sample were spread on a microscope slide immediately after manufacture and visualized by fluorescence with an Olympus (VANOX-S) microscope using an oil immersion 100× objective, or with an Olympus IX70 inverted microscope with a 40× objective. Images were collected with an Olympus camera and digitized from the 35-mm slides. The particle diameter of the emulsions was determined by measuring 50 emulsion droplets from photomicrographs prepared by fluorescence microscopy using oil immersion 100× objective.

Immunization Studies—BALB/c mice (five mice per group) were immunized intramuscularly (posterior thigh) with 100 μL of either liposome-encapsulated PSA (PSA-liposomes) or PSA-liposomes formulated in o/w emulsions. Each of these formulations contained 25 μg of antigen and 25 μg of LA per dose. Mice were bled by tail vein at 0, 2, and 5 weeks and the serum samples were analyzed for antibody titers by ELISA as described in detail elsewhere.[22]

For splenic T-lymphocyte proliferation studies, mice were euthanized by CO_2 followed by cervical dislocation and bleeding by cardiac puncture. The freshly removed spleens were placed in Hank's balanced salt solution [HBSS (Gibco Laboratories, Grand Island, NY)], and a single cell suspension was prepared. Following centrifugation, the cells were resuspended at 5 × 10^6 cells/mL in RPMI-1640 (Gibco Laboratories, Grand Island, NY):EHAA (Whittaker Bioproducts, Walkersville, MD) (1:1, v/v) supplemented with 8 mM L-glutamine, 100 units/mL pencillin, 100 μg/mL of streptomycin, 1 mM sodium pyruvate, 1X MEM nonessential amino acids, 100 μM 2-mercaptoethanol, and 0.5% BALB/c normal mouse serum. Subsequently, the cells were seeded at a density of 5 × 10^5 cells/well in 96-well fat-bottom tissue culture plates (Costar, Cambridge, MA) containing several dilutions of PSA in triplicate, in a total volume of 0.2 mL. Following 4 days of incubation at 37 °C in 5% CO_2 and 95% relative humidity, the cells were labeled with 1 μCi/well of [^{3}H]Thymidine [TdR, specific activity 6.7 Ci/mM (NEN, Boston, MA)]. The plates were then incubated overnight at 37 °C and the cell cultures harvested onto glass fiber filters with the Skatron cell harvester and processed for scintillation spectrometry. Radioactivity was quantified using the Pharmacia LKB liquid scintillation counter. Data were expressed in stimulation index, i.e., the ratio of counts from cells incubated with PSA over counts from the control.

Results

Rheological Properties of Liposomal Emulsions—The flow characteristics of liposomal emulsions were established using samples formulated with 42.5% (v/v) of mineral oil and liposomes containing different concentrations of phospholipid. Figure 1 shows that values of the viscosity increased slowly, but gradually with the lipid composition from 0.03 × 10^3 cP to 1.53 × 10^3 cP in the range 25−100 mM. Further increase of liposomal phospholipid concentration to from 125 mM to 150 mM resulted in an abrupt change of viscosity from the value of 7.80 × 10^3 cP to the value of 98.10 × 10^3 cP.

Figure 2 shows that with samples containing 50 mM and 100 mM liposomal phospholipid, values of the viscosity were very low and their increase with the oil concentration was negligible, with the change being from the mean of

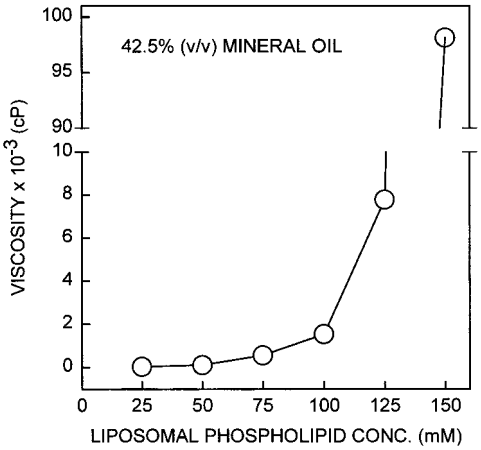

Figure 1—Viscosity characteristics of emulsions versus liposomal phospholipid concentration. The emulsions were formulated by extrusion with 42.5% (v/v) of mineral oil and liposomes containing different concentrations of phospholipid. The final liposomal phospholipid concentration used in the emulsions varied from 25 to 150 mM. Viscosity values were measured at 25 °C immediately after manufacture of the emulsions. The data were the mean of two to four determinations with a variability of ≤5%.

