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Immune response mediated by liposome-associated protein antigens

I. POTENTIATION OF THE PLAQUE-FORMING CELL RESPONSE

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Summary. Mice, immunized with liposome-associated bovine serum albumin (LSM-BSA), showed a significantly higher BSA-specific plaque-forming cell (PFC) response than did mice injected with fluid BSA (fBSA). Physical association between the liposome carrier and the protein antigen is imperative for potentiating the PFC response, since the injection of empty liposomes, together with fBSA, was found to be ineffective in inducing an immune response. Liposome-associated protein antigen was found to be a potent stimulator of immunological memory, as demonstrated by the ability of LSM-BSA primed animals to generate a vigorous PFC response upon challenge with the weakly immunogenic fBSA. The injection of congenitally athymic homozygous nude (Nu/Nu) mice with LSM-BSA failed to induce significant antibody formation, whereas the heterozygous (Nu/+) littermates gave a normal PFC response to the same LSM-BSA preparation. Thus, BSA remains a T-cell-dependent antigen, despite its entrapment within liposomes, and T lymphocytes appear to play an obligatory role in providing synergistic interactions for eliciting a BSA-specific PFC response to the LSM-BSA.

Abbreviations: A cell, accessory cell; B cell, bone marrow-derived cell; BSA, bovine serum albumin; BSS, balanced salt solution; Con A, concanavalin A; fBSA, fluid bovine serum albumin; H/H, Biozzi strain high responder mice; i.p., intraperitoneal; L/L, Biozzi strain low responder mice; LSM, liposome; LSM-BSA, liposome-associated bovine serum albumin; LPS, lipopolysaccharide; Nu/Nu, BALB/c homozygous nude mice; PBS, phosphate-buffered saline; PFC, plaque-forming cell; PHA, phytohaemagglutinin; SC, spleen cells; SRBC, sheep red blood cells; T cell, thymus-derived cell.

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INTRODUCTION

When dry polar lipids, such as phosphatidylcholine, phosphatidic acid and cholesterol, are confronted with an aqueous environment, they undergo a series of molecular rearrangements resulting in the formation of multi-lamellar structures called liposomes (Bangham, 1978). These structures are characterized by the presence of concentric lipid bilayers alternating with aqueous compartments. Water-soluble substances, like enzymes (Gregoriadis & Ryman, 1971; Sessa & Weissmann, 1970; Gregoriadis & Buckland, 1973) and proteins (Gregoriadis & Allison, 1974; Heath, Edwards & Ryman, 1976; Tyrrell, Heath, Colley & Ryman, 1976) may be entrapped within the hydrophilic pockets of the liposomal vesicles.

Initially it was anticipated that the entrapment of proteins within liposomes might render them non-immunogenic. However, when Allison & Gregoriadis (1974) reported that antibody formation to diphtheria toxoid was enhanced in animals injected with liposome-

entrapped antigen, the immunopotentiating properties of liposomes were first recognized. Since that time, the majority of studies in which liposomes have been used as carriers for protein antigens, have evaluated the immune response in terms of circulating antibody titres (Heath *et al.*, 1976; Allison & Gregoriadis, 1974; Van Rooijen & Van Nieuwmegen, 1977). Only relatively recently have attempts been made to examine some of the cellular events involved in the immune response to haptenated liposomes (Yasuda, Tadakuma, Pierce & Kinsky, 1979; Van Houte, Snippe & Willers, 1979). In the present report, the cellular aspect of the potentiated humoral response was studied in relation to the change in the number of antibody-forming cells in the spleen of the immunized host. The effectiveness of liposome-entrapped antigen in priming animals for subsequent challenge by free antigen was also explored. Finally, the requirement for the participation of T lymphocytes in eliciting an antibody response to a liposome-entrapped protein antigen was investigated.

MATERIALS AND METHODS

Animals

Female A/J mice were purchased from the Jackson Laboratories, Bar Harbor, Maine, while BALB/c nude mice and their heterozygous littermates were purchased from the Canadian Breeding Laboratories, Montreal, Quebec. Biozzi high and low responder mice, which had been inbred for at least forty-six generations, were supplied by the Laboratory Animal Group of our Institute. All mice used in this study were between 6 and 10 weeks old. Animals were kept in plastic cages and allowed free access to laboratory mouse chow and drinking water.

