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Liposomes as adjuvants with immunopurified tetanus toxoid: influence of liposomal characteristics

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SUMMARY

The effect of various manipulations of liposomes composed of equimolar phospholipid and cholesterol on immune responses to the incorporated immunopurified tetanus toxoid was investigated in BALB/c mice. In studies designed to establish proper dosage for immunization and to reveal the roles of the liposomal phospholipid to toxoid mass ratio, gel-liquid crystalline transition temperatures (Tc) of the liposomal phospholipids and mode of antigen incorporation into liposomes on immune responses, animals were injected intramuscularly with various amounts of the toxoid, free, entrapped in multilamellar dehydration–rehydration vesicles (DRV) or covalently linked to the surface of multilamellar vesicles (MLV) prepared by the classical procedure. Two identical injections separated by 4 weeks were given and IgG1 and IgG2b antibodies specific for the toxoid assayed in sera by an enzyme-linked immunosorbent assay. Results suggest that (i) the adjuvant effect of liposomes is improved considerably when liposomal phospholipid to toxoid mass ratios are as high as 2049:1; however, adjuvanticity is reduced to reach very low levels for much higher ratios (e.g. 90,361:1); (ii) antibody responses are similar for liposomes (phospholipid to toxoid mass ratios: 14·3–33·1) made of a variety of phospholipids with Tcs ranging from −32° to 41·5° but are low or non-existent when liposomes are made of distearoyl phosphatidylcholine (Tc, 54°) (however, see Discussion); (ii) there are no differences in antibody responses between liposomes with entrapped and surface-linked toxoid.

INTRODUCTION

Numerous studies have shown that liposomes act as immunological adjuvants for a wide spectrum of incorporated bacterial and viral antigens relevant to human and veterinary immunization (for reviews see Alving *et al.*, 1980; Gregoriadis, 1985). These include diphtheria (Allison & Gregoriadis, 1974) and tetanus (Davis, Davies & Gregoriadis, 1986) toxoids, *Streptococcus pneumoniae* serotype 3 (Snippe *et al.*, 1983), hepatitis B surface antigen (Manesis, Cameron & Gregoriadis, 1979) and derived polypeptides (Sanchez *et al.*, 1980), and Epstein–Barr virus antigen (Epstein *et al.*, 1985). The structural versatility of liposomes in terms of vesicle size, lamellarity, surface properties and composition and their ability to accommodate antigens in a variety of ways (e.g. passively entrapped within the aqueous phase, embedded in the lipid bilayers and adsorbed or covalently linked onto the liposomal surface) suggest, in turn, variability in immunoadjuvant action, which could be tailored to satisfy particular needs (Gregoriadis, 1986). However, in spite of the significant amount of work carried out to date in this area, there exists considerable controversy as to the liposomal characteristics deemed optimal for such action. For instance, opposing views have been expressed with regard to the roles of liposomal membrane fluidity and antigen localization in promoting adjuvanticity (Gregoriadis, 1985; Hedlund, Jansson & Sjogren, 1984; Kinsky, 1978; Dancey, Yasuda & Kinsky, 1978; Shek & Sabiston, 1982; van Rooijen & van Nieuwmegen, 1980).

In an attempt to understand liposome adjuvanticity further we have recently (Davis *et al.*, 1986; Gregoriadis, Davies & Davis, 1987a) carried out studies of antigen entrapment in (multilamellar) dehydration–rehydration vesicles (DRV), a procedure that combines simplicity and high yield of solute entrapment (Kirby & Gregoriadis, 1984). Immunopurified tetanus toxoid, used as the model antigen, was entrapped into such liposomes in yields of 39–82% of the toxoid present, depending on the phospholipid composition. The toxoid was also covalently linked to conventionally prepared MLV liposomes with high (63%) coupling efficiency (Gregoriadis *et al.*, 1987a; Davis *et al.*, 1986). In immunization studies using BALB/c mice, both DRV and MLV liposomes acted as immunological adjuvants for the entrapped or covalently linked toxoid respectively (Gregoriadis *et al.*, 1987a; Davis *et al.*, 1986; Davis, Davies & Gregoriadis, 1987). These preparations have now been used in studies on the effect of various manipulations of toxoid-incorporating liposomes on their adjuvanticity. Here we report