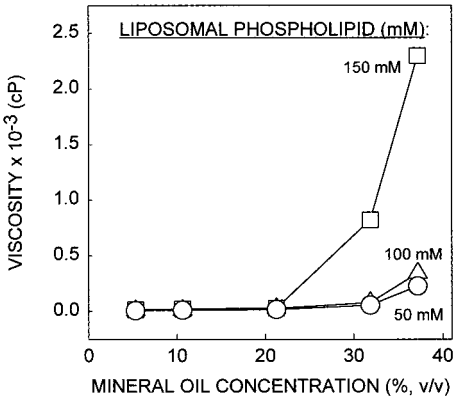

Figure 2—Viscosity characteristics of emulsions versus mineral oil concentration. The emulsions were formulated by extrusion with increasing amounts of oil in the range 5.3–37.5% (v/v) and liposomes containing different concentrations of phospholipid. The final liposomal phospholipid concentrations in the emulsions were 50 mM, 100 mM, and 150 mM as indicated in the figure. Viscosity values were measured at 25 °C immediately after manufacture of the emulsions. The data were the mean of two to four determinations with a variability of ≤5%.

0.01×10^3 cP to the mean of 0.23×10^3 cP in the range 5.3−37.5% (v/v). However, for samples formulated with 150 mM phospholipid, the viscosity which was also low at 0.02×10^3 cP did increase significantly with the oil concentration only beyond 21.3% (v/v) until it reached the value of 2.30×10^3 cP at 37.5% (v/v).

Stability Characteristics of Liposomal Emulsions—Emulsions containing 42.5% (v/v) of mineral oil and 25 mM to 150 mM of liposomal phospholipid were prepared by vortex-mixing at 2500 rpm and incubated at 37 °C. Figure 3 shows the time course of oil separated in percent of the total added, and Table 1 summarizes the rates of separation of the samples versus phospholipid concentration. Samples containing 25 mM and 50 mM liposomal phospholipid were rapidly destabilized with the percent oil separated increasing with time at rates of 1.434% and 1.184% separation per minute to complete separation after 1 and 5 h, respectively. Emulsions formulated with 100 mM and 125 mM phospholipid were slowly destabilized up to 48 h at rates of 0.003% and 0.002% separation per minute; they were thereafter destabilized rapidly to complete separation after 96 h. Interestingly, sample emulsion

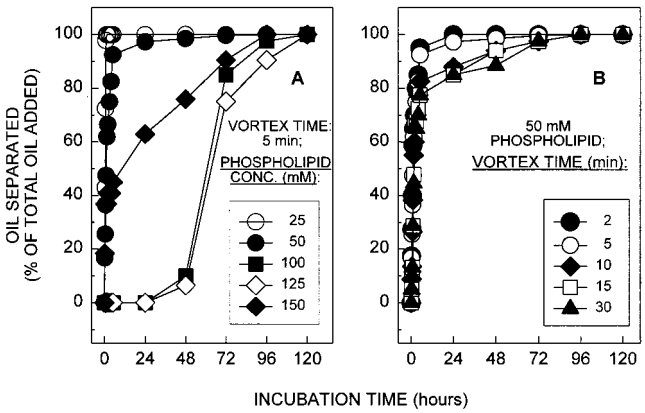

Figure 3—Time course of the degree of stability of emulsions formulated by vortex-mixing with 42.5% (v/v) of mineral oil and liposomes containing different concentrations of phospholipid. The final liposomal phospholipid concentration in the emulsions was varied from 25 mM to 150 mM as indicated. Emulsion samples were incubated at 37 °C, and their stability was assessed by detection of phase separation with time. The data were the means of two determinations with variability of ≤5%.

Table 1—Rates of Separation in % Oil Per Minute versus Phospholipid Concentration for o/w Liposomal Emulsions Prepared by Vortex-Mixing [a]

liposomal phospholipid concentrationin in o/w emulsions (mM)	rate of separation (in % oil per min)
25	1.434
50	1.184
100	0.003
125	0.002
150	1.227

[a] Liposomal emulsions were prepared with 42.5% (v/v) of mineral oil and liposomes containing different concentrations of phospholipid. The resulting samples were incubated at 37 °C, and their stability was assessed by the appearance of phase separation with time. The rates of separation were calculated from the slopes of the time of the % of oil separated using initial velocity data.

containing 150 mM liposomal phospholipid was destabilized at a rate of 1.227% per minute to complete separation after 96 h, suggesting that at the higher phospholipid concentration used where the viscosity of the sample was substantially increased, vortex of the sample was inefficient to adequately promote mixing of the components. Thus, none of the emulsions formulated by vortex-mixing was stable.

