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IMMUNOLOGIC RESPONSE TO PROTEIN IMMOBILIZED ON THE SURFACE OF LIPOSOMES VIA COVALENT AZO-BONDING

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A new method for immobilizing protein on the surface of liposomes is described. Inclusion of *N*-(*p*-aminophenyl)stearylamide in the lipid composition of vesicles resulted in liposomes that could be 'activated' by diazotization with $NaNO_2/HCl$, and subsequently coupled with protein. Using this method 39.7 ± 7.5 μg egg albumin / μmol phospholipid has been coupled to multilamellar vesicles composed of phosphatidylcholine, cholesterol, and *N*-(*p*-aminophenyl)stearylamide in a molar ratio of 15:7.5:1.1. Furthermore, when the immunologic response of mice to egg albumin that was encapsulated in, nonspecifically adsorbed, or covalently linked to liposomes was investigated, only the covalent protein-liposome conjugates elicited pronounced and sustained elevations in antibody titers. These results suggest that the immunoadjuvant effects of liposomes can be maximized by covalently linking protein antigens to their surface.

Introduction

Previous investigations have shown that the antigenicity of various proteins can be substantially enhanced when these proteins are encapsulated in phospholipid vesicles called liposomes [1,2]. For example, the significant adjuvant effect that liposomes can elicit has been demonstrated using such diverse liposome-entrapped proteins as bovine serum albumin [3], L-asparaginase [4], diphtheria toxoid [1] and malaria antigens [5]. Because liposomes are composed of biodegradable lipids normally found in cell membranes they are, by themselves, relatively nontoxic and nonimmunogenic [2]. For these reasons liposomes offer great promise as carriers of vaccines [6]. Recently Van Rooijen and Van Nieuwmegen [7] have advanced the postulate that the adjuvant effect of liposomes results from the surface exposition of partially entrapped or adsorbed antigens and that fully encapsulated proteins are nonimmunogenic. This conclusion was based on the finding that approximately equivalent antibody titers were observed in rabbits following challenge with either liposome 'encapsulated' proteins or proteins that were nonspecifically adsorbed to the surface of preformed vesicles.

In contrast to the aforementioned studies, Shek and Sabiston [8] compared the effectiveness of vesicle-entrapped and surface-associated antigen and found that encapsulated protein was more immunogenic than surface-adsorbed. The notion that surface exposition is not required for liposome immunopotentiation is also supported by studies showing that antigens that 'appear' to be fully encapsulated proved to be very effective in eliciting an immune response [9,10].

The amount of protein that can be nonspecifically adsorbed on the surface of liposomes is severely limited in most cases [7,8,11]. Moreover,

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proteins that are strongly adsorbed may have their antigenic determinants 'masked' by association with the liposomal membrane while those that are weakly bound may lack sufficient in vitro stability to have their immunogenicity enhanced. For these reasons immunopotentiation studies that are based on nonspecific adsorption of antigens by liposomes could conceivably yield conflicting results.

In the present communication we describe a convenient technique for coupling proteins to the surface of multilamellar liposomes. In addition, we present evidence that protein covalently linked to liposomes is substantially more antigenic than liposome-encapsulated or surface-adsorbed protein.

Materials and Methods

Cholesterol, egg yolk derived L-α-phosphatidylcholine, *p*-phenylenediamine and *p*-nitrophenyl stearate were obtained from Sigma Chemical Co., St. Louis, MO, and used without further purification. Egg albumin (5 × recrystallized) was a product of Calbiochem, La Jolla, CA. Radiolabelled $Na^{125}I$ was supplied by New England Nuclear, Boston, MA. All buffers were prepared using reagent grade chemicals.

Synthesis of N-(p-aminophenyl)stearylamide. 2 g *p*-nitrophenyl stearate ($4.9 \cdot 10^{-3}$ mol) and 0.80 g ($7.4 \cdot 10^{-3}$ mol) *p*-phenylenediamine were dissolved in 30 ml of dimethylsulfoxide. After adding 1 drop of triethylamine, the mixture was heated to 50°C for 2 h. The solution was poured into 200 ml of ice-water made basic by the addition of 2 ml of 6 M NaOH. The solid product was collected by filtration and recrystallized twice from 95% ethanol, m.p. 125–126°C. The product had ultraviolet ($\lambda_{max} = 385$, $\epsilon = 1280$, chloroform) and infrared spectra consistent with the assigned structure.

