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Immunology

Stimulation and inhibition of anti-hapten responses in guinea pigs immunized with hybrid liposomes

(model membranes/phosphatidylethanolamine derivatives/immunogenicity)

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ABSTRACT **Guinea pigs were immunized with liposomal model membranes containing phosphatidylethanolamine (PE) or glycerophosphorylethanolamine (GPE) derivatives in which the amino function was substituted with either dinitrophenylaminocaproyl (Dnp-Cap) or mono(*p*-azobenzenearsonic acid)tyrosyl (ABA-Tyr) residues. Previous studies have demonstrated that hapten-specific antibodies are elicited by Dnp-Cap-PE or ABA-Tyr-PE sensitized liposomes and that cell-mediated immunity is induced by ABA-Tyr-PE (but not Dnp-Cap-PE) sensitized liposomes. These liposomes differ from conventional immunogens in which haptens are covalently attached to immunogenic carriers. This investigation describes two new aspects of liposomal immunogenicity in animals immunized with hybrid liposomes containing both Dnp-Cap-PE and ABA-Tyr-PE. (*1*) Stimulation of the anti-Dnp response by incorporation of increasing amounts of ABA-Tyr-PE; (*2*) inhibition of anti-ABA antibody formation by incorporation of increasing amounts of Dnp-Cap-PE. The two phenomena are dependent on the presence of each determinant in the same lipid bilayer. Thus, entrapment of the water-soluble deacylated derivative of ABA-Tyr-PE (i.e., ABA-Tyr-GPE) in the aqueous compartments of Dnp-Cap-PE sensitized liposomes does not enhance anti-Dnp antibody production. Similarly, entrapment of the non-amphipathic derivative of Dnp-Cap-PE (i.e., Dnp-Cap-GPE) within ABA-Tyr-PE sensitized liposomes does not suppress anti-ABA antibody formation. Furthermore, mixtures of Dnp-Cap-PE sensitized liposomes and ABA-Tyr-PE sensitized liposomes neither stimulated nor inhibited the anti-hapten responses. These results indicate that preparation of hybrid liposomes with different *N*-substituted PE derivatives provides an extremely convenient method for controlling hapten and/or immunologic carrier determinant density.**

We have recently described (1–3) humoral and cellular responses in guinea pigs immunized with liposomal model membranes. The liposomes contained a phosphatidylethanolamine derivative in which the amino group was conjugated with either a 2,4-dinitrophenyl-ε-aminocaproyl (Dnp-Cap) or a mono(*p*-azobenzenearsonic acid)tyrosyl (ABA-Tyr) residue. Anti-Dnp or anti-ABA antibodies were elicited, respectively, by Dnp-Cap-phosphatidylethanolamine (PE) or ABA-Tyr-PE sensitized liposomes. In contrast, no antibodies were produced by immunization with liposomes prepared in the presence of the corresponding deacylated water-soluble homologs, Dnp-Cap-glycerophosphorylethanolamine (GPE) or ABA-Tyr-GPE. Cell-mediated immunity was induced by ABA-Tyr-PE (but not Dnp-Cap-PE) sensitized liposomes.

Abbreviations: CFA, complete Freund's adjuvant; Dnp, 2,4-dinitrophenyl; ABA, *p*-azobenzenearsonyl; Dnp-Cap, 2,4-dinitrophenyl-ε-aminocaproic acid; ABA-Tyr, mono(*p*-azobenzenearsonic acid)tyrosine; PE, phosphatidylethanolamine; GPE, glycerophosphorylethanolamine. Dnp-Cap-PE, ABA-Tyr-PE, Dnp-Cap-GPE, and ABA-Tyr-GPE represent the corresponding amino(*N*)-substituted derivatives.

