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Immunology

Immunogenicity of liposomal model membranes in mice: Dependence on phospholipid composition

(phosphatidylcholine/sphingomyelin/transition temperature/lipid A)

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ABSTRACT **This investigation demonstrated that antigenic expression in liposomal model membranes may be markedly influenced by phospholipid composition. Incorporation of dinitrophenylaminocaproylphosphatidylethanolamine (Dnp-Cap-PE) into egg lecithin/cholesterol/dicetylphosphate bilayers did not significantly enhance the response of AKR mice to this synthetic amphipathic antigen. In contrast, the immunogenicity of Dnp-Cap-PE was increased (as measured by either plaque-forming cell frequency or hemagglutination titer) after its insertion into liposomes prepared with beef sphingomyelin instead of egg lecithin. We also show that the response to the Dnp-Cap determinant can be stimulated by the presence of lipid A in the same bilayers without altering the relative immunogenic potency of the sphingomyelin and lecithin liposomes; similarly, incorporation of this mitogen into sphingomyelin liposomes produced a greater polyclonal (nonspecific) response. The response to Dnp-Cap-PE-sensitized liposomes (with or without lipid A) prepared with a series of synthetic phosphatidylcholines (distearoyl-, dipalmitoyl-, dimyristoyl-, dilauroyl-, dioleoyl-) suggests a direct correlation between liposomal immunogenicity and transition temperature of the phospholipid.**

Dinitrophenyl - ϵ - aminocaproylphosphatidylethanolamine (Dnp-Cap-PE) and mono(*p*-azobenzenearsonic acid)tyrosyl-phosphatidylethanolamine (ABA-Tyr-PE) are prototypes of a novel class of synthetic lipid antigens (1–3). When administered to guinea pigs in complete Freund's adjuvant, these *N*-substituted PE derivatives induce formation of hapten-specific antibodies and, in the case of ABA-Tyr-PE, delayed hypersensitivity reactions. The ability to elicit antibodies can be further enhanced by prior incorporation of either Dnp-Cap-PE or ABA-Tyr-PE into liposomal model membranes containing beef sphingomyelin, cholesterol, and dicetylphosphate. These liposomes therefore provide a unique opportunity to study the dependence of a humoral response on the chemical environment in which a hapten is situated because the PE derivatives are inserted noncovalently into lipid bilayers which function as the carrier (4, 5).

Preliminary experiments in guinea pigs revealed that Dnp-Cap-PE-sensitized liposomes prepared with egg lecithin instead of beef sphingomyelin did not induce a greater antibody response than did free Dnp-Cap-PE (1). This observation suggested a pronounced influence of phospholipid composition on liposomal immunogenicity and, in the present investigation, we have undertaken a more extensive examination of this phenomenon in mice.

Abbreviations: PE, phosphatidylethanolamine; Dnp-Cap, 2,4-dinitrophenyl-ϵ-aminocaproic acid; ABA-Tyr, mono(*p*-azobenzenearsonic acid)tyrosine; Dnp-Cap-PE, ABA-Tyr-PE, derivatives of PE in which the amino group has been substituted with a Dnp-Cap or ABA-Tyr residue; PFC, plaque-forming cells; SRBC, sheep erythrocytes; Tnp-SRBC, sheep erythrocytes sensitized with 2,4,6-trinitrophenyl residues.

MATERIALS AND METHODS

Liposomes were generated by essentially the procedure described elsewhere (1, 2); the sources of all materials except the synthetic phosphatidylcholines were as in those papers. The phosphatidylcholines were either purchased (Applied Science Labs., State College, Pa.; Serdary Research Labs., London, Ont., Canada) or donated (Dr. Craig M. Jackson, Department of Biological Chemistry, Washington University). A chloroform solution of the appropriate phospholipid, cholesterol, dicetylphosphate, and Dnp-Cap-PE (in molar ratios of 2:1.5:0.2:0.1, respectively) was added to a small conical flask; when required, this mixture was supplemented with 0.8 nmol of lipid A phosphate per μmol of phospholipid (lipid A was derived from *Salmonella paratyphi* lipopolysaccharide which was a generous gift of Dr. Naohisa Kochibe, Gunma University, Japan; 1 μg of lipid A contains approximately 0.7 nmol of phosphate). After removal of the chloroform, the dried mixture was suspended by vortex agitation in sufficient phosphate-buffered saline (5 mM potassium phosphate/145 mM NaCl, pH 7.2) to give a concentration of 25 mM phospholipid and then diluted with an equal volume of this buffer.