Preparation of liposomes. Phosphatidylcholine (15 μmol), cholesterol (7.5 μmol) and *N*(*p*-aminophenyl)stearylamide(1.1 μmol) in chloroform were placed in a 100 ml round bottom flask. A thin film consisting of 14.2 mg total lipid was deposited on the walls of the flask by rotoevaporation. Liposomes were dispersed in 10 ml of 0.17 M NaCl/0.01 M borate buffer (pH 8.0) by a combination of hand shaking and mechanical agitation using a Vortex mixer. After suspension, liposomes were allowed to stand for an additional hour at room temperature before activating and coupling to protein.

Radiolabelling of egg albumin. Radioiodination of egg albumin was accomplished by the chloramine-*T* method [12] using carrier-free $Na^{125}I$ (New England Nuclear). Iodinated protein was separated from iodide ion by dialysis. The specific activity of the labelled egg albumin was approx. $4 \cdot 10^6$ cpm/mg. Counts were performed on a Beckman Gamma-300 system.

Covalent coupling of proteins to liposomes. The liposome suspension prepared above was centrifuged at 20000 × *g* for 10 min using a RC2 Sorval medium speed refrigerated centrifuge. The liposome pellet was resuspended in 2.0 ml cold (4.0°C) 0.2 M $NaNO_2$ followed by 2.0 ml of cold 0.2 M NaCl/0.2 M HCl. The diazotization reaction was carried out for 5 min at 0–4°C in an ice-bath and liposomes were centrifuged (4.0°C) at 20000 × *g* for 5 min. The diazotized liposome pellet was immediately resuspended in a cold solution of egg albumin (1.0–5.0 mg/ml) in 0.05 M borate buffer (pH 10). The mixture was placed in an ice-bath and allowed to come to room temperature overnight. The resulting tan-colored liposomes were washed thrice by centrifugation with 0.17 M NaCl/0.01 M borate buffer (pH 8.0). For nonspecific adsorption the same procedure was fol-

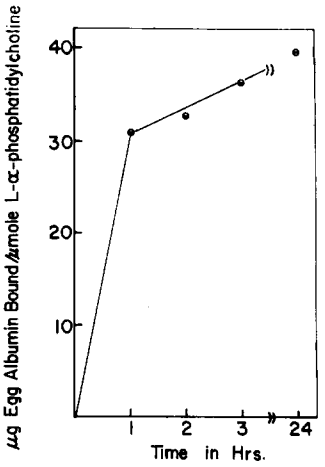

Fig. 1. Kinetics of egg albumin coupling to diazotized liposomes.

lowed except that the diazotization step was eliminated. The amount of liposome associated protein was calculated on the basis of μmoles of phospholipids originally dispersed and the percentage of the radiolabelled protein bound.

Animals. Female CAF_1 mice (Jackson Laboratories, Bar Harbor, ME), 14–16 weeks old, were used for the immunization experiments. Mice were immunized by intravenous injection with 0.10 ml of egg albumin or liposomes suspended in 0.17 M NaCl/ 0.01 M borate buffer (pH 8.0). Blood samples were obtained from the retro-orbital plexus, and serum samples were frozen at −20°C prior to use in the ELISA.

Microtiter plate enzyme-linked immunosorbent assay (ELISA). The antibody responses to egg albumin in immunized mice were assayed by a microplate ELISA following the general procedure developed for antibodies against rickettsial antigens [13,14].