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The hapten determinants (i.e., the *N*-substituted PE derivatives) in liposomes are inserted *noncovalently* within a *nonimmunogenic* carrier (i.e., lipid bilayers of sphingomyelin, cholesterol, and dicetylphosphate; compare *Discussion*). These liposomes therefore differ in two important respects from conventional immunogens in which haptens are *covalently* attached to *immunogenic* carriers such as proteins or certain synthetic amino-acid polymers. Because of these differences, preparation of liposomes provides an extremely convenient method for controlling epitope density, molecular complexity of the determinants, and their chemical environment, since these parameters can be simply regulated by varying the composition of the lipid mixture used to generate the model membranes. The future potential of liposomal immunogens as tools in immunological research nevertheless depends on whether they can be shown to duplicate a variety of responses obtained with classical hapten-carrier conjugates.

In the present communication, we describe two additional features of liposomal immunogenicity that fulfill this goal. The first is the stimulation of an anti-hapten response by the simultaneous incorporation of immunogenic carrier determinants into the lipid bilayers. This was revealed by enhanced formation of anti-Dnp antibodies in guinea pigs immunized with hybrid liposomes containing both Dnp-Cap-PE and ABA-Tyr-PE. This is the liposome equivalent of the finding by Alkan *et al.* (4) that ABA-Tyr can serve as a carrier for Dnp when the two determinants are covalently attached via one or more aminocaproyl spacers. The second is the demonstration of hapten competition. Thus, the presence of Dnp-Cap-PE in hybrid liposomes inhibits the formation of anti-ABA antibodies. This is the analog of the phenomenon described by Amkraut *et al.* (5) that covalent linkage of Dnp groups to various immunogenic carriers could partially or completely suppress the rabbit antibody response to ABA when immunized with the doubly conjugated carriers. Stimulation and inhibition of these anti-hapten responses was only obtained with liposomes in which both Dnp-Cap and ABA-Tyr groups were confined to the same lipid bilayers. These results indicate that the determinants must be associated with a common carrier but that covalent bonding is not required.

DEFINITIONS

Dnp and Dnp-Cap derivatives of PE and GPE were originally synthesized for the purpose of sensitizing liposomal model membranes to immune damage by antibody-complement (6, 7). These studies have demonstrated that the amphipathic PE derivatives are oriented in lipid bilayers in a manner that permits interaction between the hapten and anti-Dnp antibodies. Thus, immune complexes formed on the membrane surface initiate the classical complement se-

quence; resulting in marker (glucose) release from the liposomes. In contrast, no marker release was obtained from liposomes prepared in the presence of the nonamphipathic GPE derivatives due to the fact that the latter are trapped in the aqueous regions between the lipid bilayers and hence not accessible to antibody. On this basis, we define the following types of liposomes used in this investigation: (*a*) *Homogeneous liposomes* are those prepared in the presence of only a single *N*-substituted PE derivative (e.g., either Dnp-Cap-PE or ABA-Tyr-PE) which is localized within the lipid bilayers. (*b*) *Hybrid liposomes* are those prepared in the presence of more than one *N*-substituted PE derivative (e.g., Dnp-Cap-PE and ABA-Tyr-PE) so that each derivative is localized within the lipid bilayers of the same liposomes. (*c*) *Heterogeneous liposomes* are those prepared in the presence of one *N*-substituted PE derivative and one *N*-substituted GPE derivative (e.g., Dnp-Cap-PE and ABA-Tyr-GPE, or Dnp-Cap-GPE and ABA-Tyr-PE); in this case, one derivative is localized in the aqueous compartments, and the other in the lipid bilayers, of the same liposomes. (*d*) *Mixed liposomes* are simply combinations of homogenous liposomes, each prepared with a different *N*-substituted PE derivative; in this case, the derivatives are localized within the lipid bilayers but not in the same liposomes.

EXPERIMENTAL PROCEDURES

Liposomes. Refs. 1–3 and 6 should be consulted for sources of materials and complete details regarding the preparation of actively sensitized multi-compartment liposomes which were used for immunization. Active sensitization means that the appropriate *N*-substituted derivative was present at the time of model membrane formation (6). This was accomplished by adding Dnp-Cap-PE and/or ABA-Tyr-PE to the basic lipid mixture containing sphingomyelin, cholesterol, and dicetylphosphate (2:1.5:0.2 molar ratios) before rotary evaporation; Dnp-Cap-GPE or ABA-Tyr-GPE were added directly to the swelling solution (145 mM NaCl–5 mM potassium phosphate, pH 7.2). The dried lipid film was dispersed in sufficient phosphate-buffered saline to yield a liposome suspension with a final sphingomyelin concentration of 25 mM. Under these conditions, not all of the GPE derivative is trapped within the aqueous liposomal compartments; approximately 65% remains in the swelling solution. In the present experiments, the untrapped portion was not removed.