Emulsions prepared by syringe extrusion containing 42.5% (v/v) of mineral oil and 25 mM to 150 mM liposomal phospholipid were incubated at 37 °C and examined for the appearance of phase separation. Figure 4 illustrates the gross physical appearance of these emulsions following a 72-h incubation period, while Figure 5 depicts the phospholipid dose dependence of their degree of stability for the same time period. The degree of stability of the dispersions ranged from complete separation at lower phospholipid concentrations, i.e., 25 mM and 50 mM (Figure 4A,B and Figure 5), to complete stabilization at higher phospholipid content, i.e., 125 mM and 150 mM (Figure 4E,F and Figure 5). However, while the sample formulated with 75 mM liposomal phospholipid was near complete separation with 87.5% of the oil becoming separated (Figure 4C and Figure 5), the dispersions containing 100 mM phospholipid were relatively stable with only 5% of the oil being separated (Figure 4D and Figure 5).

Figure 6 shows the time course of oil separated in percent of the total added following incubation at 37 °C of emulsion samples formulated with liposomes lacking (Figure 6A) or containing PSA (Figure 6B), and Table 2 summarizes the

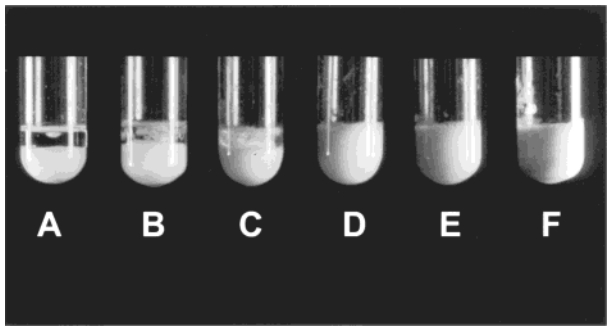

Figure 4—Gross physical appearance of emulsions formulated by extrusion with 42.5% (v/v) of mineral oil and liposomes containing different concentrations of phospholipid, following 72 h of incubation at 37 °C. The final liposomal phospholipid concentrations in the emulsions were (A) 25, (B) 50, (C) 75, (D) 100, (E) 125, and (F) 150 mM. Phase separation was evidenced by the development of two phases: a clear oil phase at the top and a milky aqueous phase containing liposomes at the bottom.

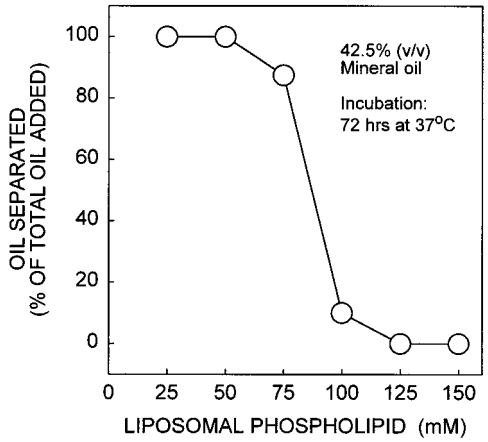

Figure 5—Phospholipid concentration dependence of the degree of stability of liposomal emulsions following 72 h of incubation at 37 °C. The emulsions were formulated by extrusion with 42.5% (v/v) of mineral oil and liposomes containing different concentrations of phospholipid. The final liposomal phospholipid concentration used in the emulsions varied from 25 to 150 mM. Stability of the emulsions was assessed by detection of phase separation.

rates of separation of the samples versus lipid composition. These rates were calculated from the slopes of the time of the percent of oil separated using the initial velocity data plotted in Figure 6A,B. Samples containing 25 mM and 50 mM liposomal phospholipid were rapidly destabilized with the percent of oil separated increasing with time (Figure 6A,B). For emulsions formulated with liposomes lacking PSA (Figure 5A), phase separation occurred at rates of 1.322% and 0.381% per minute (Table 2) to complete separation after 12 and 48 h, respectively (Figure 6A). Emulsions formulated with PSA-liposomes (Figure 6B) were destabilized more rapidly at rates of 1.895% and 0.919% per minute (Table 2) to complete separation after 2 and 24 h, respectively (Figure 6B). For the emulsions formulated with liposomes lacking (Figure 6A) or containing PSA (Figure 6B) at 75 mM phospholipid, the percent of oil separated increased gradually and almost linearly with time (Figure 6A,B), at rates of 0.012% and 0.045% per minute, respectively (Table 2), to complete separation after 96 h (Figure 6A,B). Furthermore, the samples prepared with liposomes lacking (Figure 5A) or containing PSA (Figure 6B) at 100 mM phospholipid were stable up to 72 and 24 h of incubation, respectively, after which they became destabilized toward 5% and 39% of oil separated at 120 h at rates of 0.001% and 0.003% per minute,