Microtiter plates (Imulon 'U' bottom polystyrene plates, Cooke Laboratory Products, Alexandria, VA) were coated with egg albumin. The stock egg albumin (5 × cryst., Calbiochem) solution (10 mg/ml in borate-buffered saline pH 8.0) was subjected to ten freeze-thaw cycles in a mixture of dry ice and alcohol. The wells were coated overnight at 37°C with 0.1 ml vol. of egg albumin diluted in a carbonate buffer (0.1 M sodium carbonate, 0.02% sodium azide, pH adjusted to 9.6 with hydrochloric acid). Each well contained 12 μg of egg albumin antigen for coating. After overnight incubation in a humidified incubator at 37°C, the plates were washed five times with working buffer (2.2 g boric acid, 0.2 g sodium hydroxide, 9.29 g sodium chloride, 0.09 g sodium azide, 5.0 g Tween-20 and 5.0 g bovine serum albumin fraction V per liter of solution with pH adjusted to 7.8 with hydrochloric acid). A 24 channel multiple automated sample harvester (MASH-II, Microbiological Associates, Bethesda, MD) was used to wash the plates. Volumes of 0.10 ml of serum samples diluted in working buffer were added to each well. After 2 h in the 37°C humidified incubator, the plates were washed again five times with working buffer and 0.1 ml of a suitable dilution of enzyme-antibody conjugate added to each well. The conjugate was prepared by glutaraldehyde coupling [14,15] of alkaline phosphatase (P-4502, Sigma Chemical Co.) with goat anti-mouse IgG(H + L) (Cappel Laboratories, Cochranville, PA). After incubation with the conjugate for 2 h at 37°C, the plates were washed five times with working buffer and once with diethanolamine buffer (97 ml diethanolamine, Aldrich Chemical, Co., Milwaukee, WI, 0.2 g sodium azide, 0.1 g $MgCl_2 \cdot 6\ H_2O$ and 800 ml water). Volumes of 0.10 ml of substrate solution at 37°C were added to each well. The substrate solution contained 1 mg/ml of *p*-nitrophenylphosphate (Sigma Chemical Co.) dissolved in the diethanolamine buffer. After incubation at 37°C for 1 h, 0.15 ml of 2 M sodium hydroxide was added and adsorbance read at 405 nm in a Titertek Multiskan (type 310C) plate reader, Flow Laboratories, Inc., Rockville, MD.

Results

Kinetics and extent of covalent attachment of protein to liposomes

Egg albumin has been coupled to the surface of liposomes by a diazonium salt technique similar to that used previously for immobilizing proteins on the surface of polymers [24]. The kinetics of protein binding to diazotized liposomes as determined by association of radiolabelled protein with the sedimentable liposomal fraction is presented in Fig. 1. The coupling of protein to liposomes was observed to be biphasic with 75–80% of the reaction taking place within 1 h, followed by a much slower increase in protein binding. Evidence that conjugation of protein to liposomes was enhanced as a result of specific covalent bonding of proteins to the surface of liposomes is presented in Table I. Ligand-free liposomes (PC/C) that were pretreated with $NaNO_2$/HCl bound approximately the same amount of egg albumin as untreated ligand-free liposomes. Conversely, ligand-containing liposomes (PC/C/L) not diazotized with $NaNO_2$/HCl also showed no increase in bound protein. However, ligand-containing liposomes that were diazotized showed enhanced binding relative to the controls. Using this method, the amount of liposome-associated protein was increased approximately 3-fold, from 12.8 ± 0.7 to 39.7 ± 7.5 μg protein/μmol phospholipid. Further evidence of the formation of specific 'azo-type' covalent

TABLE I

COMPARISON OF EGG ALBUMIN BOUND TO DIAZOTIZED OR NON-DIAZOTIZED LIPOSOMES WITH OR WITHOUT LIGAND INCORPORATED

Liposome composition [a]	Diazotized [b]	Protein bound [c] (μg/μmol PC)	n [d]
PC/C	–	12.8±0.7	3
PC/C	+	13.8±1.0	3
PC/C/ligand	–	13.7±0.3	3
PC/C/ligand	+	39.7±7.5	8
PC/C/DDMB [e]	–	6.0±0.6	3

[a] Liposomes were composed of 15 μmol L-α-phosphatidylcholine(PC), 7.5 μmol cholesterol(C) and 1.1 μmol *N*-(*p*-aminophenyl)stearylamide (ligand).

[b] Liposomes were activated by suspension in 2 ml 0.2 M $NaNO_2$ + 2 ml 0.2 M HCl/0.2 M NaCl for 5 min at 4°C.

[c] Diazotized liposomes were suspended in 3 ml of egg albumin at 5.0 mg/ml, pH 10, 0.05 M sodium borate at 0–4°C. The suspension was allowed to come to room temperature overnight.

[d] n, number of determinations.

[e] Didodecyldimethylammonium bromide (DDMB) was used in a molar ratio of 15:7.5:1.1.

bonds is suggested by the characteristic tan color of the egg albumin-liposome conjugates.

In order to relate the efficiency of the coupling procedure to protein concentration, liposomes were suspended in egg albumin at various concentrations. The lower curve in Fig. 2 shows nonspecific adsorption of egg albumin to untreated liposomes, the upper curves shows the amount of protein bound to diazotized liposomes. Both nonspecific adsorption and covalent attachment of egg albumin to liposomes were concentration dependent, however, this dependence was greater the in latter case.