Immunization. Homogeneous, hybrid, and heterogeneous liposome preparations were emulsified with an equal volume of complete Freund's adjuvant (CFA). When the guinea pigs were immunized with mixed liposomes, equal volumes of homogeneous liposomes (already emulsified with CFA) were mixed prior to injection. As before (1–3), each animal (white, random bred, females weighing approximately 300 g) received 100 μl per footpad of liposome-adjuvant combination. The dose of *N*-substituted derivative varied with the amount present when the liposomes were generated; the exact values are specified in the figures. Blood was withdrawn by cardiac puncture 21 days after immunization on the basis of previous results (3) indicating that guinea pigs immunized with homogeneous Dnp-Cap-PE sensitized liposomes show a peak in anti-Dnp antibody levels at this time. The sera were heated for 30 min at 56° and absorbed twice with one fifth their volume of washed packed sheep erythrocytes before hemagglutination assay.

Antibody Titration. Hemagglutination assay for anti-Dnp antibodies was performed as described previously (1) using

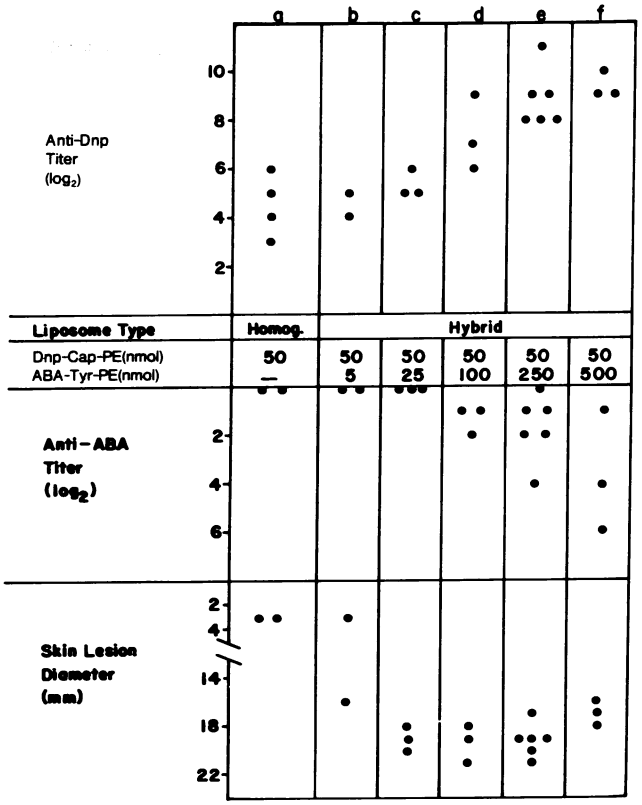
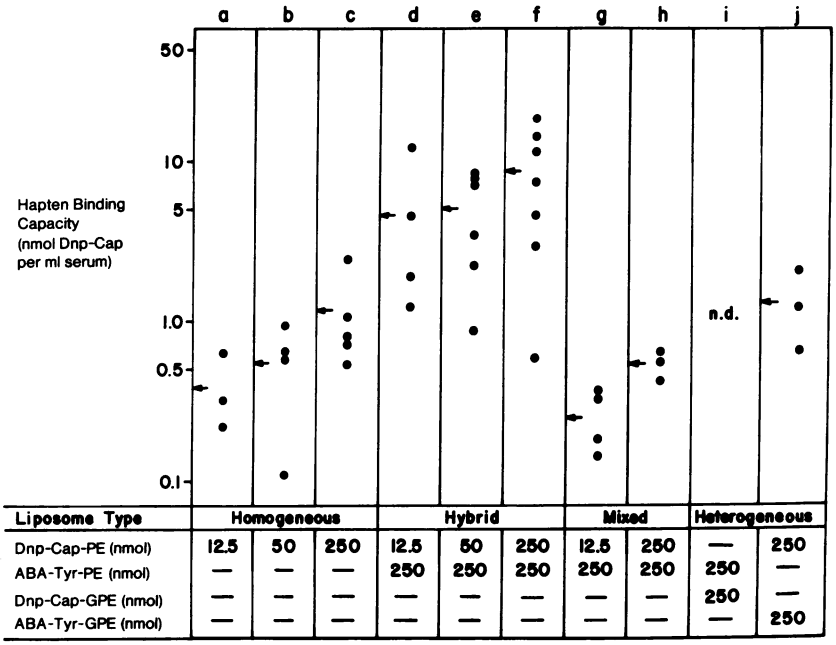