Chemicals and biologicals

Bovine serum albumin (BSA), phosphatidylcholine, cholesterol and phosphatidic acid were purchased from the Sigma Chemical Co., St. Louis, M. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI) was obtained from the Ott Chemical Company, Muskegon, Mich. Citrated sheep red blood cells (SRBC) were received weekly from Woodlyn Laboratories, Ltd, Guelph, Ontario. Guinea-pig complement was obtained from Cedarlane Laboratories Ltd., Hornby, Ontario. Agarose was purchased from L'Industrie Biologique Francaise S.A. Gennevilliers, France, and Sepharose 4B was purchased from Pharmacia Ltd, Dorval, Quebec. A polyspecific rabbit anti-mouse IgG antiserum was a gift from Dr C.W. Pierce of the Jewish Hospital of St Louis, St. Louis, M. ^{125}I, protein iodination grade, was obtained from new England Nuclear Canada Ltd, Lachine, Quebec.

Preparation of liposomes

The procedure used for the preparation of protein-containing liposomes was essentially the same as that described by Gregoriadis & Ryman (1971). Briefly, a dry lipid film was prepared by the rotary evaporation, at 37°, of a chloroform solution containing 15 mg egg phosphatidylcholine, 2·2 mg of cholesterol, and 2·12 mg of phosphatidic acid, i.e. in a molar ratio of 7:2:1. The lipid film was resuspended by gentle swirling in 2 ml of 3·3 mM phosphate buffer, pH 7·2, containing 50 mg of BSA. For the preparation of empty liposomes, the lipid film was resuspended in the same buffer but without protein. For the purpose of estimating the percentage of protein entrapment, 200 μg of ^{125}I-labelled BSA (specific activity: 0·45 μCi/μg) were also added to the protein solution. After being allowed to equilibrate at room temperature for 2 hr, the liposomal suspension was sonicated in an ice-bath for 30 sec at 100 W in a Braunsonic 1510 sonicator and was then kept at room temperature for another 2 hr. The BSA-containing liposomes were separated from non-entrapped BSA by passage through a Sepharose 4B column (1·5 × 30 cm) equilibrated with 0·15 M phosphate-buffered saline (PBS), pH 7·2. The BSA-containing liposome fractions were pooled and washed three times by centrifugation at 100,000 *g* for 30 min each time in a Beckman ultracentrifuge (Model L2-65). The final pellet was resuspended in PBS and kept at 4°.

Preparation of cell suspensions and haemolytic plaque assay

Mice were killed by cervical dislocation. The spleens were harvested and gently teased with sharp forceps in balanced salt solution (BSS; Mishell & Dutton, 1967). The dispersed spleen cells were filtered through stainless steel screens and were washed three times in BSS by centrifugation at 200 *g* for 10 min each time. The washed spleen cells were assayed for antibody-forming cells by a modified method (Mishell & Dutton, 1967) of the Jerne haemolytic plaque technique (Jerne & Nordin, 1963). For the preparation of indicator red cells, BSA was covalently linked to SRBC by the coupling reagent, ECDI, according to the method of Golub, Mishell, Weigle & Dutton (1968). For the

development of IgG plaque-forming cells (PFC), a previously determined optimal dilution of a polyspecific rabbit anti-mouse IgG antiserum was incorporated in the agarose mixture.

Passive haemagglutination

Serum haemagglutinating antibody levels were determined by the procedure of Stavitsky & Arquilla (1955) using the micromethod. Sheep red blood cells, covalently coupled to BSA, were used as the indicator cells (Johnson, Brenner & Hall, 1966). Antibody titres were expressed as the reciprocal (in $\log_2$ units) of the highest serum dilution that gave a positive haemagglutinating pattern.

