Immunogenicity of Liposomal Model Membranes Sensitized with Mono(*p*-azobenzenearsonic acid)tyrosylphosphatidylethanolamine Derivatives. Antibody Formation and Delayed Hypersensitivity Reaction†

Robert A. Nicolotti and Stephen C. Kinsky*

ABSTRACT: We have previously reported that hapten specific antibodies are produced in guinea pigs immunized with certain N-substituted phosphatidylethanolamine derivatives (either free or incorporated into liposomal model membranes) in complete Freund's adjuvant. In this paper, we describe the synthesis of mono(*p*-azobenzenearsonic acid)tyrosylphosphatidylethanolamine (ABA-Tyr-PE). Immunization with this compound (either free or present in liposomes) not only results in the formation of anti-azobenzenearsonyl antibodies, but also confers cellular immunity as manifested by delayed hypersensitivity reactions elicited by challenge with either azobenzenearsonyl-bovine serum albumin or sensitized liposomes. Thus, ABA-Tyr-PE immunized guinea pigs differ from those immunized with azobenzenearsonyl-bovine serum albumin which produce antibodies but do not reveal a delayed reaction. Moreover, the ABA-Tyr-PE immunized animals differ from those immunized with mono(*p*-azobenzenearsonic acid)tyrosine; this substance has been shown by other investigators to confer cellular immunity without antibody formation in guinea pigs. However, the deacylated homolog of ABA-Tyr-PE (i.e., mono(*p*-azobenzenearsonic acid)tyrosylglycerophosphorylethanolamine) has the same immunological properties as mono(*p*-azobenzenearsonic acid)tyrosine. These observations justify the further exploitation of liposomal model membranes as novel immunogens that are able to elicit both cell and humoral mediated immune responses.

Immune damage of natural cell membranes mediated by the exclusively humoral factors, antibody and complement, can be mimicked with liposomal model membranes. Because the latter have proven useful in investigations concerned with the molecular basis of complement induced lysis (reviewed in Kinsky, 1975), an elusive goal of this laboratory has been the preparation of liposomes that might also serve as "targets" for cell mediated immune damage.

In attempts to devise such liposomes, we have been guided by the same considerations (reviewed in Kinsky, 1972) that originally led to the successful generation of liposomes sensitive to antibody–complement (Haxby et al., 1968). An obvious prerequisite is the incorporation into the liposomal bilayers of a compound whose antigenic determinant(s) would remain accessible to the effector cells. Thus, to render liposomes susceptible to humoral damage, it was necessary to prepare the model membranes in the presence of amphipathic antigens such as mammalian ceramides and bacterial lipopolysaccharides. Subsequent studies (Uemura and Kinsky, 1972; Six et al., 1973) demonstrated that these naturally occurring lipid antigens could be replaced by several synthetic homologous dinitrophenylated derivatives of phosphatidylethanolamine such as Dnp-PE and Dnp-Cap-PE.[1] These investigations confirmed the basic premise that the nonpolar regions served to anchor the effective compounds in the lipid bilayers in a manner that permitted the polar regions to interact with antibodies; this resulted in immune complexes on the liposomal surface that were capable of activating the classical complement sequence.

Examination of the literature did not reveal the existence of any simple natural lipid antigen that met the following requirements: (a) elicits a delayed hypersensitivity reaction; (b) can be conveniently purified in sufficient amounts; (c) has a known chemical structure; (d) possesses properties that would guarantee incorporation into liposomal bilayers as described above. The ability to provoke a delayed reaction was of prime importance because this is generally considered to be an in vivo correlate of a cell mediated response. An alternative approach was suggested by the recent finding that certain N-substituted phosphatidylethanolamine derivatives can function as antigens and that their immunogenicity is significantly enhanced by incorporation into liposomes prepared from sphingomyelin, cholesterol, and either dicetyl phosphate or stearylamine as charged amphiphile (Uemura et al., 1974). For example, anti-Dnp antibodies are produced in guinea pigs that have been immunized with Dnp-Cap-PE sensitized liposomes in complete Freund's adjuvant. However, convincing evidence for a delayed reaction in these animals was not obtained when they were skin tested with the same liposomes, free Dnp-Cap-PE, dinitrophenylated proteins, or dinitrofluorobenzene (unpublished experiments).

Accordingly, we have undertaken the preparation of an N-substituted phosphatidylethanolamine derivative that would confer a cell mediated response alone or after incor-

† From the Departments of Pharmocology and Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110. *Received January 22, 1975.* Supported by U.S. Public Health Service Grant AI-09319 and Training Grant GM-00096.

[1] The following designations have been used for N-substituted derivatives of phosphatidylethanolamine (PE) and glycerophosphorylethanolamine (GPE): Dnp, 2,4-dinitrophenyl; Dnp-Cap-, 2,4-dinitrophenylaminocaproyl; *N-t*-Boc-Tyr-, N^{α}-*tert*-butyloxycarbonyltyrosyl; ABA-Tyr-, mono(*p*-azobenzenearsonic acid)tyrosyl. The complete structures of these compounds are illustrated in Uemura and Kinsky (1972), Six et al. (1973), and this paper. Other abbreviations: ABA-BSA, bovine serum albumin conjugated with *p*-diazoniumbenzenearsonate; ABA-Tyr, mono(*p*-azobenzenearsonic acid)tyrosine; CFA, complete Freund's adjuvant.