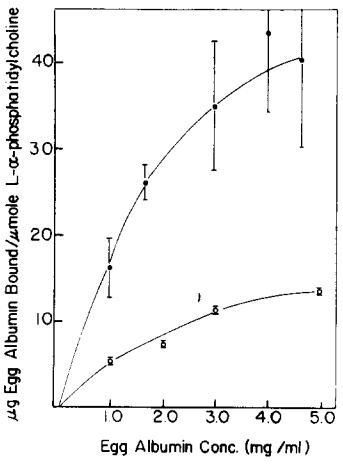

Fig. 2. Amount of egg albumin bound to diazotized (upper curve) or non-diazotized liposomes (lower curve) versus amount of egg albumin present in solution.

Stability and integrity of liposomes

Liposomes prepared with radiolabelled egg albumin coupled to their surface retained 96% of the label after storage in pH 8.0 buffer at 4°C for 2 weeks. Somewhat unexpectedly, protein that was nonspecifically adsorbed on the surface of liposomes was similarly retained.

Because liposomes are often used as carriers of drugs, it was important to demonstrate that the integrity of the liposomes is not significantly compromised during the procedure for covalent attachment of protein. To accomplish this ^{125}I iodide ion was entrapped in liposomes. Following encapsulation of radiolabelled iodide, both undiazotized and diazotized liposomes were suspended overnight in egg albumin at 5.0 mg/ml (pH 10, 0.05 M borate). The liposomes were washed by centrifugation and the amount of ^{125}I ion retained was immediately determined. The bar graphs in Fig. 3 compare the amount of radiolabelled iodide

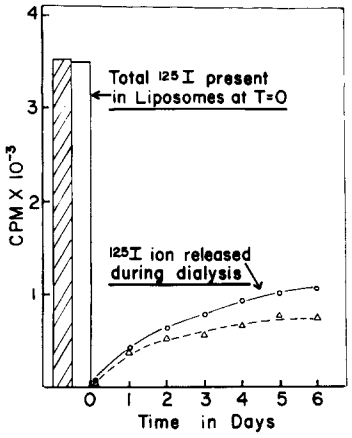

Fig. 3. Comparison of entrapped ^{125}I found in liposomes with egg albumin that is nonspecifically adsorbed (crosshatched bar) and covalently attached (open bar). Release of ^{125}I from liposomes with nonspecifically adsorbed egg albumin (upper curve) versus release from covalent liposome-egg albumin conjugates (lower curve).

TABLE II

ANTI-EGG ALBUMIN TITERS FOUND IN MOUSE SERUM AT VARIOUS INTERVALS FOLLOWING INTRAVENOUS ADMINISTRATION OF LIPOSOME ASSOCIATED EGG ALBUMIN

Antibody titers are ELISA units (EU): EU = (A_{405nm} (immune serum) − A_{405nm} (normal serum)) × dilution factor. Each value is the mean ± S.D. of five determinations. Five mice were employed at each interval. The number in parenthesis represents the number of responders. The mean value includes both responders and nonresponders. Each mouse received 0.10 ml of protein or liposomes suspended in pH 8.0, 0.17 M NaCl/0.01 M borate buffer. Mice in Groups 2, 3, 4, 5, 6, and 7 received a total of 0.5 μmol lipid. As a result of the small amount of egg albumin that could be nonspecifically adsorbed by liposomes, it was necessary to use a total of 1.5 μmol in order to deliver 20 μg egg albumin in Group 5.

Group description	Anti-egg albumin titers at week		
	2	4	6
1. 20 μg free egg albumin	0	0	0
2. Empty liposomes	0	0	0
3. 20 μg egg albumin 'encapsulated' in liposomes [a]	19 ± 17 (3)	0	0
4. 7 μg egg albumin nonspecifically bound to surface of preformed liposomes	0	0	0
5. 20 μg egg albumin nonspecifically bound to surface of preformed liposomes	0	0	54 ± 52 (2)
6. 20 μg egg albumin mixed with preformed liposomes	0	0	0
7. 20 μg egg albumin covalently coupled to surface of preformed liposomes	247 ± 125 (5)	471 ± 155 (5)	348 ± 146 (5)

[a] Results from binding experiments using preformed liposomes indicated that approx. 3 μg of the liposome-associated protein in this group was adsorbed. No attempt was made to remove surface bound egg albumin.

ion found in liposomes after covalently attaching egg albumin to their surface (open bar) to that found in liposomes with nonspecifically adsorbed albumin (crosshatched bar). Since retention of entrapped iodide was the same in both cases, it does not appear that diazotization and covalent coupling of protein to liposomes results in any significant amount of membrane damage. In a further test of stability, the two groups of liposomes were placed in dialysis bags that were immersed in isotonic buffer. The amount of radioactivity escaping into the dialysate was then determined as a function of time over a period of several days. Counts were corrected for natural decay. Surprisingly, the rate of iodide escape from undiazotized liposomes was somewhat greater than that observed for liposomes that had protein covalently bound to their surface (Fig. 3).