Assay of mitogen-stimulated proliferative response

Aseptically prepared spleen cells were suspended in sterile alpha minimal essential medium (Media Preparation Division, University of Toronto) supplemented with streptomycin and penicillin. A volume of 100 μl of the cell suspension containing 4×10^5 viable nucleated cells was added to the appropriate well of a plastic microculture plate (Falcon Plastics, Oxnard, Calif.). Each well contained 100 μl of medium with or without mitogen. For each mitogen tested, a range of concentrations was used for the stimulation of splenic lymphocytes. The range of final concentration in the culture was 0·1 to 1·0 μg/ml for concanavalin A (Con A; Miles Laboratories Inc.), 0·4 to 4·0 μg/ml for phytohaemagglutinin (PHA; Pharmacia Fine Chemicals) and 5–60 μg/ml for *E. coli* lipopolysaccharide W (LPS; Difco Laboratories). The cells were cultured at 37° in a humidified atmosphere with 5% CO_2 and 95% air for 96 hr. A volume of 20 μl of [^{3}H]-thymidine (1 μCi, specific activity: 2·0 Ci/mmole, Amersham Corporation) was added to each well 18–24 hr before the culture was terminated. At the end of the culture period, cells were harvested on glass-fibre filters (Reeve Angel) using a multiple cell harvester (MASH II, Microbiological Associates). After appropriate drying, radioactivity was determined by liquid scintillation counting in a Beckman scintillation counter (Model LS 9000). Each cell culture was performed in triplicate and the response to the optimal dose of each mitogen was expressed as the net (i.e. experimental −control) c.p.m. per culture.

Preparation of iodinated BSA

BSA was iodinated with ^{125}I by a chloramine-T method (McConahey & Dixon, 1966; Sonada & Schlamowitz, 1970). Freshly prepared chloramine-T (0·1 ml; 1·45 μmol) was injected rapidly into a chilled and stirred mixture of 10 mg BSA (0·5 ml) and 5 mCi ^{125}I (0·2 ml) in phosphate buffer (pH 7·4; 0·05 M). After the mixture had reacted for 10 min at 0°, sodium metabisulphite (0·1 ml; 1·74 μmol) was injected rapidly to stop the reaction. Five minutes later, carrier KI (0·1 ml; 0·1 M) was added and the entire mixture transferred to a column of Sephadex G-25. Following elution with phosphate buffer (pH 7·4; 0·05 M), the BSA protein-bound iodine was 98%. The average level of substitution was $0{\cdot}88 \times 10^{-2}$ atoms ^{125}I per molecule BSA, resulting in an absolute level of substitution of one atom I per molecule BSA (Sonada & Schlamowitz, 1970).

RESULTS

Primary response

Mice were injected with 50 μg of LSM-BSA, fluid BSA (fBSA), or fBSA together with empty LSM. At different times after injection, spleen cells were obtained from the immunized animals and were used, in the haemolytic plaque assay, for the determination of the number of BSA-specific antibody-forming cells. As shown in Fig. 1, animals immunized with fBSA or

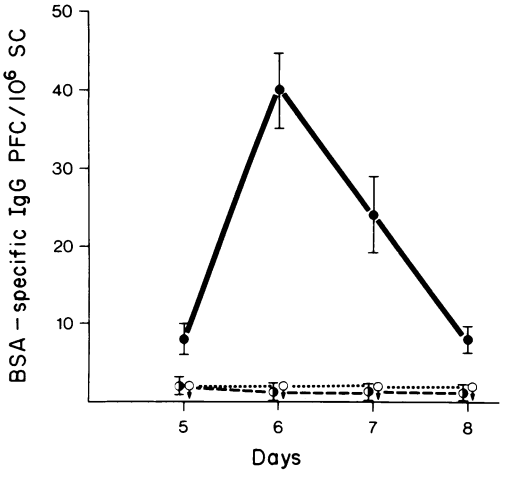

Figure 1. Kinetics of the primary BSA-specific PFC response induced by LSM-BSA. A/J mice were immunized intraperitoneally with 50 μg of either LSM-BSA (●———●), fBSA (o. . . . . .o), or fBSA together with empty LSM (o – – – o). Spleen cells (SC) of the immunized animals were harvested for PFC assay 5–8 days after the injection of antigen. Each point represents the mean response ± SEM of five to ten animals. The inverted arrow indicates no detectable PFC response.

with empty LSM and fBSA gave practically no detectable PFC response. On the other hand, the injection of LSM-BSA elicited a well-defined anti-BSA PFC response which peaked on day 6 after immunization.