FIG. 1. Effect of ABA-Tyr-PE incorporation into hybrid liposomes on anti-Dnp and anti-ABA antibody titers, and delayed hypersensitivity reaction to ABA-Tyr-PE sensitized liposomes. Challenge with azobenzenearsonyl-bovine serum albumin also produced skin lesions of comparable size to those obtained with ABA-Tyr-PE sensitized liposomes (2); no animals revealed delayed reactions when challenged with unsensitized or Dnp-Cap-PE sensitized liposomes (results not shown).

sheep erythrocytes coated with 2,4,6-trinitrophenyl groups. Essentially, the same protocol was used to detect anti-ABA antibodies with target cells prepared as follows. Three milligrams of *p*-diazoniumbenzenearsonate (tetrafluoroborate salt) were dissolved in 0.5 ml of phosphate-buffered saline which was added dropwise to 3 ml of a 10% suspension of washed sheep erythrocytes in 100 mM NaCl–50 mM sodium carbonate, pH 9.5. This pH was maintained with 50 mN NaOH–150 mM NaCl during gentle stirring at 2° for 1 hr. After extensive washing with 75 mM NaCl–75 mM potassium phosphate, pH 7.2, the cells were suspended in this buffer (supplemented with 1% fetal calf serum) at a 1% concentration. Each point in the figures denotes the anti-Dnp or anti-ABA titer of serum from a single animal expressed as the logarithm (base 2) of the minimum dilution required for cell agglutination.

Dnp-Cap Binding Capacity of Serum. The procedure of Stupp *et al.* (8) was modified to determine binding of tritiated Dnp-Cap by Farr assay. Experimental tubes contained (in order of addition) 60 μl of phosphate-buffered saline, 20 μl of hapten solution, and 20 μl of immune serum. When derived from animals immunized with hybrid liposomes, the serum was diluted 2- to 5-fold with normal guinea pig serum. Control tubes were similar except that 20 μl of normal guinea pig serum was substituted for immune serum. For each serum tested, seven concentrations of Dnp-Cap were employed (6, 9, 14, 32, 61, 130, and 160 pmol/100 μl of assay mixture); initial input of radioactivity varied from 24,000 cpm (in tubes with 6–61 pmol) to 90,000 cpm (in



Liposome Type	Homogeneous			Hybrid			Mixed		Heterogeneous	
Dnp-Cap-PE (nmol)	12.5	50	250	12.5	50	250	12.5	250	—	250
ABA-Tyr-PE (nmol)	—	—	—	250	250	250	250	250	250	—
Dnp-Cap-GPE (nmol)	—	—	—	—	—	—	—	—	250	—
ABA-Tyr-GPE (nmol)	—	—	—	—	—	—	—	—	—	250

FIG. 2. Effect of homogeneous, hybrid, mixed, and heterogeneous liposomes on hapten binding capacity. Each point indicates Dnp-Cap binding by serum from an individual animal; the arithmetic mean of these values is shown by the arrows. (n.d. denotes that hapten binding could not be detected under the conditions of assay.)

tubes with 130 and 160 pmol). After 1 hr at room temperature, 200 μl of cold 66% saturated ammonium sulfate were added; following further incubation at 5° for 1 hr, the reaction mixture was centrifuged (2000 × *g* for 10 min). One hundred microliters of the supernatant solution were mixed with 10 ml of Aquasol (New England Nuclear; Boston Mass.) and counted in a Beckman, model L-230, liquid scintillation spectrometer.