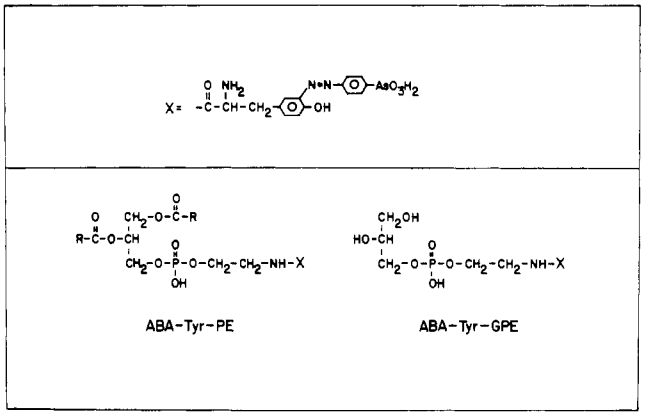

FIGURE 1: Structures of ABA-Tyr-PE and ABA-Tyr-GPE.

poration into liposomes. The present paper describes the results obtained with mono(*p*-azobenzenearsonic acid)tyrosylphosphatidylethanolamine whose structure is depicted in Figure 1. This compound was synthesized in view of the extensive studies (see, e.g., Leskowitz et al., 1966; Alkan et al., 1972; Becker et al., 1973; and references cited therein) which have demonstrated that immunization with mono(*p*-azobenzenearsonic acid)tyrosine, as well as certain related analogs, in CFA induces cellular immunity without antibody production in guinea pigs. We find that immunization with ABA-Tyr-PE or the sensitized liposomes in CFA not only provokes a strong delayed reaction but also formation of hapten specific antibodies. This difference between ABA-Tyr and ABA-Tyr-PE could be attributed to the amphipathic character of the latter compound because ABA-Tyr-GPE (the deacylated homolog of ABA-Tyr-PE; Figure 1) produced only a delayed reaction. These observations set the stage for further exploitation of liposomal model membranes as novel immunogens capable of eliciting both cell and humoral mediated immune responses.

Experimental Procedures

Preparation of Derivatives. *p*-Diazoniumbenzenearsonate (tetrafluoroborate salt) and ABA-Tyr were made by slight variation of established procedures (Wofsy et al., 1962; Koo and Cebra, 1974).

N-t-Boc-tyrosyl-PE, ABA-Tyr-PE, and ABA-Tyr-GPE were synthesized as described in the following paragraphs. Thin-layer chromatography on silica gel plates with fluorescent indicator (Brinkmann Instruments, Des Plaines, Ill.) was employed to determine the extent of the reaction (analytical plates: 0.25 mm thickness) and to isolate the desired compound (preparative plates: 2 mm thickness) using the solvent systems indicated below. Compounds were visualized by 260 nm uv light, iodine vapor, or ninhydrin spray. In the case of preparative chromatography, appropriate areas (ca. 8 mm^2) were removed and analyzed for total phosphate by minor modification of the Gerlach and Deuticke (1963) procedure. Spectra of *N-t*-Boc-Tyr-PE and ABA-Tyr-PE in acid were determined in a single phase mixture of chloroform–methanol–0.1 *N* HCl (1:2:0.8) and in alkali in an analogous system containing 0.1 *N* NaOH.

N-t-Boc-tyrosyl-PE. The reaction mixture in 38 ml of benzene–dioxane (1:1) contained 0.5 mmol of egg PE (Cyclo Chemical, Los Angeles, Calif.), 5 mmol of redistilled triethylamine, and 1 mmol of *N-t*-Boc-L-tyrosyl-*N*-hydroxysuccinimide ester (Bachem, Inc., Marina del Rey, Calif.). No ninhydrin positive spot was detected after incubation under a N_2 atmosphere for 2 hr at room temperature indicating complete substitution of the amino group on PE. The reaction mixture was taken to dryness by rotary evaporation at 40° and the residue redissolved in 15 ml of chloroform–methanol (1:1); 1-ml aliquots were chromatographed on preparative plates in chloroform–methanol–7 *N* NH_4OH (70:30:5). After migration of the solvent to the top, only one band (R_f of 0.54) was found that absorbed uv light and possessed significant amounts of phosphate. Pooled bands from three plates were transferred to a small column bed of Unisil (Clarkson Chemical, Williamsport, Pa.) that had been previously washed with chloroform–methanol (1:1). Approximately 250 ml of the same solvent was employed for elution; the eluate was dried under reduced pressure and the residue was redissolved in 40 ml of chloroform–methanol (1:1). If necessary, any silica gel fines passing through the Unisil bed were removed by centrifugation at 27,000 *g* for 30 min.

Phosphate determination indicated 80% yield (based on the amount of PE added) of a compound whose spectra showed a single peak at 278 nm in acid and at 294 nm in alkali. The peak for tyrosine in acid is at 274 nm with a molar extinction coefficient of 1340 and in alkali at 294 nm with a molar extinction coefficient of 2330 (Little and Donahue, 1968). Using these values, we calculated a tyrosine/phosphate ratio for the compound of 1.03. Identification of this material as *N-t*-Boc-tyrosyl-PE was confirmed by its complete conversion to tyrosyl-PE. Removal of the protective group by incubation in 98% formic acid for 3 hr at room temperature produced a new ninhydrin positive phospholipid with a R_f of 0.44 and a tyrosine/phosphate ratio of 1.15.