Experimental groups consisted of at least five AKR male mice 6–9 weeks old (obtained from National Laboratory Animal Co., O'Fallon, Mo.). Each animal was immunized intraperitoneally with 200 μl of the diluted liposomal preparation containing 125 nmol of Dnp-Cap-PE and, if indicated, 2 nmol of lipid A phosphate (or the same amounts of free Dnp-Cap-PE and lipid A suspended in phosphate-buffered saline). Four days later, the number of antibody-secreting cells in individual spleen cell suspensions was determined by a modification of the plaque-forming cell (PFC) assay with untreated sheep erythrocytes (SRBC) or trinitrophenylated erythrocytes (Tnp-SRBC) as target cells to measure the nonspecific and Dnp-Cap-specific responses, respectively (6). Results are expressed as either PFC per 10^6 cells or per spleen (arithmetic mean ±SEM). Only direct PFC counts have been recorded because indirect PFC counts rarely amounted to more than 10% of direct PFC counts. Anti-Dnp-Cap antibody titer of serum (collected at the time of sacrifice) was determined by hemagglutination assay as previously described (1) except that the target cells (Tnp-SRBC) were added at a 4-fold lower concentration. Results are expressed as the geometric mean (to the log base 2) ±SEM.

RESULTS

Table 1 shows the results of an experiment in which mice were immunized with various preparations that did not contain lipid A. Egg lecithin liposomes were only 2-fold better immunogens than free Dnp-Cap-PE with PFC frequency (per 10^6 cells or per spleen) as the criterion and were not at all better on the basis of hemagglutination titer. In contrast, prior incorporation of

Table 1. Immune response to free Dnp-Cap-PE and Dnp-Cap-PE-sensitized egg lecithin or beef sphingomyelin liposomes (without lipid A)

Immunogen preparation	(Tnp-SRBC) PFC* /10^6 cells‡	(Tnp-SRBC) PFC* /spleen‡	(SRBC) PFC* /spleen‡	Titer (log_2)†
Saline alone	8 ±2	732 ±130	72 ±12	0
Free Dnp-Cap-PE	18 ±4	1,576 ±541	148 ±74	0.4 ±0.2
Dnp-Cap-PE in egg PC liposomes	37 ±10	3,408 ±1,086	150 ±43	0.4 ±0.2
Dnp-Cap-PE in beef SM liposomes	268 ±31	35,078 ±7,469	130 ±24	3.0 ±0.7

* (Tnp-SRBC) and (SRBC) indicates that the PFC assay was performed with trinitrophenylated or unsensitized SRBC, respectively. PC = phosphatidylcholine; SM = sphingomyelin.
† Geometric mean ±SEM.
‡ Arithmetic mean ±SEM.

Table 2. Immune response to free Dnp-Cap-PE and Dnp-Cap-PE-sensitized egg lecithin or beef sphingomyelin liposomes (with lipid A)

Immunogen preparation	(Tnp-SRBC) PFC /10^6 cells	(Tnp-SRBC) PFC /spleen	(SRBC) PFC /spleen	Titer (log_2)
Lipid A alone	7 ±2	738 ±312	132 ±20	0
Free Dnp-Cap-PE	63 ±13	9,345 ±2,329	660 ±398	4.5 ±0.3
Dnp-Cap-PE in egg PC liposomes	288 ±62	52,448 ±10,845	1,220 ±521	6.0 ±0.4
Dnp-Cap-PE in beef SM liposomes	2,031 ±466	318,600 ±55,227	5,040 ±1,895	7.5 ±0.3

Abbreviations and calculations as in Table 1.