Antibody response of mice to liposome-coupled egg albumin

Mice were injected once intravenously with free egg albumin, or egg albumin that was either encapsulated in, mixed with, nonspecifically adsorbed, or covalently bound to liposomes. Mice were bled at 2-, 4- and 6-week intervals following antigen challenge, and their serum anti-egg albumin titer was determined using an ELISA technique. The results of these experiments are presented in Table II. Significantly, only egg albumin that was covalently coupled to the surface of liposomes elicited a marked immunologic response that was sustained. A relatively weak transient response was oberved using liposome-encapsulated egg albumin.

Discussion

Proteins have been bound directly to the surface of liposomes by a variety of methods. Several investigators have used crosslinking reagents such as glutaraldhyde [16], carbodiimide [17], or suberimidate [18]. A second approach is based on Schiff's base formation between proteins and periodate oxidized vesicles made with glycolipids [19]. More recently, proteins have been bound to liposomes using the heterobifunctional reagent SPDP [20,21], or by including the sulfhydryl-reactive phospholipid derivative *N*-(4-(*p*-maleimidophenyl)butyryl)phosphatidylethanolamine (MPB-

PE) in the membrane surface [22]. While large amounts of protein (Fab' fragments) have been bound to large unilamellar liposomes using the SPDP or MPB-PE methods [21,22], both suffer from the limitation that proteins not bearing free sulfhydryl groups must be thiolated before they can be coupled to SPDP or MPB-PE modified liposomes. Although apparently not as efficient as the SPDP or MPB-PE techniques, the method reported herein appears to be at least as efficient as those employing crosslinking reagents without the inherent disadvantage of producing homopolymers. In addition, it is a very simple procedure to perform and, therefore, lends itself readily for the routine preparation of liposomes bearing surface-bound protein antigens.

The egg albumin-liposome conjugates generated by the azo-coupling technique described herein appear to be quite stable. Approx. 96% of the bound protein remained associated with the liposomes after incubation in pH 8.0 borate buffer at 4°C for two weeks. Liposomes that have protein coupled to the surface by this method also maintained their membrane structural integrity as indicated by the results presented in Fig. 3. Protein-coated vesicles retained approx. 80% of the ^{125}I iodide ion entrapped within them after dialysis for 6 days.

The mechanism by which liposomes enhance the immunogenicity of encapsulated or surface-associated proteins is unclear at the present time. In this study we failed to observe a primary humoral response using free egg albumin, 'encapsulated egg albumin', or nonspecifically adsorbed egg albumin. Only when egg albumin was covalently-linked to the liposome surface was a significant and reproducible response observed (Table II). These results are consistent with the suggestion of Van Rooijen and Van Nieuwmegen [7] that surface exposure may be a critical factor. Failure to observe a pronounced primary immune response in this as well as in other studies using either 'encapsulated' or liposome-adsorbed proteins could then be rationalized on the basis of insufficient antigen to induce a detectable response or that essential antigenic determinants were 'masked' as a consequence of the particular protein-liposome interactions involved. The importance of the assessability of antigenic determinants on the surface of liposomes as a decisive factor for eliciting an immune response has been clearly demonstrated using liposomes that included dinitrophenylated phosphatidylethanolamine derivatives of various lengths in their lipid bilayers [23]. No immune response was observed unless an adequate 'spacer' group was interposed between the lipid and the hapten. Furthermore, the magnitude of the response correlated with the extent to which the haptenic determinant was removed from the surface of the liposome.

In conclusion, although the present study raises questions with respect to the use of antibody coated liposomes for bioselective drug delivery, the longstanding notion that liposomes have potential application as antigen carriers is reinforced and extended. Furthermore, our results strongly suggest that the immunogenicity of liposome-associated proteins is most effectively potentiated when these proteins are covalently linked to the vesicle surface.

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