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results with liposomes composed of equimolar phospholipid and cholesterol suggesting that (i) adjuvanticity of DRV increases with increased liposomal phospholipid:toxoid mass ratio; however, adjuvanticity returns to low levels for very high ratios (90,361:1 or above); (ii) antibody responses are similar for DRV (phospholipid to toxoid ratios: 14·3–33·1) made of a variety of phospholipids with gel-liquid crystalline transition temperatures (Tc) ranging from −32° to 41·5° but are low or non-existent when DRV are made of distearoyl phosphatidylcholine (DSPC) (Tc, 54°) (however, see Discussion for DSPC liposomes of higher phospholipid to toxoid ratios); (iii) there are no differences in antibody response between liposomes with entrapped and surface-linked toxoid.

MATERIALS AND METHODS

Sources and grades of dilinoleoyl phosphatylcholine (DLPC) dioleyl phosphatylcholine (DOPC), egg phosphatylcholine (PC), dimyristoyl phosphatylcholine (DMPC), dipalmitoyl phosphatylcholine (DPPC), distearoyl phosphatylcholine (DSPC) and cholesterol have been described elsewhere (Gregoriadis *et al.*, 1987a; Senior & Gregoriadis, 1982). Immunopurified tetanus toxoid (1·2–6·3 mg per ml) was obtained from Wellcome Biotechnology Ltd (Beckenham, Kent) freed of aggregates and labelled with ^{125}I as described elsewhere (Gregoriadis *et al.*, 1987a). Synthesis of *N*-amino-(*p*-phenyl)stearylamide (APSA) was carried out using *p*-phenylenediamine and *p*-nitrophenylstearate (Sigma, Poole, Dorset) (Snyder & Vannier, 1984; Gregoriadis *et al.*, 1987a). Horseradish peroxidase-labelled rabbit anti-mouse IgG1 serum was from Nordic (Maidenhead, Berks) and similarly labelled rabbit anti-mouse IgG2b serum was from Miles Laboratories (Slough, Berks). All other reagents were of analytical grades.

Entrapment of tetanus toxoid in DRV liposomes

The general procedure for solute entrapment into DRV liposomes has been described elsewhere (Kirby & Gregoriadis, 1984). Entrapment of tetanus toxoid into DRV composed of a variety of phospholipids and cholesterol was carried out at appropriate temperatures, according to the type of phospholipid used (Gregoriadis *et al.*, 1987a). In brief, small unilamellar vesicles (SUV) in distilled water, composed of equimolar phospholipid (16 μmols) and cholesterol were prepared (Senior & Gregoriadis, 1982; Wolff & Gregoriadis, 1984) by probe sonication at 18° (DLPC, DOPC, PC and DMPC) and 40° (DPPC and DSPC liposomes) and were used to generate DRV (Kirby & Gregoriadis, 1984) in the presence of 1·0 mg ^{125}I-labelled toxoid. The final DRV preparations with entrapped toxoid were suspended in 0·45 mM Na phosphate buffer, pH 7·4, containing 0·8% NaCl and 0·02% $MgCl_2$ (PBS). The suspension was diluted with 7 ml PBS and centrifuged at 10,000 *g* for 40 min to remove free toxoid. The liposome pellet was washed twice in 8 ml PBS by centrifugation and then resuspended in 1 ml PBS. Toxoid entrapment values, estimated on the basis of ^{125}I radioactivity, varied (39–82% of toxoid used) depending on the phospholipid (Gregoriadis *et al.*, 1987a; Davis *et al.*, 1986). In some experiments, DRV were prepared in the absence of toxoid or the amount of toxoid used was varied so as to achieve a range of liposomal phospholipid to protein mass ratios. The extent of non-specific adsorption of toxoid to the surface of DRV during the entrapment procedure, estimated by treating toxoid-containing DRV with protease (Gregoriadis *et al.*, 1987a), was very low (7% of the total liposomal toxoid).