ABA-Tyr-PE. Solution A: *N-t*-Boc-Tyr-PE (0.2 mmol) was dissolved in 5 ml of a mixture containing 1 volume of 10 *mM* sodium borate in 1.5 *M* NaCl and 1 volume of 2-propanol; the solution was cooled and the pH adjusted to approximately 10. Solution B: *p*-Diazoniumbenzenearsonate (0.25 mmol) was dissolved in 2 ml of cold 10 m*M* sodium borate–1.5 *M* NaCl followed by 2 ml of cold 2-propanol. Solution B was slowly added with continuous stirring to solution A while maintaining the pH near 10.7 with 1 *N* NaOH–2-propanol (1:1). The reaction, which was characterized by the appearance of a deep yellow color, was terminated after 15 min by neutralization with cold 5 *N* HCl.

$CaCl_2$ (10 ml of a 100 m*M* solution), chloroform (10 ml), and methanol (5 ml) were then added to the reaction mixture because preliminary experiments indicated that the desired product could be more easily purified by thin-layer chromatography as the calcium salt. After thorough mixing, the lower phase (intensely yellow) was removed; the upper phase (slightly yellow) was extracted three times with 5-ml portions of chloroform. The combination of lower phase and chloroform extracts was taken to dryness under reduced pressure and the residue dissolved in 20 ml of chloroform–acetic acid (3:1). The protective group was cleaved by addition of 8 ml of redistilled boron trifluoride etherate (Eastman Chemicals, Rochester, N.Y.) using a modification of the procedure of Hiskey et al. (1971) for *N-t*-Boc peptides. Following incubation under a dry N_2 atmosphere for 20 min at room temperature, the reaction mixture was shaken with 20 ml of 1 *M* calcium acetate and 10 ml of methanol. The upper phase was extracted three times with 10-ml portions of chloroform and these extracts were combined with the lower phase. After removal of organic solvents and acetic acid by rotary evaporation and desiccation,

the residue was dissolved in 8 ml of chloroform–methanol (1:1).

One-milliliter aliquots were chromatographed on preparative plates in chloroform–methanol–8.7 *N* acetic acid (50:40:10). After migration of the solvent to the top, the major yellow band (R_f of 0.34), which also contained appreciable amounts of phosphate and gave a positive ninhydrin reaction, was scraped from the plates. ABA-Tyr-PE was isolated as described for *N-t*-Boc-Tyr-PE (see above) except that the Unisil bed was previously washed with a mixture of chloroform–methanol–25 m*M* sodium ethylenediaminetetraacetate (pH 7.5) (50:50:8); this mixture was also employed for elution. The chelating agent was present to reconvert ABA-Tyr-PE to the sodium salt and eliminate free Ca ions (the calcium salt could not be used in the preparation of liposomes). To assure complete removal of Ca ions, the combined eluates were diluted to 540 ml with the eluting mixture and 185 ml of 25 m*M* sodium ethylenediaminetetraacetate (pH 7.5) was added. The lower phase (250 ml) was collected and washed by shaking with methanol (250 ml) and H_2O (225 ml). The bottom layer, which contained all of the yellow compound, was taken to dryness and the residue dissolved in 25 ml of chloroform.

Phosphate determination indicated 30% yield (relative to the amount of *N-t*-Boc-Tyr-PE added) of a compound whose spectrum in alkali revealed two distinct peaks with maxima at 328 and 488 nm. In the characterization of ABA-Tyr-PE, we assumed a phosphate/arsonic acid ratio of 1 and therefore phosphate assays were corrected for color contributed by equimolar amounts of arsenate. The molar extinction coefficients on the basis of phosphate content were 13,200 and 9,800 at 328 and 488 nm, respectively. These values are in excellent agreement with those reported by Tabachnick and Sobotka (1959) for the reference compound, mono(*p*-azobenzenearsonic acid)chloroacetyltyrosine, in 0.1 *N* NaOH: 14,300 at 328 nm and 10,500 at 490 nm. Purity of ABA-Tyr-PE was also confirmed by the fact that it moved as a single yellow spot upon thin-layer chromatography in several solvent systems and no contaminants could be detected with either iodine vapor or ninhydrin spray.

ABA-Tyr-GPE. Synthesis of this derivative was accomplished by mild alkaline hydrolysis of ABA-Tyr-PE using a modification of the method described by Brokerhoff (1963). ABA-Tyr-PE (0.1 mmol) was suspended in 5 ml of methanol and 5 ml of 0.4 *N* LiOH in methanol was slowly added. Complete solubilization occurred after incubation for 60 min at room temperature with intermittent shaking. ABA-Tyr-GPE was isolated by the same steps employed earlier in the preparation of dinitrophenylglycerophosphorylethanolamine (Uemura and Kinsky, 1972); these involved conversion to the H^+ form by passage through an Amberlite IRC-50 column followed by organic solvent extraction to remove free fatty acids.

The yield was 85% of a compound that differed from ABA-Tyr-PE in being water soluble but whose spectrum in 0.1 *N* NaOH was similar to the latter under alkaline conditions (see above). In 20 m*M* potassium phosphate (pH 6.2), ABA-Tyr-GPE revealed a peak at 325 nm with a shoulder at 380 nm; in this regard, its absorption characteristics are identical with mono(*p*-azobenzenearsonic acid)chloroacetyltyrosine (Tabachnick and Sobotka, 1959). On the basis of phosphate content, we calculated a 325 nm molar extinction coefficient of 20,700 at pH 6.2; this is also in excellent agreement with the value of 22,200 reported for the reference compound.