Secondary response

Animals were given two intraperitoneal injections of 50 μg of LSM-BSA, fBSA, or empty LSM together with fBSA, 14 days apart. The splenic anti-BSA PFC response of the immunized animals was assayed 3 to 6 days after the second injection of antigen. A vigorous secondary PFC response was observed in animals immunized with LSM-BSA (Fig. 2). Antibody-forming cells were detectable as early as day 3 after antigenic challenge and peak PFC activity occurred on day 4 of the secondary response. In contrast, animals immunized with fBSA alone, or fBSA together with empty liposomes, gave no appreciable PFC response. Similar unresponsiveness was obtained when the period of assay was extended up to 15 days after antigenic challenge.

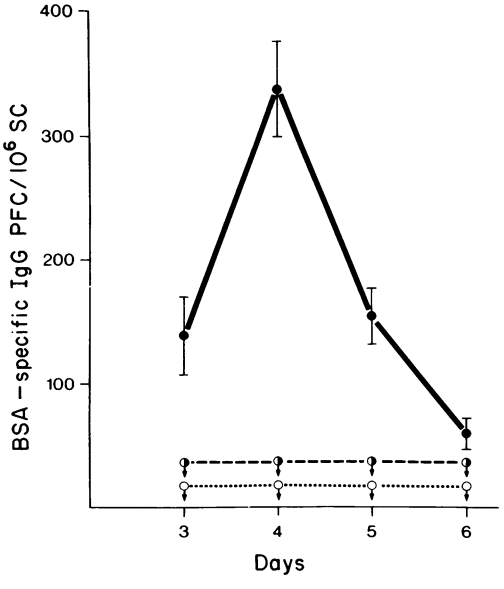

Figure 2. Potentiation of the secondary BSA-specific PFC response by LSM-BSA. A/J mice were given two intraperitoneal injections of 50 μg of either LSM-BSA (●——●), fBSA (○.....○), or fBSA together with empty LSM (●– – –●), 2 weeks apart. Spleen cells of the immunized animals were harvested for the PFC assay 3–6 days after the second injection of antigen. Each point represents the mean response ± SEM of seven to ten animals. The inverted arrow indicates a mean response of less than 10 PFC per 10^6 spleen cells.

Comparison of the effectiveness of LSM-BSA and fBSA in immunological priming

Mice were divided into two groups. One group was primed with an injection of 50 μg of fBSA; the other, with the same dose of LSM-BSA. After a resting period of 5 weeks, all animals were challenged with 50 μg of fBSA. It can be seen from Fig. 3 that fBSA is an extremely poor antigen for priming, whereas LSM-BSA is very effective.

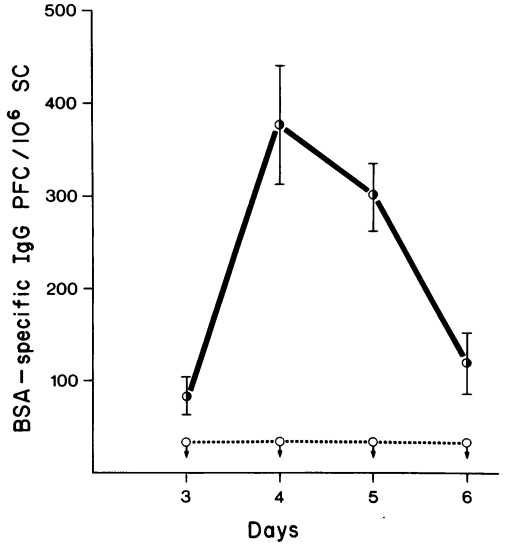

Figure 3. Effectiveness of LSM-BSA in priming animals for a secondary antibody response. A/J mice were divided into two groups and each group was injected intraperitoneally with 50 μg of either LSM-BSA (●——●), or fBSA (○.....○). After a resting period of 5 weeks, all animals were challenged with 50 μg of fBSA. The spleen cells of the immunized animals were harvested for PFC assay 3–6 days after antigenic challenge. Each point represents the mean response ± SEM of five to six animals. The inverted arrow indicates a mean response of less than 10 PFC per 10^6 spleen cells.