Covalent coupling of tetanus toxoid to MLV liposomes

MLV liposomes were prepared (Gregoriadis *et al.*, 1987a) by bath sonication at the appropriate temperatures (see above) for 2 min (10-min bursts) from equimolar PC or DSPC (16 μmols) and cholesterol supplemented with 3·2 μmols of APSA (4·975:4·975:0·05, molar ratio). In brief, the dry layer of the lipids obtained following rotary evaporation of the solvent $CHCl_3$ was suspended by vigorous shaking into 0·17 M NaCl/0·01 M sodium borate buffer, pH 8·0. The suspension was then centrifuged at 20,000 *g* for 10 min and the liposomal pellet resuspended as described by Snyder & Vannier (1984) in 2·0 ml cold (4°) 0·2 M $NaNO_2$ and 2 ml 0·2 M HCl/NaCl. To the diazotized liposomes a cold (4°) solution of ^{125}I-labelled toxoid (0·5 mg) was added and the resultant tan-coloured liposomes were washed with PBS. The proportion of toxoid coupled to the surface of liposomes (63·1 ± 8·3% of the amount used) was estimated on the basis of ^{125}I radioactivity and, again, non-specific adsorption was very low (1·7%) (Gregoriadis *et al.*, 1987a). In some experiments the amounts of toxoid used for coupling varied so as to achieve a range of liposomal to protein mass ratios.

Animal experiments

Male BALB/c mice (Clinical Research Centre, Harrow, Middlesex), 4–8 weeks old, were used in immunization experiments. In studies designed to establish proper dosage for immunization in dose–response protocols and to reveal the effects of the phospholipid Tc, mode of antigen incorporation into liposomes (entrapped versus surface-linked) and phospholipid to toxoid mass ratio on immune responses, animals were injected intramuscularly in groups of five with 0·1 ml of various amounts of toxoid, free, entrapped in liposomes, or covalently linked to their surface. Four weeks later, mice were boosted with the same dose of antigen and, when appropriate, liposome form. Blood samples were obtained from the tail vein 1–3 days before priming and 9–10 days after the second injection. Serum samples were kept at −20° until assayed for anti-toxoid IgG1 and IgG2b.

Enzyme-linked immunosorbent assay (ELISA)

Antibody responses to tetanus toxoid were monitored by a microplate ELISA technique (Gregoriadis *et al.*, 1987a; Davis *et al.*, 1987). Immunopurified tetanus toxoid was the solid phase on plastic microelisa plates (Dynatech, Detroit, MI). The antigen was then incubated for two 3-hr periods at room temperature, first with a 1 in 100 dilution of the mouse serum and secondly with a horseradish peroxidase-labelled antiserum against mouse immunoglobulins IgG1 or IgG2b. Wells were washed three times between incubations. Subsequently, the colour change after 30 min incubation at room temperature with the substrate (O-phenylenediamine) was measured spectrophotometrically at 492 nm. ELISA values were determined by subtracting the mean of two duplicate readings obtained with serum taken before the first injection of a given mouse, from the mean of two duplicate readings obtained with serum taken from the same mouse following antigenic stimulation (Davis *et al.*, 1987). Since the IgG1 responses of some mice were so high as to give maximal ELISA readings, the mouse sera were titrated by

doubling dilutions. Results are expressed as the highest dilution of serum that yielded an optical density of 0·200.

Statistical analyses

Comparison of ELISA values, when more than two groups of mice were involved, was made according to the method of Kruskall-Wallis as described by Campbell (1974). This technique yields a statistic H, the significance of which may be determined using χ^2 tables. When only two groups of mice were included in a single experiment, ELISA values were compared using the two sample rank test.