ABA-BSA. The procedures of Gelewitz et al. (1954) were used in the preparation and characterization of this conjugate. The reaction mixture initially contained a 150-fold molar excess of *p*-diazoniumbenzenearsonate over protein (500 mg) in 70 ml of cold 100 m*M* NaCl–50 m*M* sodium borate (pH 9.2); this pH was maintained with 1 *N* NaOH during incubation for 4 hr. The product was lyophilized after extensive dialysis in the cold against 100 m*M* NaCl followed by distilled H_2O. Spectral analysis indicated 18.5 mol of azobenzenearsonyl groups/mol of protein.

Liposome Preparations. All experiments were performed with liposomes prepared from a basic lipid mixture containing sphingomyelin, cholesterol, and dicetyl phosphate (2:1.5:0.2 molar ratio) and sensitized under active conditions. As defined previously (cf. Uemura and Kinsky, 1972), active sensitization means that the N-substituted phosphatidylethanolamine derivative was present at the time of model membrane formation.

Undialyzed liposomes, sensitized with 10% ABA-Tyr-PE, were used for immunization. These were generated as described elsewhere for liposomes sensitized with Dnp-Cap-PE (Uemura et al., 1974, 1975) except that the dried lipid film was dispersed in 150 m*M* NaCl–5 m*M* potassium phosphate (pH 7.2) (phosphate-buffered saline) instead of 150 m*M* NaCl. When ABA-Tyr-PE was replaced by ABA-Tyr-GPE, the latter was dissolved in the swelling solution at a final ratio of 0.1 μmol/μmol of sphingomyelin.

Dialyzed liposomes, sensitized with 5% ABA-Tyr-PE, were employed in the spectrophotometric assay for antibody. These were prepared by the same procedure described previously for liposomes sensitized with Dnp-PE or Dnp-Cap-PE (Uemura and Kinsky, 1972; Six et al., 1973) except that the dried lipid film was dispersed in 290 m*M* glucose–5 m*M* Tris (pH 7.5) instead of 300 m*M* glucose.

Undialyzed liposomes, sensitized with 5% ABA-Tyr-PE, were used for skin testing. These were made similarly to the liposomes employed for antibody detection except that phosphate-buffered saline was the dispersing medium. After formation, the liposomal suspension was sonicated for 2 hr under a N_2 atmosphere and centrifuged to remove any lipid aggregate (cf. Six et al., 1974), and then diluted five-fold with phosphate-buffered saline.

Immunization.[2] Adult (ca. 300 g), white, female, random-bred guinea pigs were obtained from Eldridge Rabbitry, Antonia, Mo. As in earlier studies (Uemura et al., 1974, 1975), liposome suspensions (25 m*M* in sphingomyelin and 2.5 m*M* in ABA-Tyr-PE or ABA-Tyr-GPE) were mixed with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Each animal received 100 μl/footpad of liposome–adjuvant combination corresponding to a total dose of 0.5 μmol of ABA-Tyr-PE or ABA-Tyr-GPE. When the guinea pigs were immunized with the free derivatives or ABA-Tyr, the immunogens were prepared by mixing equal quantities of 2.5 m*M* solutions in phosphate-buffered saline with CFA. In the case of ABA-BSA, the animals received 500 μg (0.13 μmol of *p*-azobenzenearsonyl groups) in CFA.

[2] As before (Uemura et al., 1974), the shorthand notation used to specify the composition of various immunogen preparations indicates the antigen (e.g., N-substituted phosphatidylethanolamine derivative) in the "numerator" and if followed by (L) means that it was incorporated into liposomes under conditions of active sensitization; the adjuvant is indicated in the "denominator".

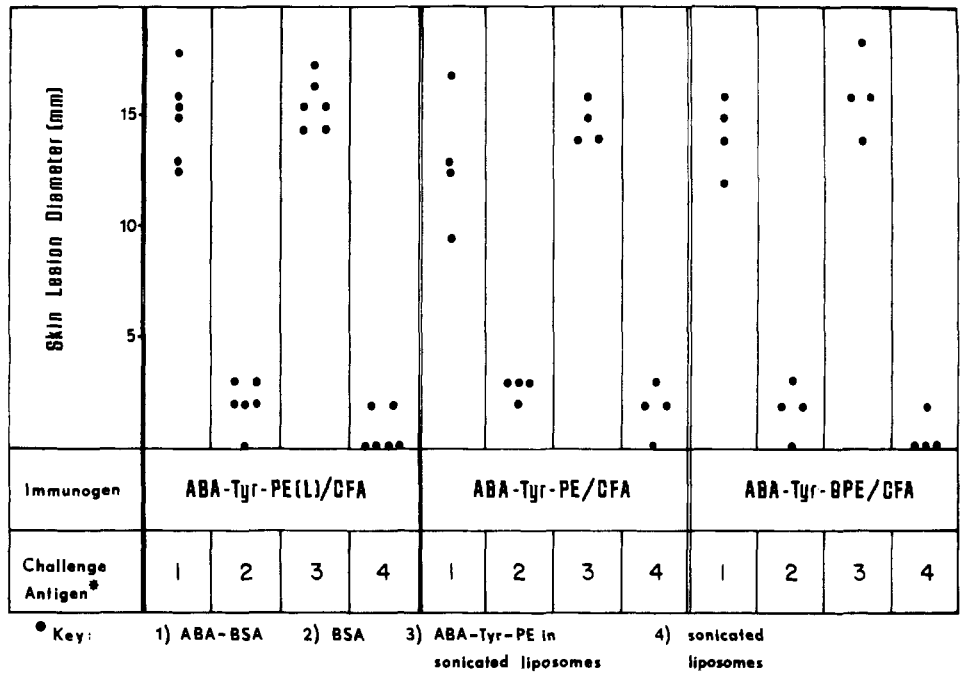

FIGURE 2: Delayed hypersensitivity reactions in guinea pigs immunized with ABA-Tyr-PE (either free or incorporated into liposomes) or ABA-Tyr-GPE. The antigens used to elicit the response are specified in the figure; see text for additional details.