Dnp-Cap-PE into beef sphingomyelin/cholesterol/dicetylphosphate liposomes led to a 6-fold greater response (in comparison to that induced by the free derivative) on the basis of antibody titer, 15-fold on the basis of PFC per 10^6 cells, and 22-fold on the basis of PFC per spleen. Therefore, mice, like guinea pigs, produce significantly more anti-Dnp-Cap antibodies when the liposomal immunogens are made with beef sphingomyelin than with egg lecithin. The specificity of these responses for the Dnp-Cap determinant is indicated by the fact that PFC per spleen (and also PFC per 10^6 cells; not shown) were at least an order of magnitude less when SRBC replaced Tnp-SRBC as target cells.

Table 2 presents the results of an analogous experiment in which mice were immunized with preparations that contained lipid A. Incorporation of this mitogen into each of the immunogens markedly stimulated the anti-Dnp-Cap response as measured by either PFC frequency (Tnp-SRBC as target cells) or antibody titer. However, in the context of this investigation, it is particularly noteworthy that the presence of lipid A did not alter the relative immunogenic potencies of these preparations: regardless of the parameter chosen (titer, PFC per spleen, PFC per 10^6 cells), the order remained liposomes prepared with beef sphingomyelin > liposomes prepared with egg lecithin > free Dnp-Cap-PE > no derivative (i.e., lipid A alone).

Table 2 further shows that incorporation of lipid A did not alter the relative specificity of the response: PFC frequency was still much lower when assays were done with SRBC instead of Tnp-SRBC as target cells. Nevertheless, the absolute number of PFC per spleen detected with SRBC was considerably higher when lipid A was included in each of the immunogens. This nonspecific response, which can be attributed to polyclonal activation of bone-marrow derived cells by lipid A, was also induced to a greater extent by incorporation of the mitogen into liposomes that contained beef sphingomyelin instead of egg lecithin (Table 2).

A conspicuous difference between egg lecithin and beef sphingomyelin is the temperature (approximately −10° and +38°, respectively) at which hydrated bilayers of each phospholipid undergo a thermotropic transition from a crystalline to a liquid-crystalline state (7, 8). Transition temperatures of a number of synthetic phosphatidylcholines have been determined and, for the ones used in this study, are in the order: distearoyl-, 57° > dipalmitoyl-, 41° > dimyristoyl-, 23° > dilauroyl-, 0° > dioleoyl-, −22°. This order is essentially in agreement with the relative immunogenicity of Dnp-Cap-PE-sensitized liposomes prepared with these phospholipids (Fig. 1). Both curves suggest a threshold phenomenon—i.e., incorporation of Dnp-Cap-PE into lipid bilayers does not significantly enhance the immune response unless the bilayers contain a phospholipid with a transition temperature above a critical value.

DISCUSSION

This investigation has shown that changes in the phospholipid composition of liposomes have a pronounced effect on the ability of these model membranes to induce antibody formation (an afferent immune response) in mice. Our emphasis of an apparent causal relationship between an increase in liposomal immunogenicity and the transition temperature of the phospholipid present in the bilayer does not mean that the latter should be regarded as the only important factor. For example, the possibility that the other liposomal constituents (e.g., cholesterol) play a role remains to be determined. Indeed, this study also has demonstrated that additional considerations must eventually be taken into account. Thus, beef sphingomyelin and dipalmitoylphosphatidylcholine have nearly the same transition temperature, yet liposomes prepared with the sphingophospholipid were more potent immunogens than those prepared with the glycerophospholipid.

Phospholipid composition may exert an influence on liposomal immunogenicity by various means that are not mutually exclusive. An increased humoral response could, for example, simply reflect greater bilayer stability at mouse body temperature or a slower rate of clearance of the more immunogenic liposomes from the animal. However, at the present time, three alternatives are particularly attractive because of the available evidence that closely related phenomena are also controlled by liposomal composition. Liposomes prepared with beef sphingomyelin or distearoyl- or dipalmitoylphosphatidylcholine may elicit more antibodies than those prepared with egg lecithin or dilauryl or dioleoylphosphatidylcholine because: (*a*) Greater accessibility of the antigenic determinants enhances binding to immunocompetent cells in lymphoid tissues. (*b*) The liposomes are bound more avidly to lymphocytes due to an altered distribution and/or mobility of antigenic determinants within the plane of the bilayer. (*c*) After binding to cells that participate in the immune response, the liposomes are processed differently.