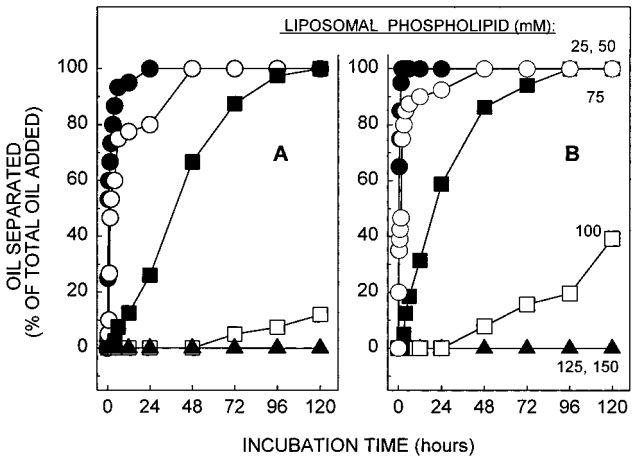

Figure 6—Time course of the degree of stability of emulsions formulated by extrusion with 42.5% (v/v) of mineral oil and empty liposomes (Figure 6A) or PSA-liposomes (Figure 6B), containing different concentrations of phospholipid. The final liposomal phospholipid concentration used in the emulsions varied from 25 to 150 mM as indicated in the figure. Emulsion samples formulated with PSA-liposomes contained 6.5 μg PSA per 100 μL. The samples were incubated at 37 °C, and their degree of stability was assessed by detection of phase separation with time. The data were the mean of two to three determinations with a variability of $\leq$8%.

Table 2—Rates of Separation in % Oil Per Minute versus Phospholipid Concentration for o/w Liposomal Emulsions[a]

liposomal phospholipid concentration in o/w emulsions (mM)	rate of separation (in % oil per min) of emulsions formualted with	
	empty liposomes	PSA-liposomes
25	1.322	1.895
50	0.381	0.919
75	0.012	0.045
100	0.001	0.003
125	0.000	0.000
150	0.000	0.000

[a] Liposomal emulsions were formulated with 42.5% (v/v) of mineral oil and empty liposomes or PSA-liposomes containing different concentrations of phospholipid. Emulsion samples formulated with PSA-liposomes contained 6.5 μg PSA/100 μL. The samples were incubated at 37 °C, and their stability was assessed by the appearance of phase separation with time. The rates of separation were calculated from the slopes of the time of the % of oil separated using initial velocity data.

respectively. However, emulsions formulated with 125 mM and 150 mM liposomal phospholipid were stable over the 120-h incubation period regardless of the lack or addition of PSA.

Effect of Mineral Oil Concentration on the Stability of the Emulsions—The oil concentration dependence of the degree of stability of the dispersions was established using emulsion samples containing various amounts of mineral oil and 25−150 mM liposomal phospholipid. Figure 7 shows that samples containing 25 mM and 50 mM liposomal phospholipid were unstable regardless of the oil concentration and the incubation time, with 91−100% of the oil becoming separated following 6 to 72 h of incubation at 37 °C. For emulsions formulated with 75 mM liposomal phospholipid, the percent oil separated increased with time and was inversely proportional to mineral oil concentration, until it reached the plateau of 88% after 72 h over the range 10−42.5% (v/v) of oil. The 100 mM-liposomal phospholipid sample was stable at all mineral oil concentrations following 6 and 24 h of incubation, but was relatively stable after 72 h, with only 5−15% of the oil being separated over the range 5−42.5% (v/v) of oil. However, samples containing 125 mM and 150 mM liposomal phospholipid were stable up to 72 h regardless of the oil content.