Skin Testing. Fourteen to 18 days after immunization, depilated back sites were injected intradermally with 100 μl of phosphate-buffered saline solutions of ABA-BSA (50 μg of protein containing 13.5 nmol of *p*-azobenzenearsonyl groups) or sonicated sensitized liposomes (10 nmol of ABA-Tyr-PE). Control sites received corresponding amounts of BSA or unsensitized liposomes. Each point in the figures denotes the average of the minimum and maximum diameter of the erythematic lesion measured on individual animals 24 hr after challenge. Because of the absence of noticeable induration in lesions whose diameter was 5 mm or less, we have generally discounted their significance.

Antibody Detection. Blood was withdrawn by cardiac puncture a day before skin testing to minimize any possible booster effect of the challenge antigen. The presence of antibodies was determined by the ability of sera to initiate complement dependent glucose release from liposomes sensitized with ABA-Tyr-PE. This was measured by the same spectrophotometric assay used in all previous studies (see, e.g., Uemura and Kinsky, 1972; Six et al., 1973) except that Veronal-buffered saline was replaced by a solution of 150 m*M* NaCl, 0.5 m*M* $MgCl_2$, 0.15 m*M* $CaCl_2$, and 20 m*M* Tris (pH 7.5) (Six et al., 1974). Control cuvets contained either 5 μl of unsensitized liposomes or 125 μl of guinea pig serum that had been heated at 56° for 30 min to destroy hemolytic complement activity. On the basis of past experience, glucose release less than 5% over controls was usually not considered significant.

Results

Immunogenicity of ABA-Tyr-PE(L) and ABA-Tyr-PE. Figure 2 (left and middle panels) shows that guinea pigs, which had been immunized with ABA-Tyr-PE sensitized liposomes or free ABA-Tyr-PE and subsequently skin tested with ABA-BSA, manifest a strong delayed hypersensitivity reaction (columns 1). Most importantly, with either immunogen, a response of similar magnitude could be elicited by sensitized sonicated liposomes in place of ABA-BSA (compare columns 3 with columns 1). We have also noted marked central induration at sites that received intradermal injections of multicompartment (unsonicated) sensitized liposomes or free ABA-Tyr-PE although, in these instances, the diameter of the erythematic areas was significantly smaller (approximately 10 and 7 mm, respectively). This is consistent with previous experiments showing that sonication of liposomes results in a greater accessibility of the antigenic determinants (Six et al., 1974). Specificity for azobenzenearsonyl groups was indicated by the fact that neither BSA nor unsensitized liposomes could provoke the delayed reaction (compare columns 2 with columns 1, and columns 4 with columns 3).

Figure 3 (right panel) shows that immunization with control liposomes, which contain PE substituted for ABA-Tyr-PE, does not induce a cell mediated response. No significant delayed reaction was obtained with ABA-BSA (middle panel), but was observed in animals that had been immunized with ABA-Tyr (left panel). These results are in agreement with the earlier observations of Leskowitz et al. (1966), Alkan et al. (1972), and Becker et al. (1973) (and references cited therein). Degrand and Raynaud (1973) have subsequently confirmed the fact that induction of cellular immunity by ABA-Tyr requires administration of the antigen in complete Freund's adjuvant. In the present investigation, we have also found that ABA-Tyr-PE sensitized liposomes are not effective immunogens when given in incomplete adjuvant (results not shown).

Although immunization of guinea pigs with ABA-BSA in complete Freund's adjuvant does not confer an ABA-Tyr specific cell mediated response, it is known to result in the production of anti-azobenzenearsonyl antibodies. Table I shows that sera from such animals promote glucose release from liposomes sensitized with ABA-Tyr-PE in the presence of native (but not heated) guinea pig serum as complement source. Complement dependent marker release was also observed with sera from animals immunized with the sensitized liposomes and, to a lesser extent, with sera from