RESULTS

Previous studies (Davis *et al.*, 1987) indicated that injection of BALB/c mice with 10 μg of tetanus toxoid, free or entrapped in DRV liposomes composed of equimolar PC and cholesterol, produced IgG1 responses with serum titres in excess of $\log_{10}$ 5·0. An adjuvant effect of liposomal toxoid in comparison with the free toxoid was only seen with the IgG2b subclass. A reduction of the amount of the injected antigen to 2 μg did not change the titre of IgG1 in the serum, nor did it produce an adjuvant effect. A further reduction, however, to 0·1 μg lowered the titre of IgG1 specific for tetanus toxoid but allowed the observation of an adjuvant effect. Adjuvant effects were seen with the IgG2b subclass at all doses of antigen. Since adjuvant effects were lost as the dose of liposomal tetanus toxoid was increased, the design of further experiments depended on the knowledge of the shape and position of the dose–response curve. Full dose–response curves were therefore generated in two independent experiments for the toxoid entrapped in either PC or DSPC DRV liposomes (Fig. 1). These data were used to determine toxoid doses in the remainder of the present studies.

The effect of liposomal phospholipid to tetanus toxoid mass ratio on antibody respose

Three separate experiments were carried out, each with a variety of liposomal phospholipid to toxoid mass ratios ranging from 2:1 to 553,097:1. When ELISA values were analysed statistically (see legend to Fig. 2), both IgG1 and IgG2b responses showed significant differences between the different phospholipid to protein ratios in two of the experiments (Fig. 2). In the third experiment (which included control mice immunized with corresponding amount of free toxoid), there was a similar significant difference for the IgG2b response but not for IgG1 (legend to Fig. 2). However, when sera from this experiment were titrated for IgG1 antibody, a significant difference ($P<0{\cdot}05$) was observed between the responses of mice injected with liposomes of a 30:1 ratio ($\log_{10}$ $3{\cdot}67 \pm 0{\cdot}12$) and mice injected with free toxoid ($\log^{10}$ $2{\cdot}42 \pm 0{\cdot}36$). In further work a phospholipid to protein ratio of 30:1 was aimed at in preparing liposomes containing toxoid.

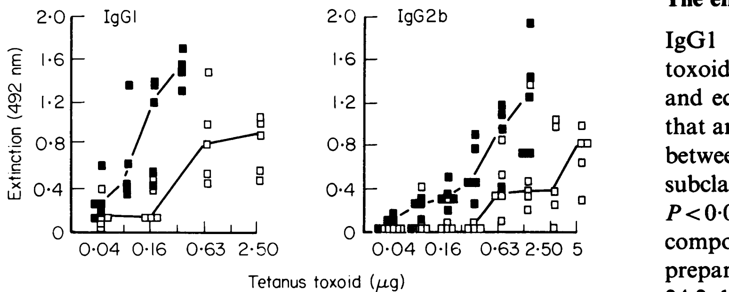

Figure 1. Dose–response to liposomal tetanus toxoid. BALB/c mice (in groups of five) were injected with increasing amounts of tetanus toxoid entrapped in DRV liposomes composed of equimolar PC (■) or DSPC (□) and cholesterol. Animals were bled 9–10 days after an identical booster injection and analysed for IgG1 and IgG2b by the ELISA immunosorbent assay. The phospholipid to toxoid mass ratios were 24·6:1 for DSPC and 30:1 for PC DRV. Results are individual values for each of the treated mice. For other details see text.

