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A Novel Positively-Charged Lipid 1, 2-Bis (Hexadecylcycloxy)-3-Trimethyl aminopropane (BisHOP) Enhances the Adjuvant Effect of Liposomes on Encapsulated Tetanus Toxoid

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SUMMARY **A novel positively charged lipid, 1,2-bis(hexadecylcycloxy)-3-trimethylaminopropane-HCl (BisHOP), when incorporated into the bilayers of phosphatidylcholine (PC) and distearoyl phosphatidylcholine (DSPC) dehydration-rehydration vesicles (DRV), was shown to have a powerful effect in enhancing the IgG_1 response to tetanus toxoid encapsulated within the liposomes. The adjuvant effect was significantly greater when 20% BisHOP was incorporated as compared to 10% incorporation and to control PC and DSPC DRV. Plain, uncharged DSPC DRV were found to have a greater adjuvant effect than plain PC DRV on the entrapped tetanus toxoid after a single intramuscular injection. Even though antibody levels at 8 weeks post-injection were similar for 20% BisHOP PC and DSPC DRV, the rate of rise of antibody titres was more rapid for 20% BisHOP DSPC than for 20% BisHOP PC DRV. These results suggest that faster and higher titers of antibodies may be obtained by optimal manipulation of the charged and non-charged lipid components of liposomes.**

(Abbreviations: SUV, small unilamellar vesicles; DRV, dehydration-rehydration vesicles; PC, phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; Chol, cholesterol; BisHOP, 1,2-bis(hexadecylcycloxy)-3-trimethylaminopropane-HCL; T_c, gel-to-liquid crystalline transition temperature; ELISA, enzyme-linked immunosorbent assay.)

One of the reasons for the widespread applicability of liposomes in diverse fields of biological research is the eminent amenability to alteration of their bilayer characteristics, enabling them to be tailored for specific needs. Parameters such as hydrophobicity, fluidity, charge, size, surface-associated molecules and encapsulated material may be varied to form a truly protean complex.

Since the discovery that liposomes function as adjuvants to entrapped diphtheria toxoid,[1] many other investigators have confirmed this effect for a wide range of bacterial and viral products,[2] giving rise to the hope that liposomes may eventually be used clinically as versatile, non-toxic carriers and adjuvants in a new generation of vaccines. Owing to the relatively complex structure and components of various liposomal formulations, Davis *et al.*[3] attempted to elucidate the importance of parameters such as phospholipid composition, antigen dose and lipid to antigen ratio by using tetanus toxoid as a model antigen in association with liposomes.

In this study, the effects of using phospholipids of differing liquid crystal-to-gel transition temperatures (T_c), and surface charge imparted by a novel positively-charged lipid, 1, 2-Bis(hexadecylcycloxy)-3-trimethylaminopropane-HCl (BisHOP; structure shown in Fig. 1) on the immune response to encapsulated tetanus toxoid are investigated. Results indicate that antibody responses to the entrapped antigen may be greatly enhanced by optimal manipulation of bilayer phospholipid composition and charge. It is proposed that this represents an effective strategy for maximising the immunogenicity of an antigen in a liposome-based vaccine.

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MATERIALS AND METHODS

Entrapment of tetanus toxoid in neutral and positively-charged DRV liposomes

Thirty-two μmoles egg PC or DSPC (Lipoid), or phospholipid incorporating 5 and 10% (on a molar basis) BisHOP (Syntex Research, Palo Alto, Ca., U.S.A.) and 32 μmoles cholesterol (British Drug House, U.K.) were mixed in a 50 ml round-bottomed flask (Quickfit). The lipids were dried to a thin film by evaporation of the solvent at a low speed in a rotary evaporator (Buchi) connected to a running tap water pump. Two ml of phosphate-buffered saline (PBS, pH 7.4) was added to the dried lipid film. The flask was lowered into a bath sonicator (Kerry) and subjected to bursts of sonication while being manually rotated to form MLV. To convert MLV into small unilamellar vesicles (SUV), sonication of the MLV samples using a probe sonicator was performed at room temperature.[4] One ml of the SUV formed was mixed with 1 ml of ^{125}I-labelled tetanus toxoid at a concentration of 50 μg/ml water to generate neutral and positively-charged DRV by overnight lyophilization followed by two-step rehydration with 0.1 ml distilled water and 0.9 ml PBS (pH 7.4).[5] The amount of tetanus toxoid encapsulated in uncharged and charged liposomes was determined by measuring ^{125}I radioactivity in the pellets after washing three times in 8 ml PBS by centrifugation at 10,000 × g for 30 min.