T-cell requirement in the antibody response to LSM-BSA

Biozzi low responder strain (L/L) mice were found to have a significantly lower response to stimulation with the T-cell mitogens, Con A and PHA, than Biozzi high responder (H/H) mice (Fig. 4). In contrast, the two strains had a similar response to stimulation with the B-cell mitogen, LPS (Fig. 4). Thus, the two strains

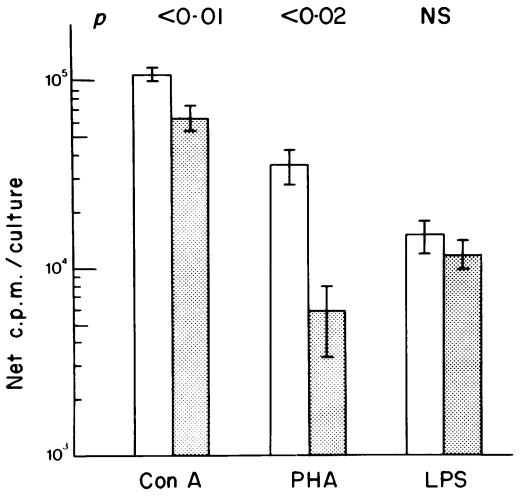

Figure 4. Responsiveness of splenic lymphocytes of Biozzi high (□) and low (■) responder mice to T-cell and B-cell mitogens. Spleen cells obtained from untreated animals were cultured in the absence or presence of a certain dose range (see Materials and Methods) of Con A, PHA and LPS for 72 hr. [^{3}H]-thymidine was added 18–24 hr before the termination of each cell culture. The proliferative response to each mitogen was measured by the quantitative incorporation of [^{3}H]-thymidine and is expressed as net c.p.m./culture. Each vertical bar represents the mean peak response ± SEM of three experiments.

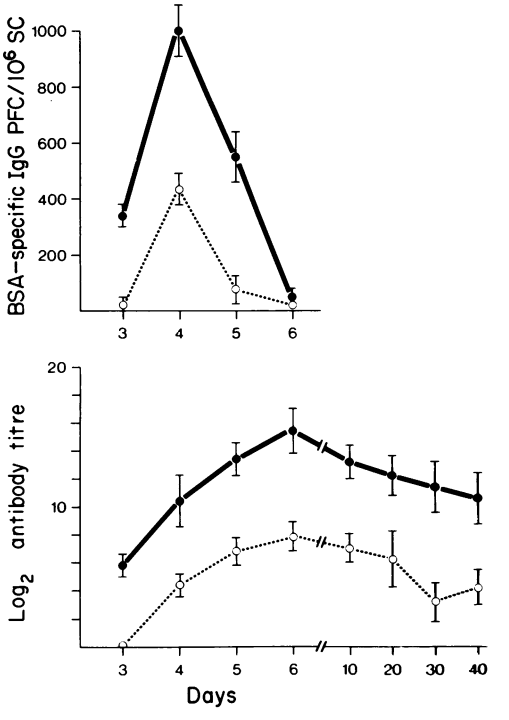

Figure 5. The antibody response of Biozzi high and low responder mice to LSM-BSA. Biozzi high (●——●) and low (o . . . . . o) responder mice were given two intraperitoneal injections of 50 μg of LSM-BSA each time, 3 weeks apart. The immunized animals were assayed for their BSA-specific PFC response (upper panel) on days 3–6, and for their serum antibody titres (lower panel) 3–40 days after the second injection of antigen. Each point represents the mean response ± SEM of five animals.

appeared to provide a potentially useful model for studying the relative T-cell dependency of the LSM-mediated response. When mice of the two strains were immunized with LSM-BSA (two intraperitoneal injections of 50 μg each, 14 days apart), the magnitude of the antibody response of the L/L mice was less than that of H/H animals (Fig. 5). This diminished response was reflected both in a lower number of BSA-specific PFC (Fig. 5, upper panel) and in lower titres of circulating haemagglutinating antibody (Fig. 5, lower panel). When congenitally athymic nude (Nu/Nu) mice, their heterozygous (Nu/+) littermates, and normal BALB/c mice were immunized with LSM-BSA, the Nu/Nu animals were incapable of mounting a significant antibody response while the Nu/+, as well as the normal animals, responded well (Table 1).