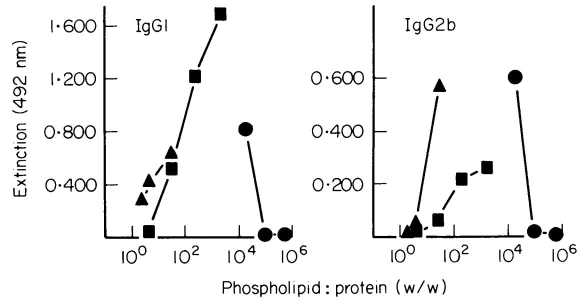

Figure 2. Immune responses to tetanus toxoid given in liposomes of varying phospholipid to toxoid mass ratio. In three separate experiments BALB/c mice (in groups of five) were injected with 0·1 (▲), 0·025 (■) or 0·005 (●) μg of tetanus toxoid per mouse, free or entrapped in DRV liposomes composed of equimolar PC and cholesterol and with the phospholipid to protein ratios shown. Animals were bled 9–10 days after an identical booster injection and the sera analysed for IgG1 and IgG2b by the ELISA immunosorbent assay. Results are expressed as median readings for each of the treated groups. Phospholipid to toxoid mass ratios are plotted on a logarithmic scale. Readings (not shown) for sera of control mice injected with free toxoid in one of the experiments (▲) were below 0·1 or nil. For other details see text. Results from statistical analysis (carried out by the Kruskall-Wallis non-parametric test) of differences between the various phospholipid to toxoid mass ratios in each of the experiments were: (▲) $H=4{\cdot}16$, $P>0{\cdot}1$ (IgG1) and $H=8{\cdot}93$, $P<0{\cdot}01$ (IgG2b); (■) $H=15{\cdot}61$, $P<0{\cdot}01$ (IgG1) and $H=11{\cdot}30$, $P<0{\cdot}01$ (IgG2b); (●) $H=12{\cdot}02$, $P<0{\cdot}01$ (IgG1) and $H=11{\cdot}18$, $P<0{\cdot}01$ (IgG2b).

The effect of liposomal phospholipid Tc on antibody response

IgG1 and IgG2b responses in mice immunized with tetanus toxoid entrapped in DRV liposomes of varying phospholipid and equimolar cholesterol are shown in Fig. 3. Results show that antibody response is similar when phospholipid Tc ranges between −32° and 41·5°, but decreases significantly for both subclassses ($H=14{\cdot}37$, $P<0{\cdot}01$ for IgG1 and $H=11{\cdot}23$, $P<0{\cdot}05$ for IgG2b) when DSPC (Tc = 54°) is the phospholipid component of DRV. The lipid to protein ratios for the preparations used in this experiment were 21·2:1 (DLPC), 24·2:1 (DOPC), 23·0:1 (PC), 33·1:1 (DMPC), 29·0:1 (DPPC) and 14·3:1 (DSPC).

Antibody responses to entrapped and surface-linked tetanus toxoid

Results from five independent immunization experiments (two with surface-linked and three with entrapped tetanus toxoid), in

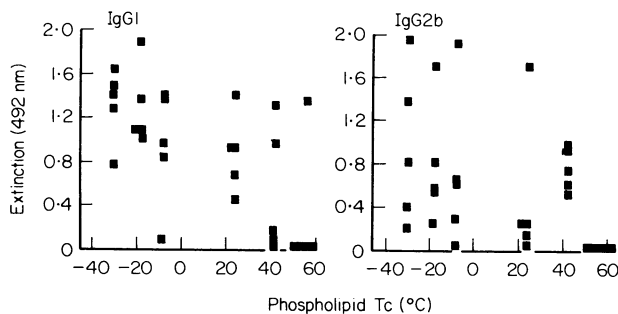

Figure 3. Effect of the liposomal phospholipid Tc on liposome adjuvanticity. BALB/c mice in groups of five were injected twice with 0·25 μg of tetanus toxoid entrapped in DRV liposomes composed of equimolar phospholipid and cholesterol. Animals were bled 9–10 days after the booster injection and analysed for IgG1 and IgG2b by the ELISA immunosorbent assay. The phospholipid to toxoid mass ratios are given in the text. Readings for individual mice are plotted against the liquid-crystalline phase transition temperatures (given in parentheses) of the DLPC (−32°), DOPC (−20°), PC (−10°), DMPC (23°), DPPC (41·5°) and DSPC (54°) components of DRV. Differences in response between DSPC DRV and the other DRV preparations were significant (IgG1, H = 15·37, $P < 0{\cdot}01$; IgG2b, H = 11·23, $P < 0{\cdot}05$, as determined by the Kruskall-Wallis non-parametric test. For other details see text.