Animal immunization experiments

BALB/c mice were injected intramuscularly in groups of five with 1 μg of tetanus toxoid encapsulated in the various formulations of charged and uncharged DRV. Blood samples were obtained from the tail veins 1 day before and every 14 days after administration of the liposomal preparations. Serum samples were assayed for anti-toxoid IgG_1 by the enzyme-linked immunosorbent assay (ELISA) as described.[3]

RESULTS

Encapsulation of tetanus toxoid in BisHOP-containing DRV

To immunize mice with various formulations of tetanus toxoid in liposomes composed of phospholipids of widely differing transition temperatures and varying amounts of the novel positively-charged lipid, equimolar phospholipid (egg PC or DSPC containing 0%, 10% and 20% of the charged lipid BisHOP as a percentage of the total phospholipid used) and cholesterol were used to form liposomes entrapping tetanus toxoid by the DRV method.

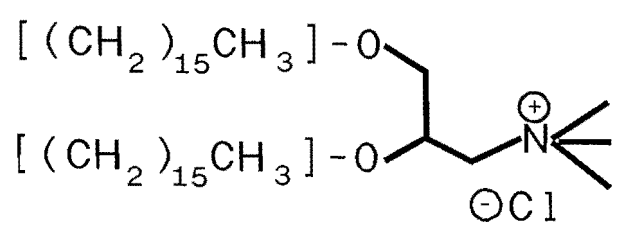
In keeping with previously established data,[6] entrapment values for tetanus toxoid in PC and DSPC DRV were high and reproducible (40.0 ± 0.4% and 42.6 ± 1.2% respectively). However, it was found that incorporation of increasing amounts of the novel positively-charged lipid BisHOP into the structure of liposomes led to progressively diminishing entrapment values, falling to 28.7 ± 2.3% for 20% BisHOP : PC DRV and to 27.4 ± 2.2% for 20% BisHOP : DSPC DRV.

Primary IgG_1 responses to tetanus toxoid encapsulated in neutral and BisHOP-containing DRV

In an experiment designed to compare the effect of adding increasing amounts of the positively-charged lipid BisHOP to PC and DSPC DRV on the primary immune response to 1 μg encapsulated tetanus toxoid, BALB/c mice in groups of five were administered a single intramuscular injection of various liposomal formulations of tetanus toxoid and bled every two weeks for 56 days. IgG_1 levels in appropriate dilutions of sera were monitored by ELISA. Fig. 2 compares the primary antibody responses to the various neutral and positively-charged liposomal preparations over a period of eight weeks.

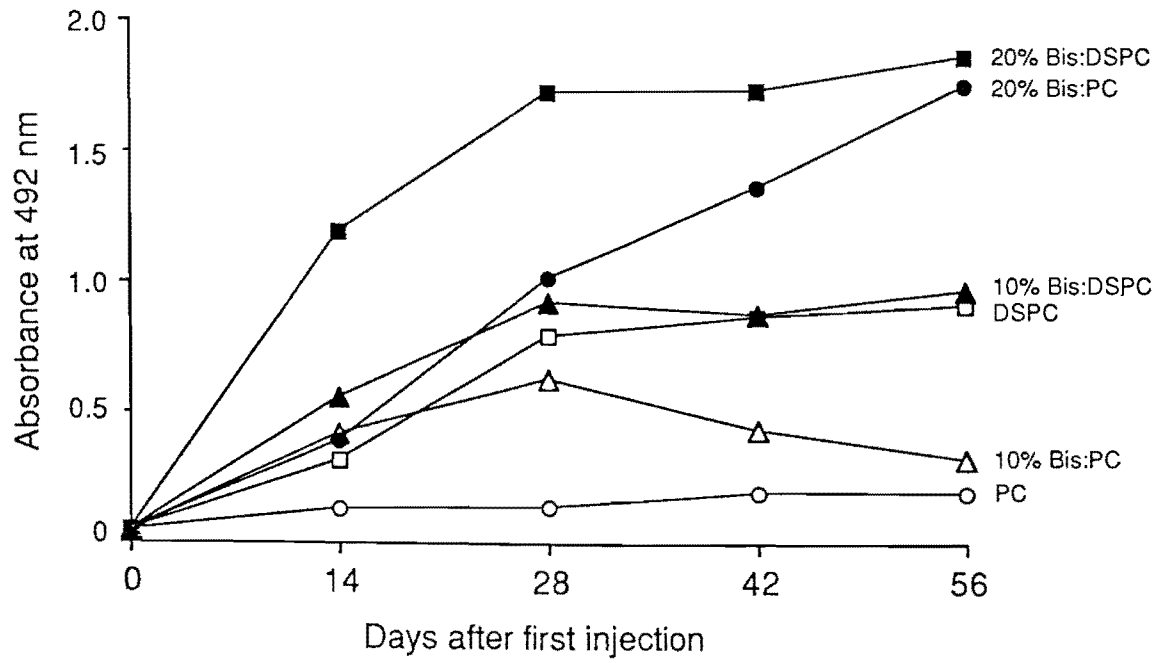
DISCUSSION

Results observed for the antibody responses against control neutral PC and DSPC DRV were surprising in that DSPC liposomes showed a greater initial adjuvant effect than PC liposomes with IgG_1 levels in the DSPC DRV group rising steadily and more steeply than for the PC DRV group (Fig. 2). This is in direct contrast with previously established data[3] comparing the secondary responses obtained against tetanus toxoid encapsulated in 'fluid' (PC) and 'solid' (DSPC) liposomes, which showed that PC DRV had a much greater adjuvant effect than DSPC DRV where the effect was negligible, in mice bled 10 days after the booster injection. However, it must be borne in mind that the primary response



BisHOP

Fig. 1 Structure of 1, 2–Bis (hexadecylcycloxy)—3—trimethylaminoprepane (BisHOP).