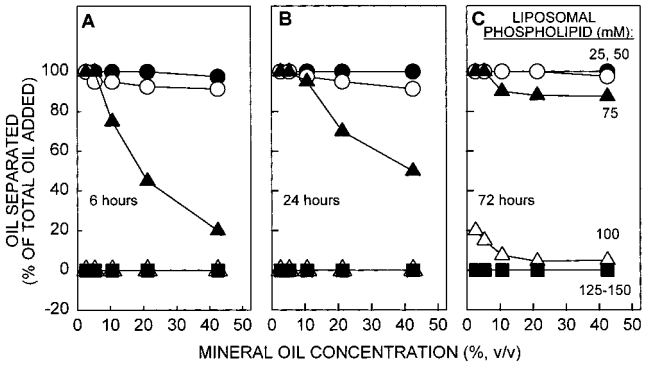

Figure 7—Mineral oil concentration dependence of the stability of liposomal emulsions. The emulsions were formulated by extrusion with increasing amounts of mineral oil in the range 2.5–42.5% (v/v) and liposomes containing different concentrations of phospholipid. The final liposomal phospholipid concentration in the emulsions was varied from 25 to 150 mM as indicated in the figure. The samples were incubated at 37 °C, and their stability was assessed by detection of phase separation following 6, 24, and 72 h of incubation. The data were the mean of two determinations with a variability of ≤5%.

Appearance of Fluorescent Liposomes in Emulsions—Fluorescent liposomes that were nearly spherical in shape were easily observed surrounding the surface of oil droplets in the emulsions (Figure 8B−D). Figure 8A shows, as a control experiment, the photomicrograph of fluorescent, nonemulsified liposomes.

Stability of Liposomes As Determined by Release of Trapped Glucose—In the previous experiment with N-NBD-PE, liposome-like structures appeared to be present in the emulsions (Figure 8B−D), but the experiment could not determine whether intact liposomes remained that retained their permeability properties. Figure 9A shows that release of liposomal trapped glucose, measured immediately after manufacture of the emulsions, increased gradually with the liposomal phospholipid concentration from 16% to 43% in the range 25−125 mM. Furthermore, Figure 9B depicts the shelf life characteristics of the liposomes formulated in the emulsions at selected lipid compositions following incubation at 37 °C. The increase of release of trapped glucose with time was not substantial irrespective of the phospholipid composition, with the average increase being in the range 5.5−7% over the 20-day incubation period.

Antibody Response to PSA Encapsulated in Emulsified and Nonemulsified Liposomes—PSA-liposomes formulated in emulsions were tested for their ability to induce antibodies to PSA. Figure 10 shows that immunization of mice with the unencapsulated PSA formulation did not induce antibodies to PSA over a period of 5 weeks. In contrast, the use of liposome-encapsulated PSA resulted in a level of serum IgG that increased gradually over 5 weeks to reach a final concentration of approximately 120 μg/mL. When mice were immunized with the liposomal emulsion formulations, the serum IgG titers were in the range 35−137 μg/mL depending on the phospholipid composition at 5 weeks (Figure 10). It is clear that the liposomes in the emulsion retained their potent activities as carriers of antigen.

Proliferative Response of Splenic T-Lymphocytes—The ability of PSA to induce splenic T-lymphocyte proliferation in vitro was investigated by immunizing mice with unencapsulated PSA, liposome-encapsulated PSA, and PSA-liposomes formulated in the emulsion containing 150 mM phospholipid. Figure 11 shows the PSA concentration dependence of stimulation index in vitro. There was no