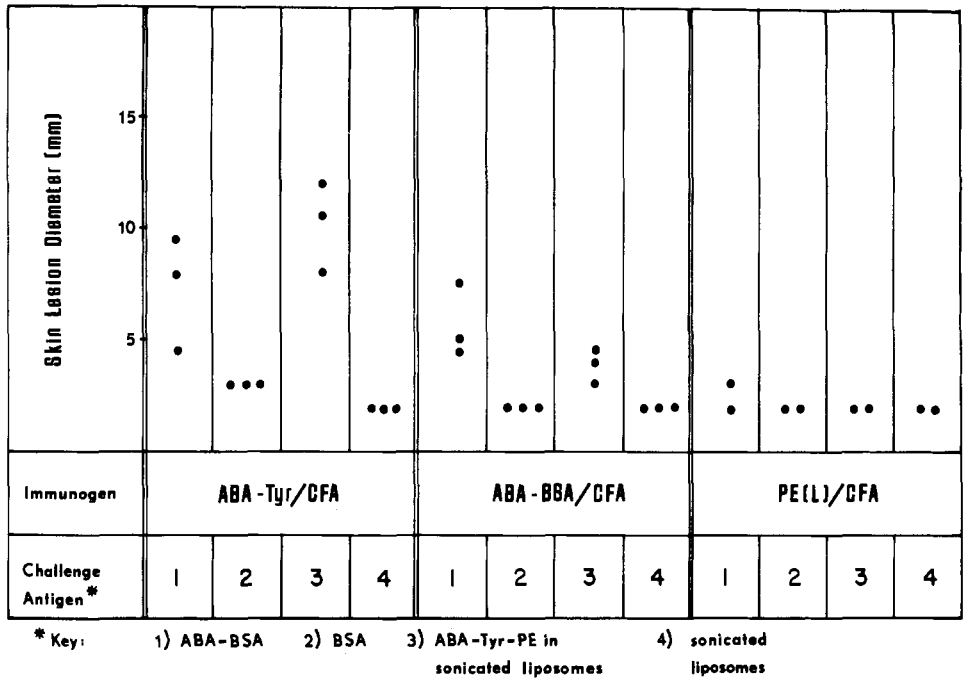

FIGURE 3: Delayed hypersensitivity reactions in guinea pigs immunized with ABA-Tyr, ABA-BSA, or control liposomes. The antigens used to elicit the response are specified in the figure; see text for additional details.

Table I: Presence of Anti-azobenzenearsonyl Antibodies in Guinea Pigs Immunized with ABA-BSA or ABA-Tyr-PE (Either Free or Incorporated into Liposomes).[a]

Sera from Guinea Pigs Immunized with	% Glucose Released in 30 min in Presence of	
	Native GPS	Heated GPS
ABA-BSA/CFA	57.5	0
	44.8	1.5
	37.0	2.4
ABA-Tyr-PE(L)/CFA	56.9	0
	44.7	2.7
	44.5	0
	44.2	2.9
	43.2	1.9
	37.8	0
	29.5	0
	19.7	0
ABA-Tyr-PE/CFA	19.7	0
	19.0	1.8
	12.0	2.0
	7.1	0.6
	5.6	2.5
	2.8	0

[a] Serum (decomplemented by incubation at 56° for 30 min) was obtained from individual animals that had been immunized as indicated in the table. Each serum sample (100 μl) was assayed for its ability to initiate glucose release from ABA-Tyr-PE sensitized liposomes (5 μl) in the presence of native, i. e., unheated, guinea pig serum (125 μl) as source of complement; the latter was derived from nonimmunized animals. Control cuvets were identical except that the complement source was heated as described above.

Table II: Complement Dependent Glucose Release from Sensitized and Unsensitized Liposomes.[a]

Serum No.	% Glucose Released in 30 min from		
	Sensitized L plus Native GPS	Unsensitized L plus Native GPS	Sensitized L plus Heated GPS
1–8	40.1 ± 11.2	2.6 ± 3.1	0.9 ± 1.3
9	61.6	40.8	0
10	48.5	33.4	0
11	58.2	15.9	0

[a] Serum (decomplemented by incubation at 56° for 30 min) was obtained from animals that had been immunized with ABA-Tyr-PE (L)/CFA. The assays were performed as described in the legend to Table I and included cuvets containing unsensitized liposomes (no ABA-Tyr-PE) prepared from the basic lipid mixture of sphingomyelin, cholesterol, and dicetyl phosphate. Row 1 is mean ± standard deviation for the eight sera listed individually in Table I. See text for further discussion.

guinea pigs that had been administered free ABA-Tyr-PE. None of these sera produced glucose release from liposomes prepared in the absence of ABA-Tyr-PE. However, it should be noted (Table II) that, in the case of three animals immunized with ABA-Tyr-PE(L)/CFA, we did obtain significant marker loss from unsensitized liposomes although the extent of glucose release was still greater from sensitized liposomes. Because the basis of this phenomenon is not yet understood, these sera were not employed in preliminary experiments designed to characterize the antibodies produced as a consequence of liposomal immunization.

Figure 4 illustrates the dependence of glucose release on the concentration of antibodies precipitated by ammonium sulfate from pooled sera derived from animals that had been immunized with ABA-Tyr-PE(L)/CFA. A comparable percentage of marker loss was observed with smaller amounts of an analogous fraction obtained from ABA-BSA/CFA immunized guinea pigs, suggesting that the protein was a more potent immunogen for antibody formation than the liposomes.

Table III summarizes the ability of various materials to inhibit complement dependent glucose release. Of particular significance is the fact that arsanilic acid, as well as the substances not substituted by azobenzenearsonyl groups, was ineffective. These results indicate that the specificity of

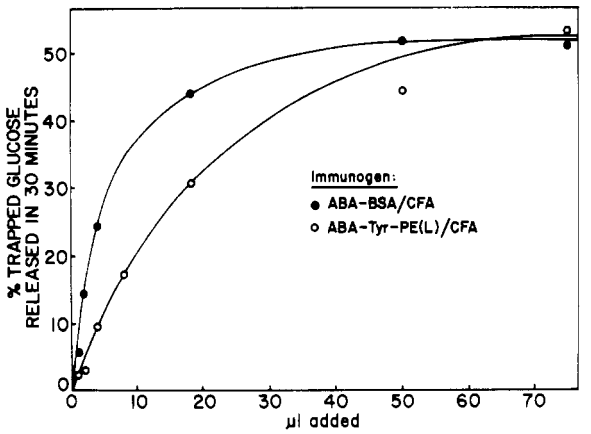

FIGURE 4: Ability of immune sera to initiate complement dependent glucose release from liposomes sensitized with ABA-Tyr-PE. Pooled sera from animals immunized with either ABA-BSA/CFA or ABA-Tyr-PE(L)/CFA were mixed with an equal volume of saturated ammonium sulfate at pH 7.8. The resulting precipitate was recovered by centrifugation at 3000*g* for 30 min at 2°, dissolved in a small volume of 150 m*M* NaCl–5 m*M* Tris (pH 7.8) and extensively dialyzed against the same buffer in the cold. Before assay, the dialyzed protein solutions were adjusted to give the same absorbancy at 280 nm (A = 0.365 for a 1:100 dilution in Tris-saline).