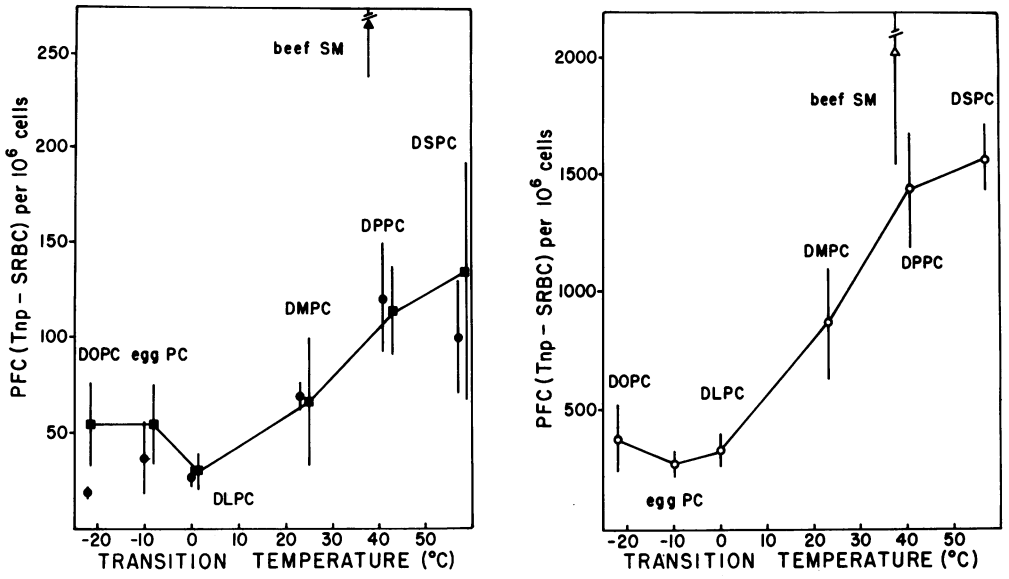

FIG. 1. Immune response to Dnp-Cap-PE-sensitized liposomes prepared with phospholipids of different transition temperature. *Left.* Without lipid A. Results of two experiments (using phosphatidylcholines from different sources) are shown. *Right.* With lipid A. PC = phosphatidylcholine; DO = dioleoyl-; DL = dilauroyl-; DM = dimyristoyl-; DP = dipalmitoyl-; DS = distearoyl-.

In regard to *c*, numerous studies (see ref. 9 and references cited therein) suggest that liposomes composed of phospholipids that are "fluid" (low transition temperature) are primarily taken up by cells as a consequence of fusion with the plasma membrane, whereas "solid" liposomes (prepared with phospholipids of high transition temperature) are incorporated into cells mainly via an endocytotic process. In regard to *a* and *b*, it should be mentioned that liposomes have already been extensively used to study the mechanism of humoral mediated immune cytolysis (an efferent response). These investigations (reviewed in ref. 5) have shown that the ability of antibodies to initiate complement-dependent marker release from antigen-sensitized liposomes is a function not only of phospholipid composition (10, 11) but also of cholesterol content (12). Other studies (13, 14) have demonstrated that these two factors, and also antigen density, regulate complement fixation by immune complexes on the liposomal surface.

Finally, we note that liposomes have attracted so much attention since their discovery (reviewed in ref. 15) because of the fact that many properties associated with natural membranes can be duplicated with these model membranes. One of the more important immunological phenomena is antigenic expression because the extent to which this membrane characteristic occurs partially determines whether foreign cells are recognized as such. The present experiments strongly suggest that this phenomenon may also be mimicked with liposomal immunogens and that continued use of the latter may clarify the role played by different lipid constituents.

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