Data reduction was performed with a Wang, series 600, computer equipped with graphical display (Wang Laboratories; Tewksbury, Mass.); the program was kindly provided by Dr. Craig M. Jackson, Department of Biological Chemistry, Washington University. After correction for dilution by ammonium sulfate, the excess of radioactivity in control over experimental supernatant solutions was used to calculate the concentration of Dnp-Cap bound specifically by anti-Dnp antibodies (B). Free hapten concentration (F) was determined by subtraction of (B) from the original concentration of Dnp-Cap in each tube. A Scatchard plot of (B)/(F) versus (B) was constructed and the total concentration of hapten binding sites was obtained by extrapolation of the curve to the abscissa. Results are expressed as nmol of Dnp-Cap bound per ml of undiluted serum.

Skin Testing. This was performed as described elsewhere (2) by intradermal injection into depilated back sites (after blood samples were collected) of 10 nmol of ABA-Tyr-PE in sonicated homogeneous liposomes. Each point in the figures denotes the average of the minimum and maximum diameter of the erythematous lesion on individual animals 24 hr after challenge. Lesions with diameters of 5 mm or less were not considered significant because induration was generally absent.

RESULTS

Effect of ABA-Tyr-PE incorporation into hybrid liposomes on anti-Dnp and anti-ABA antibody titers, and delayed hypersensitivity reaction (Fig. 1)

Hybrid liposomes containing 100 nmol of ABA-Tyr-PE and 50 nmol of Dnp-Cap-PE produced a significant increase in anti-Dnp titer over that observed in animals immunized with homogeneous liposomes containing 50 nmol of Dnp-Cap-PE (upper panel; column d versus column a). Stimulation of approximately 16-fold was obtained with hybrid liposomes that contained 250 nmol of ABA-Tyr-PE (column e). Incorporation of more ABA-Tyr-PE did not further enhance anti-Dnp titer (column f).

It should be emphasized that there was no quantitative correlation between the ability of ABA-Tyr-PE in hybrid liposomes to stimulate anti-Dnp titer and the capacity to confer cell-mediated immunity. Thus, immunization with hybrid liposomes containing 25 nmol of ABA-Tyr-PE (column c) did not increase anti-Dnp titer and yet gave maximal delayed reactions (lower panel; columns c–f). Also, less ABA-Tyr-PE was required for elicitation of the cellular than the humoral (anti-ABA antibody) response (lower and middle panels; columns b and c). However, as discussed below, this may be partly due to the fact that the amount of Dnp-Cap-PE incorporated in the hybrid liposomes did cause a slight inhibition of anti-ABA antibody production. Guinea pigs immunized with homogeneous Dnp-Cap-PE sensitized liposomes did not reveal a delayed reaction upon challenge with ABA-Tyr-PE sensitized liposomes (column a).

Effect of hybrid liposomes on hapten binding capacity

To confirm the preceding results, the total hapten (Dnp-Cap) binding capacity was measured in sera of guinea pigs immunized with homogeneous or hybrid liposomes (Fig. 2). With each of three different levels of Dnp-Cap-PE, incorporation of a constant amount of ABA-Tyr-PE increased binding capacity by approximately an order of magnitude (columns d versus a, e versus b, f versus c).

Effect of mixed and heterogeneous liposomes on hapten binding capacity

Fig. 2 further demonstrates that stimulation of anti-Dnp antibody formation is a unique property of hybrid liposomes; i.e., this phenomenon depends on the presence of ABA-Tyr determinants in the same lipid bilayers in which