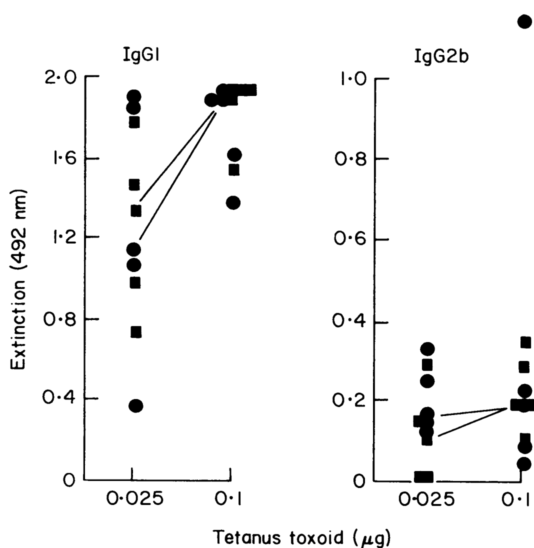

Figure 4. Antibody response to entrapped and surface-linked tetanus toxoid. BALB/c mice in groups of five were injected twice with 0·025 or 0·1 μg tetanus toxoid entrapped in (multilamellar) DRV liposomes (■) or covalently linked to conventional multilamellar liposomes (●). Animals were bled 9–10 days after the booster injection and analysed for IgG1 and IgG2b by the ELIZSA immunosorbent assay. The phospholipid to toxoid mass ratios were 24·7:1 (entrapped) and 38·7:1 (surface-linked toxoid). Results are individual values for each of the mice treated. Statistical analysis was carried out by the two-sample rank test. For other details see text.

which the liposomal phospholipid (PC) to antigen mass ratios were similar (about 30:1) and amounts of injected antigen identical (0·1 μg), were used initially to derive estimates of the median (with 95% confidence interval) IgG1 and IgGb levels. ELISA readings of the sera of the immunized mice corresponding to the IgG1 response were 0·798 (0·420–1·436, $n = 14$) for entrapped and 1·090 (0·650–1·454, $n = 9$) for surface-linked toxoid. The corresponding IgG2b responses were 0·297 (0·060–0·522) and 0·224 (0·050–0·767). In view of the similarity of these values, an experiment was carried out to compare entrapped and surface-linked toxoid within a single experiment.

Figure 4 shows the immune responses (IgG1 and IgG2b) in mice immunized with the toxoid entrapped in, or covalently linked to, the surface of liposomes composed of equimolar PC and cholesterol. Using two different doses of the toxoid (0·025 and 0·1 μg) no significant difference in responses for the two liposomal preparations was observed for either IgG1 or IgG2b. The phospholipid to protein ratios for the preparations were 24·7:1 (entrapped) and 38·7:1 (surface-linked toxoid).

DISCUSSION

In contrast to conventional adjuvants, liposomes are uniquely versatile in structural characteristics and mode of antigen accommodation. This, in turn, allows for a wide range of options in designing effective vaccines. Such versatility may also account for the variability of data obtained with regard to the role of a number of liposomal parameters in adjuvanticity (Gregoriadis, 1986). In the present work we set out to examine some of these parameters using multilamellar liposomes incorporating tetanus toxoid either (passively) entrapped or covalently linked to their surface. The choice of dehydration–rehydration procedure was based on previous experience (Kirby & Gregoriadis, 1984; Gregoriadis *et al.*, 1987a) of realistically and reproducibly high antigen entrapment values. On the other hand, the method of diazotization provides a rapid and efficient means for the coupling of antigen to the liposomal surface (Snyder & Vannier, 1984; Gregoriadis *et al.*, 1987a; Davis *et al.*, 1986). Both types of liposomal antigen preparations can be freeze-dried for storage, with most of the antigen being retained upon reconstitution in water (Gregoriadis *et al.*, 1987a; Davis *et al.*, 1986).