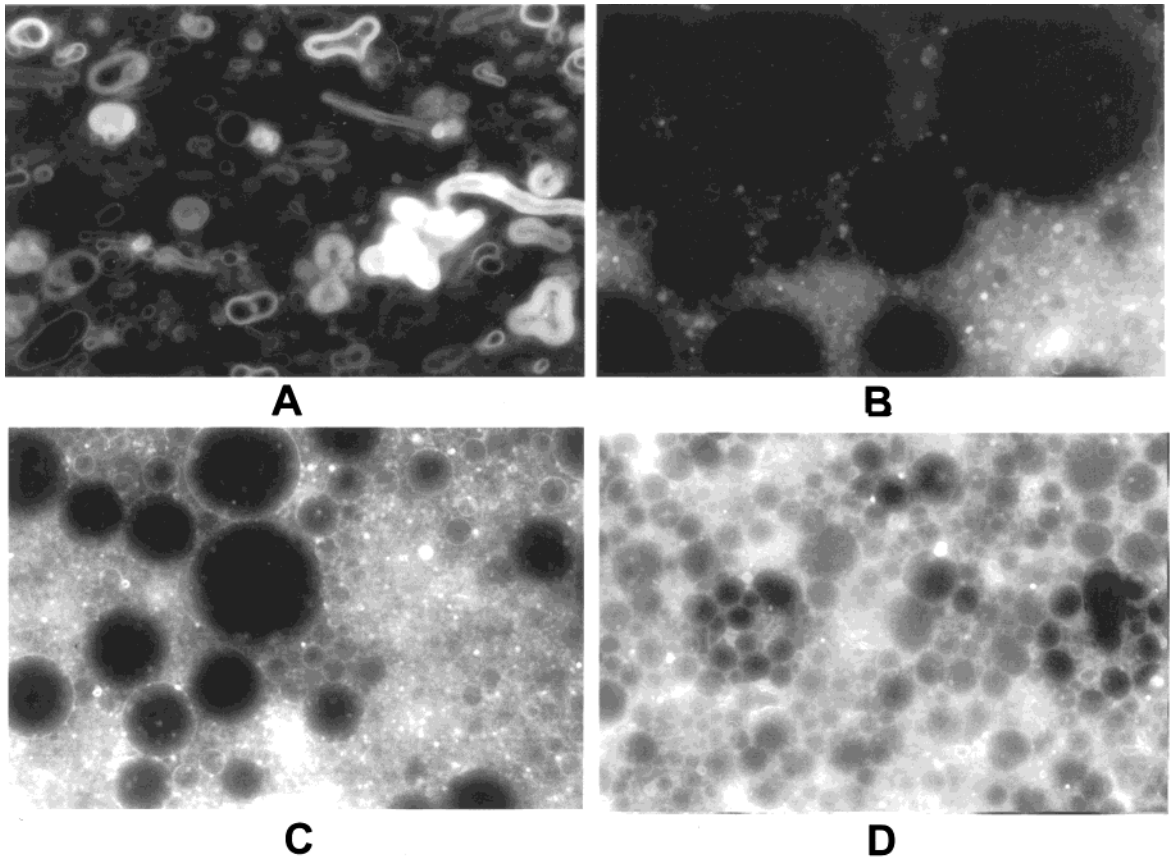

Figure 8—Photomicrographs of nonemulsified fluorescent liposomes (Figure 8A), and emulsions (Figure 8B–D) formulated with 42.5% (v/v) of mineral oil and liposomes containing trace amounts of N–NBD-PE. The final liposomal phospholipid concentrations in the emulsions were 25 (Figure 8B), 50 (Figure 8C), and 125 mM (Figure 8D). Fluorescent microscopy was performed immediately after manufacture of the samples.

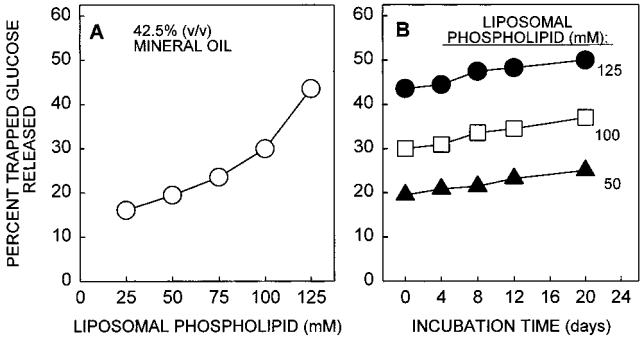

Figure 9—Phospholipid concentration (Figure 9A) and time (Figure 9B) dependencies of the stability of liposomes within the emulsions as determined by measurement of release of liposomal trapped glucose. The emulsions were formulated with 42.5% of mineral oil and liposomes containing different amounts of phospholipid. The final liposomal phospholipid concentration in the emulsions was varied from 25 to 125 mM. Liposomal trapped glucose released was determined immediately after manufacture (Figure 8A) and following incubation of the samples at 37 °C up to 20 days (Figure 8B). The data were the mean of 2 determinations with a variability of ≤5%.

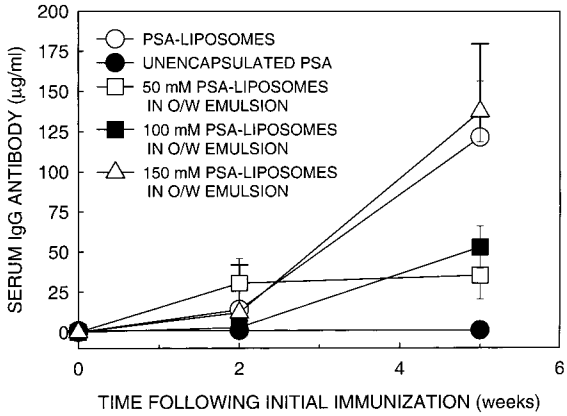

Figure 10—Antibody response to PSA; PSA was encapsulated in emulsified and nonemulsified liposomes. BALB/c mice (n = 5) were immunized intramuscularly with the indicated formulations each of which contained 25 μg of antigen and 25 μg of LA per dose. The final liposomal phospholipid concentrations in the emulsions were 50 mM, 100 mM, and 150 mM as indicated in the figure. Sera were collected at weeks 0, 2, and 5, and analyzed for antigen-specific antibodies by ELISA.