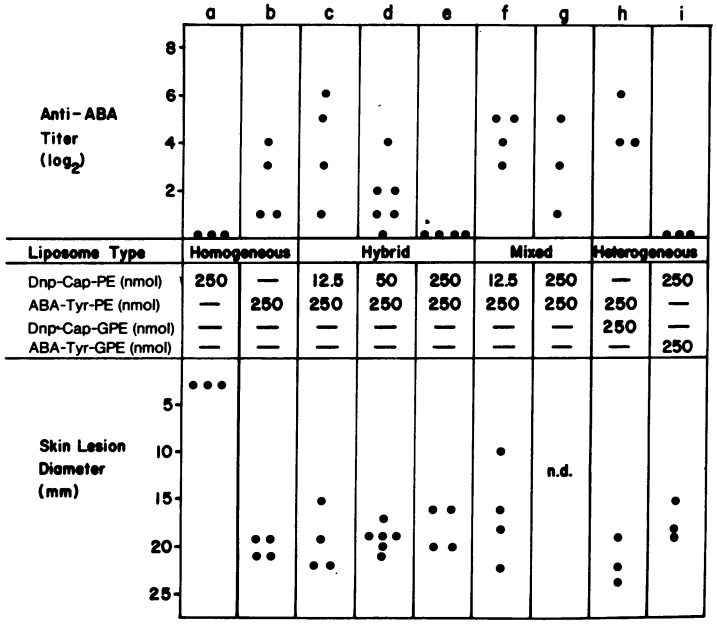


FIG. 3. Effect of homogeneous, hybrid, mixed, and heterogeneous liposomes on anti-ABA antibody titer, and delayed hypersensitivity reaction to ABA-Tyr-PE sensitized liposomes. (n.d. denotes that skin testing was not done.)

Dnp-Cap-PE is incorporated. Thus, immunization with mixed liposomes did not enhance the response produced by homogeneous Dnp-Cap-PE sensitized liposomes (columns g versus a, and h versus c); the presence of ABA-Tyr-GPE in heterogeneous liposomes containing Dnp-Cap-PE did not stimulate hapten binding capacity (column j versus c). As noted in the introduction, previous experiments (1) have shown that liposomes generated in the presence of Dnp-Cap-GPE (instead of Dnp-Cap-PE) are not able to induce a significant anti-Dnp antibody response in guinea pigs. This also applies to heterogeneous liposomes with ABA-Tyr-PE; even in Farr assay tubes containing the highest concentration of Dnp-Cap, hapten binding could not be detected ("n.d."; column i).

Effect of homogeneous, hybrid, mixed, and heterogeneous liposomes on anti-ABA antibody titer, and delayed hypersensitivity reaction

In the initial study (2) concerning the immunogenicity of ABA-Tyr-PE and ABA-Tyr-GPE, the ability of sera to initiate complement-dependent glucose release from ABA-Tyr-PE sensitized liposomes was used as the criterion for anti-ABA antibody formation. The experiments described in Fig. 3, which employ a hemagglutination assay (upper panel), confirm and extend our earlier observations. Thus, homogeneous ABA-Tyr-PE sensitized liposomes induced antibody production in guinea pigs (column b); no anti-ABA antibodies were detected after immunization with either homogeneous Dnp-Cap-PE sensitized liposomes (column a) or heterogeneous liposomes prepared with Dnp-Cap-PE and ABA-Tyr-GPE (column i). In contrast, both types of heterogeneous liposomes were as effective as the homogeneous ABA-Tyr-PE sensitized liposomes in conferring cellular immunity (lower panel; columns h and i versus b). ABA-Tyr-GPE (either free or incorporated into liposomes) has been shown previously (2) to resemble ABA-Tyr in its capacity to induce a cell-mediated response but not antibody formation.

When guinea pigs were immunized with hybrid liposomes, incorporation of various amounts of Dnp-Cap-PE did not influence the intensity of the delayed reaction (columns c–e versus b). However, in these same animals, a decline in anti-ABA titer was observed which was dependent on the quantity of Dnp-Cap-PE incorporated (columns c–e); 250 nmol of Dnp-Cap-PE consistently produced complete inhibition (column e). Most importantly, no inhibition was evident in guinea pigs immunized with either mixed liposomes (columns f and g versus b) or heterogeneous liposomes prepared with Dnp-Cap-GPE and ABA-Tyr-PE (column h versus b). These results therefore demonstrate that suppression of anti-ABA antibody formation is contingent on the presence of Dnp-Cap determinants in the same lipid bilayers in which ABA-Tyr-PE is incorporated.