It was firstly reasonable to assume (Lifshitz, Gitler & Mozes, 1981) that the immunoadjuvant action of liposomes would be favoured by an increased concentration of antigen in individual vesicles as this would allow presentation of sufficient quantities of antigen to immunocompetent cells. Contrary to such expectations, our data (Fig. 2) show that the higher the phospholipid to toxoid mass ratio, the higher the immune response (IgG1 and IgG2b). Although a ratio of about 30:1 was considered high enough (Fig. 2) for the purpose of the present studies, it is clear that adjuvanticity is improved to a much greater extent when ratios as high as 2049:1 are used. However, at much higher ratios (e.g. 90,361:1 or above) adjuvanticity is reduced to reach very low levels. It thus seems that under the conditions of experimentation, liposomal adjuvanticity is related to the lipid dose up to a certain level, possibly in conjunction with a slow rate of degradation/removal of liposomes given in large amounts, from the site of injection. We are unable to explain at present the reduction in adjuvanticity at very high phospholipid to toxoid ratios, although it could be tentatively attributed to an immunosuppressive effect of excessive PC: interaction of T lymphocytes with both MHC and antigen (or antigenic fragments) on the surface of macrophages (an essential requirement for induction of immune response; Unanue *et al.*, 1984) may require a certain degree of fluidity of the domains surrounding the two antigens. If there is excessive incorporation of PC (from liposomal toxoid of a very high phospholipid to protein ratio)

into the macrophage membrane, this could alter fluidity to an extent that T lymphocyte effect is inhibited. Alternatively, or at the same time, it may be that as the ratios become greater, a situation is reached where the number of vesicles in the preparation is so great that only a small proportion of them contain antigen, probably at a concentration too low for induction of immune responses.

It thus appears that in comparing antibody responses to antigen incorporated in different liposome preparations, the mass of injected liposomal lipid (as well as that of the antigen) must be similar. Hitherto, these conditions have been difficult to fulfil since antigen entrapment values with the procedures used have been low and/or unpredictable, or the significance of an optimal lipid to antigen mass ratio had not been appreciated. These problems are reflected in the opposite conclusions reported in the literature regarding the adjuvanticity of liposomes for entrapped or surface linked antigen. Shek & Sabiston (1982), for instance, showed that liposomal surface-linked albumin was less efficient in stimulating indirect plaque-forming cells than the entrapped protein. However, the lipid to protein mass ratio was four-fold greater for the surface-linked albumin. In contrast, van Rooijen & van Nieuwmegen (1980) found liposomes coated with albumin to be better adjuvants than liposomes entrapping the protein, although the lipid to albumin ratio in the former preparation was 50-fold greater than in the latter. The use of DRV preparations (especially when generated from small unilamellar vesicles) and the diazotisation technique for linking antigen to the liposomal surface (Gregoriadis *et al.*, 1987a) in the present study gave liposomes with antigenic content sufficiently high and predictable to allow for meaningful comparative experiments. Our results (Fig. 4) with two doses of the toxoid indicate no difference in the immune response between entrapped and surface-linked antigen. This finding was confirmed in further work and was also found to apply to PC and DSPC liposomes of higher phospholipid to toxoid ratios. For instance, in experiments similar to those described in Fig. 4, BALB/c mice were immunized in groups of five with toxoid entrapped in or surface-linked to PC DRV (0·025 μg antigen) with phospholipid to toxoid ratios of 442:1 or 46,875:1 (entrapped) and 410:1 or 30,487:1 (linked) or to DSPC DRV (0·1 μg antigen) with phospholipid to toxoid ratios of 35·1:1 or 2580:1 (entrapped) and 20:1 or 2857:1 (linked toxoid). ELISA values revealed, by the two sample rank test, no statistically significant difference between entrapped and surface-linked antigen for both liposomal compositions and IgG subclasses (results not shown).