splenic T-cell proliferation when mice were immunized with the unencapsulated PSA formulation. Stimulation index values were low and remained close to 1.0 with increasing PSA concentration (Figure 11). However, when mice were immunized with liposome-encapsulated PSA and emulsified PSA-liposomes, stimulation index values increased gradually for both formulations in a PSA concentration-dependent manner toward 4.1 and 5.7, respectively. Stimulation index data obtained with the liposomal emulsion were slightly higher than those obtained with liposomes themselves (Figure 11), but the relative differences observed were not statistically significant. No proliferation occurred in a set of in vitro control experiments using ovalbumin instead of PSA (data not shown).

Discussion

This work is part of a larger effort to develop a variety of liposome-based adjuvant and carrier combinations for vaccines. It describes an approach for the creation of practical emulsified oil-in-water liposomal vaccines. The important factors for formulation were the degree of stability of the emulsions, the integrity of the liposomes

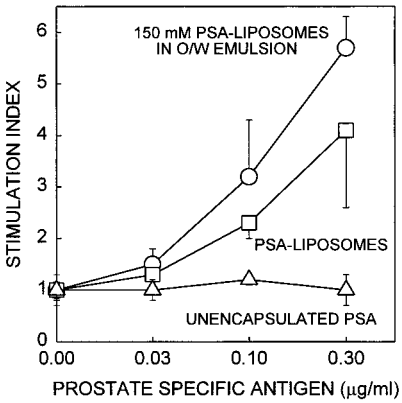

Figure 11—Lymphocyte proliferation response to PSA; PSA was encapsulated in emulsified and nonemulsified liposomes. BALB/c mice (n = 5) were immunized intramuscularly with the indicated formulations which each contained 25 μg of antigen and 25 μg of LA per dose. The final liposomal phospholipid concentration in the emulsion was 150 mM as indicated in the figure. Spleens were removed from mice at week 5 and processed for T-lymphocyte proliferation as described in the material and methods section. Data were expressed in stimulation index, i.e., the ratio of counts from cells incubated with PSA over counts from nonstimulated cells. Nonstimulated cells had about 190 to 250 counts per minute.

within the emulsion, and the ability of emulsified liposome-encapsulated antigen to exhibit immunostimulating activity.

The results show that the physicochemical properties, including viscosity and stability, of the emulsions changed with the liposomal phospholipid concentrations. At lower concentrations, over the range 25−100 mM, the emulsions were less viscous or fluid-liquidlike (Figure 1) and had different degrees of stability, ranging from complete to partial destabilization (Figures 4, 5, and 6A). Conversely, at higher concentrations, over the range 125−150 mM, they were viscous (Figure 1) and stable (Figures 4, 5, and 6A). Because rheological properties can affect the flow of emulsions through a needle or a catheter, the stable and viscous emulsions were so thickened that they could not be easily handled. This simply calls for formulation of emulsions of low viscosity for ease of injection. Our data showed that stable emulsions of low viscosity could be formulated over the range 125−150 mM liposomal phospholipid by lowering the oil concentration up to 2.5% (v/v) (Figures 2 and 7).

The rates of separation of the unstable emulsions which were inversely proportional to phospholipid concentration, were much higher with emulsified PSA-liposomes (Figure 6 and Table 2), probably due to perturbation of the bulk phase as well as the interfacial region brought about by released PSA from liposomes. Proteins are well-known to induce long-range electrostatic forces as well as variations in several physicochemical parameters, including surface potential and surface viscosity.[23] Thus, PSA could behave like a detergent displacing phospholipid molecules from the surface of oil droplets, thereby increasing the van der Waals attractive forces in the medium.