Table III: Hapten Inhibition of Glucose Release from ABA-Tyr-PE Sensitized Liposomes.[a]

Addition	% Inhibition
ABA-Tyr-GPE	37.8
GPE	0.8
ABA-TYR	25.6
Arsanilic acid	2.6
ABA-BSA	48.3
BSA	4.2

[a] Pooled sera from animals immunized with ABA-Tyr-PE(L)/CFA (60 µl of the ammonium sulfate fraction prepared as described in the legend to Figure 4) were incubated with 40 µl of the materials indicated in the table. Solutions of the latter were made in 150 m*M* NaCl–10 m*M* Tris (pH 7.5) and contained 1.25 m*M* arsanyl groups or an equivalent quantity of unsubstituted derivative. After 30 min at room temperature, the remaining components (270 µl of complement buffer, 500 µl of assay reagent, 125 µl of native guinea pig serum, and 5 µl of sensitized liposomes) were added to the assay cuvets. Percent inhibition was calculated relative to control cuvets (no addition) which showed an average of 52% glucose release in 30 min (see Figure 4).

Table IV: Absence of Anti-azobenzenearsonyl Antibodies in Guinea Pigs Immunized with ABA-Tyr-GPE (Either Free or Incorporated into Liposomes), ABA-Tyr, or Control Liposomes.[a]

Sera from Guinea Pigs Immunized with	% Glucose Released in 30 min in Presence of	
	Native GPS	Heated GPS
ABA-Tyr-GPE(L)/CFA	1.0	1.4
	0.1	1.4
	1.5	1.2
ABA-Tyr-GPE/CFA	2.3	2.3
	1.1	0.3
	0.9	0.4
	0.5	0.3
	0.1	1.3
	1.9	1.5
	0.2	0.9
ABA-Tyr/CFA	3.7	2.4
	3.5	0
	0	0
PE(L)/CFA	0.4	0
	0	0
	0	0

[a] See legend to Table I.

the antibodies produced by liposomal immunization includes the azo linkage to tyrosine. It is also noteworthy that final hapten concentrations of 50 µ*M* did not cause complete inhibition, suggesting that the antibodies formed approximately 2 weeks after immunization with liposomes have a relatively low affinity. Higher hapten concentrations could not be used in the spectrophotometric assay due to their appreciable absorbance at the wavelength (340 nm) at which glucose release was measured.

Immunogenicity of ABA-Tyr-GPE(L) and ABA-Tyr-GPE. The previous experiments indicate a marked difference in the response of guinea pigs to ABA-Tyr-PE and ABA-Tyr. Whereas both immunogens confer a cell mediated response, ABA-Tyr-PE (either free or incorporated into liposomes) also results in the formation of anti-azobenzenearsonyl antibodies. However, the immunological properties of the deacylated homolog, ABA-Tyr-GPE, resemble those of ABA-Tyr. Table IV shows that immunization with ABA-Tyr-GPE (either free or contained within liposomes) does not lead to the production of antibodies at least in amounts that could be detected within the sensitivity of the spectrophotometric assay. This is consistent with the finding that sera from animals immunized with either ABA-Tyr or control liposomes are unable to initiate significant release of glucose marker from ABA-Tyr-PE sensitized liposomes in the presence of complement.

Figure 2 shows that immunization with free ABA-Tyr-GPE confers a strong cell mediated response equivalent in intensity to that observed in animals immunized with ABA-Tyr-PE(L) or ABA-Tyr-PE. Although not indicated, a response of comparable magnitude was also detected in guinea pigs that had been immunized with liposomes prepared in the presence of ABA-Tyr-GPE. In addition, we have found that free ABA-Tyr-GPE resembles ABA-Tyr in its poor effectiveness as a challenge antigen (results not shown); the latter phenomenon has been attributed by Nauciel and Raynaud (1971) to the extremely rapid clearance of ABA-Tyr from the sites of intradermal injection.

Discussion

The present experiments confirm and extend our initial studies on the ability of N-substituted phosphatidylethanolamine derivatives to function as antigens that result in the formation of specific antibodies. Thus, anti-dinitrophenyl antibodies are produced by guinea pigs immunized with Dnp-Cap-PE in complete Freund's adjuvant and the immunogenicity of Dnp-Cap-PE is markedly enhanced by incorporation into sphingomyelin–cholesterol–dicetyl phosphate liposomes (Uemura et al., 1974). ABA-Tyr-PE behaves similarly; sera from animals immunized with the sensitized liposomes contain more anti-azobenzenearsonyl antibodies than sera from guinea pigs that had received free ABA-Tyr-PE (Table I).