The same argument holds when the adjuvanticity of liposomes composed of phosphatidylcholines with different Tcs are compared and, again, adopted procedures enabled us to use preparations of similar phospholipid to toxoid ratios. Figure 3 shows that liposomes composed of DSPC (Tc 54°) and expected to remain 'solid' at the body temperature following injection (even though they are also incorporating equimolar cholesterol) gave very much reduced antibody levels. On the other hand, liposomes composed of phospholipids with transition temperatures of 41·5° or lower and equimolar cholesterol are expected to be 'fluid' following injection, and all these preparations stimulated antibody production to the same degree (Fig. 3). The phospholipid to protein ratios of the various preparations used in this study ranged between 14·3:1 and 33·1:1. Although this ratio for the DSPC liposomes was the lowest and might therefore explain the low antibody response, similar low responses have been observed with DSPC liposomes exhibiting lipid to protein ratios of 24·6:1 (Fig. 1).

Findings of reduced or no antibody response with toxoid entrapped in DSPC liposomes of a relatively low phospholipid to antigen ratio (Fig. 3) contrast those by others (Kinsky, 1978; Dancey *et al.*, 1978; Bakouche & Gerlier, 1986) who, however, used membrane antigens. Kinsky (1978) and Dancey *et al.* (1978), for instance, showed that liposomes incorporating a hapten–phospholipid complex (estimated total lipid to complex ratio being about 20:1) induce immune response to the hapten if beef sphingomyelin (Tc 37–39°), but not egg phosphatidylcholine, was the phospholipid component. Similarly, Bakouche & Gerlier (1986) reported a broad correlation between increasing phospholipid Tc and increasing antibody response to liposomal Gross virus cell surface antigen (estimated phospholipid to protein ratio 7·8; Gerlier, Sakai & Doré, 1978).

The nature of the antigens used in this study and those quoted above could account for the opposite effects of phospholipids with high Tc on liposomal immunoadjuvant action. Membrane antigens, for instance, may pass into the plasma membrane of the antigen-presenting cells without being first processed. Indeed, dipalmitoyl phosphatidylethanolamine (Tc 63·5°) liposomes with albumin covalently linked to their surface and incorporating MHC antigens were found to stimulate T-cell clones *in vitro* in the absence of antigen-presenting cells (Walden, Nagy and Klein, 1985). Soluble antigens, on the other hand, must be taken into such cells and processed before they can be exposed on their surface (Mills, 1986; Unanue *et al.*, 1984). It is conceivable that transfer of a membrane antigen into the plasma cell membranes will depend on its mobility and/or distribution within the liposomal bilayers and also on its accessibility. All of these could be influenced by a reduced bilayer fluidity in a way that promotes antigen transfer to antigen-presenting cells. On the other hand, liposomal DSPC or other high melting phospholipids may inhibit the processing of soluble antigens.

Interestingly, in recent work (Gregoriadis, Davis & Garcon 1987b) using a much greater DSPC to toxoid ratio (4224:1), antibody (IgG1 and IgG2b) responses to DSPC DRV were as high as those obtained with PC liposomes of a similar ratio. A mixture of low ratio (12:1) PC DRV and 'empty' (toxoid-free) PC liposomes giving a high (2770:1) overall ratio also improved immune responses to levels approaching those obtained with toxoid entrapped in PC DRV of an identical (2770:1) ratio. Although the way by which liposomes exert their immunoadjuvant action is still unclear, it is probable that such action is influenced by at least two events, probably occurring at the same time: the rate of antigen release from the vesicles at the site of injection and the mode of their interaction with immunocompetent cells. Both rate of release and interaction with cells depend on liposomal fluidity. In the first case, it is known (Senior, Crawley & Gregoriadis, 1985) that solid DSPC liposomes become unstable *in vivo* at a slower rate than fluid ones. In the second, liposomal fluidity influences the extent to which liposomes fuse with or are endocytosed and processed by cells (Poste, 1980). The enhancement of immune responses by excess DSPC may, as suggested in the case of excess PC liposomes (Fig. 2; ratio 2049:1), result from a prolonged release of the antigen at

the site of injection, which presumably overrides any inhibitory effect that DSPC may have on such responses.

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