Fif. 2 Antibody (IgG_1) responses to tetanus toxoid entrapped in neutral and positively–charged liposomes.
BALB/c mice in groups of five were injected intramuscularly with 1μg of tetanus toxoid entrapped in DRV composed of PC:Chol (○), 10% BisHOP :PC:Chol (△), 20% BisHOP:PC:Chol (●), DSPC:Chol (□), 10% BisHOP : DSPC:Chol (▲), 20% BisHOP:DSPC:Chol (■). Animals were bled just before and every 14 days after the injection. Anti–toxoid IgG_1 responses were measured in sera by ELISA.

at two week intervals is being monitored in this study and that preliminary results (unpublished) indicate that this phenomenon is reversed early in the secondary response, in keeping with observations by Davis *et al.* [3]

It is seen that irrespective of the transition temperature of the lipid used to construct the liposomes, the incorporation of increasing amounts of positively-charged BisHOP leads to marked enhancement of the adjuvant effect in the primary response. When DRV are composed of 20% BisHOP as a percentage of the total phospholipid used, IgG_1 levels attained similar values for both PC and DSPC liposomes which were considerably higher than neutral DSPC DRV and very much greater than for neutral PC DRV. However, incorporation of 10% charged lipid did not lead to much increase in adjuvant activity over neutral liposomes. This implies that there may be a threshold of positive charge above which liposomal adjuvanticity is greatly increased. The initial rate of rise of IgG_1 levels is seen to be the greatest for 20% BisHOP:DSPC DRV, whilst although the initial increase in antibody levels induced by 20% PC:DRV is more gradual, it continues its rise relentlessly after IgG_1 levels of the former liposomes have reached a plateau and effectively catch up with then by day 56. Antibody levels for 10% PC DRV showed good initial rise to peak at day 28 but fell off after this period to almost PC DRV levels by day 56.

Liposomes may be formulated to possess a net neutral, negative or positive charge depending on the lipid used in the liposomes preparation. The effect of surface charge on the immunoadjuvant effect of multilamellar liposomes was first observed by Allison and Gregoriadis. [1] It was found that inoculation of diphtheria toxoid in negatively charged liposomes elicited significantly higher antibody levels than when entrapped in neutral or positively charged liposomes. A later report showed that although negatively-charged liposomes could act as adjuvants, positively charged liposomes too could do the same. [7] Similar results were obtained by van Roijen *et al.* [8] showing that positively charged and neutral liposomes have the same adjuvant activity as negatively charged liposomes.

With regard to phospholipid

composition, liposomes composed of dipalmitoyl phosphatidylcholine (DPPC) and distearoyl phosphatidylcholine (DSPC) (T_C 41.4° and 54.9°C, respectively) have been reported to be more effective immunogens than those prepared from egg PC. [9-11] It is postulated that these liposomes may have greater bilayer stability at physiological temperature and may thus persist longer *in vivo* than egg PC. In contrast, Davis *et al.* [3] found that PC DRV elicited higher antibody titers during the secondary immune response against entrapped tetanus toxoid as compared to DSPC DRV. They attributed this to the greater fluidity of the PC liposomes which facilitates antigen presentation. This study, which focuses on the primary response, shows that DSPC liposomes, especially those containing higher amounts of charged lipid, initially function as better adjuvants than egg PC causing a more rapid rise in IgG_1 levels but that as time progresses antibody levels elicited by BisHOP-containing PC liposomes rise to equal those of the DSPC liposomes. Thus, the differing efficacies of adjuvanticity afforded by different phospholipids depends not only on the size and other physical properties of the antigen causing their unique and individual modes of interaction with the liposomal bilayers/aqueous compartments, but also on the time point at which antigen-specific antibody levels are assayed. It has also been pointed out that the ratio of lipid to antigen administered is of crucial importance. [2]

This study has shown that the basic immunoadjuvanticity of liposomes to entrapped antigens may be further boosted by the incorporation of charged lipids such as BisHOP into the liposomal bilayers. Present evidence indicates that judicious inclusion of other adjuvants within the aqueous compartments like interleukin-2 and selection of phospholipid components [12] may enable one to modulate the immune response to liposomes-associated antigens to a remarkable degree.

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