Earlier experiments also suggest that liposomal immunogenicity is contingent on the localization of the appropriate derivative in the lipid bilayers of the model membranes and not in the aqueous compartments. Thus, comparable amounts of anti-dinitrophenyl antibodies are formed after immunization with liposomes sensitized by either of the amphipathic homologs, Dnp-Cap-PE or Dnp-Cap-lysoPE; no

antibodies are detected upon immunization with liposomes prepared in the presence of Dnp-Cap-GPE (Uemura et al., 1974). Similarly, anti-azobenzenearsonyl antibodies are found in the sera of animals immunized with ABA-Tyr-PE sensitized liposomes but none when the liposomes contain ABA-Tyr-GPE (compare Tables I and IV). We have reported elsewhere that the guinea pig IgM and IgG anti-dinitrophenyl antibodies produced as a consequence of liposomal immunization are considerably more restricted than the antibodies obtained after immunization with dinitrophenylated proteins (Uemura et al., 1975). Whether an analogous difference is displayed by the anti-azobenzenearsonyl antibodies elicited by sensitized liposomes as compared to ABA-BSA is currently under investigation.

Indeed, the fact that ABA-Tyr-PE (either free or incorporated into liposomes) induces antibodies at all was unanticipated. Our original expectation (see introduction) was that this N-substituted phosphatidylethanolamine derivative would resemble ABA-Tyr as an antigen capable of bestowing a pure cell mediated response in guinea pigs.[3] This anomalous behavior can be attributed to the amphipathic character of ABA-Tyr-PE because ABA-Tyr-GPE, which is similar to ABA-Tyr in being water soluble, confers only cellular immunity without antibody production (Figure 2 and Table IV).

These findings have the following significance in view of the goal that prompted this investigation. (a) ABA-Tyr-PE can be incorporated into stable liposomes containing a trapped marker because they can serve as targets for humoral mediated (i.e., antibody-complement) immune damage (see, e.g., Figure 4). (b) The possibility that such model membranes, in which the antigenic determinants are exposed, might also function as targets for cell mediated immune damage is suggested by the fact that they can elicit a delayed hypersensitivity reaction in appropriately immunized animals (see, e.g. Figure 2). (c) Effector cells to be used for studying cell mediated antibody dependent membrane damage should be derived from guinea pigs immunized with either free ABA-Tyr-PE or sensitized liposomes. (d) Animals immunized with ABA-Tyr-GPE should be the source of effector cells for examining cell mediated antibody independent membrane damage. Experiments based on the preceding considerations are now in progress.

[3] Recent experiments by Levin et al. (1974) have shown that rabbits also do not produce significant amounts of anti-azobenzenearsonyl antibodies when immunized with ABA-Tyr. However, using a sensitive phage inactivation assay, they were able to detect antibodies in rabbits that had been immunized with ABA-Tyr covalently attached to small oligotyrosine carriers (two to four amino acid residues).

Acknowledgments

We are indebted to Constance B. Kinsky and Dr. Naohisa Kochibe for their assistance with some of these experiments, and to Drs. Kochibe, Craig M. Jackson, Fred Dombrose, and Joseph M. Davie for invaluable advice.

References

Alkan, S. S., Williams, E. B., Nitecki, D. E., and Goodman, J. W. (1972), *J. Exp. Med. 135,* 1228.

Becker, M. J., Levin, H., and Sela, M. (1973), *Eur. J. Immunol. 3,* 131.

Brokerhoff, H. (1963), *J. Lipid Res. 4,* 96.

Degrand, F., and Raynaud, M. (1973), *Eur. J. Immunol. 3,* 660.

Gelewitz, E. W., Riedeman, W. L., and Klotz, I. M. (1954), *Arch. Biochem. Biophys. 53,* 411.

Gerlach, E., and Deuticke, B. (1963), *Biochem. Z. 337,* 477.

Haxby, J. A., Kinsky, C. B., and Kinsky, S. C. (1968), *Proc. Natl. Acad. Sci. U.S.A. 61,* 300.

Hiskey, R. G., Beacham, L. M., Matl, V. G., Smith, J. N., Williams, E. B., Jr., Thomas, A. M., and Wolters, E. T. (1971), *J. Org. Chem. 36,* 488.

Kinsky, S. C. (1972), *Biochim. Biophys. Acta 265,* 1.

Kinsky, S. C. (1975), in Cold Spring Harbor Symposium on Proteases and Biological Control (in press).

Koo, P. H., and Cebra, J. J. (1974), *Biochemistry 13,* 184.

Leskowitz, S., Jones, V. E., and Zak, S. J. (1966), *J. Exp. Med. 123,* 229

Levin, H., Becker, M., and Sela, M. (1974), *Eur. J. Biochem. 44,* 271.

Little, J. R., and Donahue, H. (1968), *Methods Immunol. Immunochem. 2,* 343.

Nauciel, C., and Raynaud, M. (1971), *Eur. J. Immunol. 1,* 257.

Six, H. R., Uemura, K., and Kinsky, S. C. (1973), *Biochemistry 12,* 4003.

Six, H. R., Young, W. W., Jr., Uemura, K., and Kinsky, S. C. (1974), *Biochemistry 13,* 4050.

Tabachnick, M., and Sobotka, H. (1959), *J. Biol. Chem. 234,* 1726.

Uemura, K., Claflin, J. L., Davie, J. M., and Kinsky, S. C. (1975), *J. Immunol 114,* 958.

Uemura, K., and Kinsky, S. C. (1972), *Biochemistry 11,* 4085.

Uemura, K., Nicolotti, R. A., Six, H. R., and Kinsky, S. C. (1974), *Biochemistry 13,* 1572.

Wofsy, L., Metzger, H., and Singer, S. J. (1962), *Biochemistry 1,* 1031.