DISCUSSION

Previous studies (1, 2) have shown that either free Dnp-Cap-PE or ABA-Tyr-PE can elicit hapten-specific antibodies when administered to guinea pigs in CFA although the humoral response is more pronounced upon prior incorporation of these compounds into liposomes prepared from a mixture of sphingomyelin, cholesterol, and dicetylphosphate. These earlier investigations have also demonstrated that sera from animals given unsensitized liposomes (i.e., not containing any *N*-substituted PE derivative) in CFA were unable to initiate complement-dependent glucose release from unsensitized liposomes, and challenge with unsensitized liposomes did not produce a delayed reaction. These results indicate that sphingomyelin–cholesterol–dicetylphosphate bilayers act as nonimmunogenic carriers.

ABA-Tyr is known to stimulate the effector T-cells (thymus-derived) involved in delayed hypersensitivity reactions (9). Alkan *et al.* (4, 10) have convincingly demonstrated that ABA-Tyr can function as a carrier for covalently attached haptens such as Dnp-Cap or poly(γ-D-glutamate). We have shown (2) that ABA-Tyr-PE sensitized liposomes can elicit cell-mediated immunity. Altogether, these observations suggested that noncovalent insertion of ABA-Tyr-PE into liposomes may convert the lipid bilayers into immunogenic carriers capable of stimulating helper T-cells.

This prediction was borne out by the present observations that immunization with hybrid liposomes (containing both

Dnp-Cap-PE and ABA-Tyr-PE) results in a significantly greater formation of anti-Dnp antibodies than does immunization with homogeneous liposomes (containing only Dnp-Cap-PE). Conversely, the noncovalent incorporation of Dnp-Cap-PE into hybrid liposomes partially or completely suppressed the guinea pig antibody response to ABA-Tyr-PE. This constitutes another indication that these lipid bilayers can be considered immunogenic carriers because Amkraut *et al.* (5) have previously shown similar intramolecular hapten competition when Dnp and ABA groups were simultaneously covalently bonded to T-cell-dependent immunogens such as keyhole limpet hemocyanin, sheep erythrocyte stroma, or bovine serum albumin.

That stimulation and inhibition of these anti-hapten responses are unique characteristics of hybrid liposomes is consistent with earlier data (1) indicating that liposomal structure can survive emulsification with CFA; if the model membranes had been extensively disrupted, immunization with hybrid and mixed liposomes should have produced the same response. Our results also bear on the persistent question of whether liposomal immunogenicity proceeds indirectly; e.g., as a consequence of an *in vivo* transpeptidation reaction by which the determinants (i.e., Dnp-Cap and ABA-Tyr) are removed from *N*-substituted derivatives and become covalently attached to host protein. Although such possibilities cannot be rigorously excluded, we regard them unlikely. Thus, transfer to endogenous carriers should also occur in animals immunized with mixed or heterogeneous liposomes, yet, under these conditions, enhancement of the anti-Dnp response was not observed. Indeed, the unlikelihood that transfer to proteins is a significant event was suggested initially by the fact that homogeneous Dnp-Cap-PE sensitized liposomes produced IgM and IgG_2 anti-Dnp antibodies that were considerably more restricted than those induced by either dinitrophenylated guinea pig albumin or ovalbumin (3). It remains to be determined if incorporation of ABA-Tyr-PE into hybrid liposomes results in the selective stimulation of either IgM or IgG_2 (or IgG_1) antibody formation, as well as a less restricted response.

We believe that this investigation should remove doubts that liposomes, which have been sensitized with *N*-substituted PE derivatives, can be used to study a variety of immune responses. Although differing significantly from conventional immunogens in which determinants are covalently attached to immunogenic carriers, they can nevertheless elicit many known features of humoral and cell-mediated phenomena that until now have been examined only with classical hapten-carrier conjugates. Liposomal immunogens should be further exploited to dissect the complex series of cellular events leading to an immune response.

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