Fluorescence studies showed that the emulsions retained liposomal structures (Figure 8), but the integrity of liposomes within the emulsion was not maintained completely intact, as shown by the release of as much as 16% to 43% of the trapped marker with increasing lipid composition (Figure 9A). As suggested by the shelf life characteristics of liposomes within the emulsions (Figure 9B), no substantial leakage of intact liposomes occurred during incubation. Thus, the partial degradation of liposomes in the emulsions was not due directly to interaction or collision of liposomes with the oil droplets, but solely due to shear forces of the extrusion process involved in the manufacture of the dispersions. This conclusion is also supported by the

observation that no substantial degradation of liposomes occurred when, in control experiments, the emulsion samples were prepared by vortex-mixing. As expected, none of the latter emulsions was stable regardless of the liposomal phospholipid concentration (Figure 3). However, its possible that the slight increase in the release of glucose with time (Figure 9B) could be due to diffusion from the remaining intact liposomes. Alternatively, the glucose could be released by Brownian motion induced collision of oil droplets with intact liposomes resulting in the destruction of the liposomes.

The partial degradation of liposomes in the emulsions (Figure 9A) donated phospholipid molecules that provided partial or complete emulsification of the oil. Phospholipids, which form the bulk phase of liposomes, have been classically viewed as being particularly effective as emulsifiers for oil-in-water emulsions.[24] The photomicrographs shown in Figure 8 support the view that emulsification of liposomes with the oil was more effective with increasing the proportion of liposomes, with subsequent reduction of the size of the oil drops. The mean values of the particle diameters of the emulsion containing 25, 50, and 125 mM phospholipid were 12.5, 2.5, and 0.625 μm, respectively. These values were obtained by measurement of the oil droplets. The tiny continuous dots coating the oil droplets could be part of the phospholipid monomolecular layers at the interface which have been reported to impart a significant steric stabilization to the emulsion droplets.[25,26] The protective effect of phospholipid monolayers against coalescence and coagulation would be quite analogous to the reported repulsive forces arising from purely osmotic and finite-volume restricting effects of compressed coils of polymer-covered surfaces.[27,28]

The integrity of liposomes is a critical aspect of the vaccine formulation because, as demonstrated by some reports,[29−31] ingested liposomes provide intracellular targeting in phagocytic cells, with distribution of the liposomal antigen both to the cytoplasm and Golgi apparatus via the major histocompatibility (MHC) class I antigen presentation pathway and to low pH compartments. Despite the partial degradation of liposomes in the emulsions, the data showed that both PSA-liposomes formulated in emulsions and nonemulsified PSA-liposomes exhibited a comparable immunostimulating activity with regard to induction of not only antibody titers (Figure 10), but also antigen-specific lymphoproliferative responses (Figure 11) in mice. The relative differences observed in both responses were not statistically significant. Although the three liposomal emulsion formulations tested, which were administered to mice immediately after manufacture, had different degrees of stability in vitro, they exhibited comparable immunogenic properties in vivo (Figure 11). Similar results were reported on the adjuvant activity of o/w emulsions stabilized by a combination of Tween 80/Span 80 surfactants and for which the degree of stability did not affect antibody response as long as an emulsion was formed; stable emulsions were as effective as unstable ones.[32] Moreover, experiments are currently underway to examine the influence, if any, of the degree of stability of o/w liposomal emulsions on their immunogenic properties.

Overall, the data presented in this work show that emulsification of liposomes with mineral oil resulted in the formation of o/w liposomal emulsions containing a substantial number of intact liposomes that could still exhibit some immunostimulating activity in mice. These results are very encouraging for examining further biochemical and immunogenic properties of the emulsified o/w liposomal formulation and its extension to other vaccines. Further experiments will address the effect of oil concentration, the nature of the antigen, and the integrity of the liposomes as well as the degree of stability of the emulsion on the quality of the immune response generated by these formulations. Because oil emulsions induce predominantly humoral immunity[7−10] through their capacity to concentrate and orient antigens on oil droplets for effective delivery to cells of the immune system,[33,34] and liposomes are known as the best carriers for cell-mediated immune responses[3,4] through the MHC class I pathway,[29−31] combination of both mechanisms in a single vaccine could lead to a synergistic adjuvant effect.[35,36] The PSA-liposomal vaccine formulated in the emulsion has exhibited the highest immunostimmulating activity for induction of antibody titers and antigen-specific lymphoproliferative response in current clinical trials of prostate cancer patients (unpublished data).

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Acknowledgments

This work was performed under a cooperative research and development agreement between the Walter Reed Army Institute of Research, Washington, DC, and Jenner Biotherapies, Inc., San Ramon, CA, and was partially supported by both institutions. The authors gratefully acknowledge the outstanding technical assistance of Ms. Elaine Morrison.

JS990011U