DISCUSSION

Soluble native proteins are known to be extremely weak antigens in mice (Howard & Mitchison, 1975; Dresser, 1962; Dietrich & Weigle, 1964) unless they are administered with adjuvants (Dresser & Phillips, 1973; Johnson, Gaines & Landy, 1956) or have their immunogenicity enhanced by chemical (Golub & Weigle, 1969) or physical (Gamble, 1966) aggregation. We now show that the weakly immunogenic protein, BSA, can be rendered highly immunogenic, simply by its entrapment within liposomes. Indeed, the magnitude of the BSA-specific PFC response stimulated by LSM-BSA is comparable with that obtained with the highly potent Freund's adjuvant (Golub *et al.*, 1968). Moreover, LSM-BSA proved effective in priming animals to a subsequent challenge with fBSA whereas fBSA alone was completely ineffective in inducing immunological memory (Fig. 3). This ability of liposome-entrapped antigen to confer upon the immunized host, a substantial immunological memory, may find application in potentiating the

Table 1. Comparison of antigen-specific secondary PFC response to LSM-BSA among normal BALB/c mice, homozygous athymic nude (Nu/Nu) mice, and heterozygous (Nu/+) littermates*

Group	Strain	Mice (*n*)	BSA-specific IgG PFC/spleen†	*P*
			Mean ± SEM	
(I)	BALB/c +/+	5	26,200 ± 3400	
(II)	BALB/c Nu/+	5	32,100 ± 6000	NS‡
(III)	BALB/c Nu/Nu	5	90 ± 20	< 0·001§ < 0·001¶

* Animals were given two intraperitoneal injections of 50 μg of LSM-BSA each time, 14 days apart.

† Spleen cells were harvested for the PFC assay, 4 days after the second injection of LSM-BSA.

‡ Statistically not significant in comparison with group (I).

§ *P* value (Student's *t* test) in comparison with group (I).

¶ *P* value in comparison with group (II).

efficacy of otherwise weak vaccines, useful in preventive medicine.

The fact that LSM-BSA gave a consistent increase in the PFC response while separate but simultaneous injections of empty liposomes and fBSA did not (Figs 1 and 2), suggests that the immune potentiation process requires that there be a direct physical association between the BSA molecules and the liposomal membrane. It has been shown that protein adsorbed to the surface of liposomal vesicles, can enhance titres of circulating antibody in immunized animals (van Rooijen & Nieuwmegen, 1980) and that haptenic molecules associated with liposomal membrane surfaces can elicit a hapten-specific PFC response (Uemura, Nicolotti, Six & Kinsky, 1974; Yasuda, Dancey & Kinsky, 1977). While the possibility cannot be excluded that some of the BSA molecules were adsorbed to the membrane surfaces (Hoekstra & Scherphof, 1979), the method of preparing the LSM-BSA used in the present study, favoured the entrapment of protein molecules *within* the hydrophilic compartments of the liposomal vesicles (Gregoriadis & Allison, 1974; Tyrrell *et al.*, 1976). Clearly then, antigenic determinants within, as well as on the surface of liposomal membranes, can provoke an enhanced immune response. This requirement for a physical association between the antigen and the liposomal membrane, suggests that the liposomal vesicle may facilitate the processing, and possibly the recognition, of the antigenic moieties by the interacting cells. Liposomes, injected intraperitoneally, preferentially activate peritoneal macrophages (Fidler, Hart, Raz, Fogler, Kirsh & Poste, 1980). Thus an accessory cell (A cell) might well be the first cell to encounter and process the i.p. injected LSM-BSA. A role for the participation of A cells is strengthened by the demonstrated dependence of the LSM-BSA response on T-cell function (Table 1).

Like most other serum proteins, BSA is a T cell-dependent antigen (Taylor, 1969; Allison & Davies, 1971). Its requirement for T-cell function is not bypassed even when the antigenic molecules are presented in association with phospholipid vesicles. The importance of T-cell participation in generating a BSA-specific response to LSM-BSA, is evidenced by the small PFC response of low responder mice having impaired T-mitogen responsiveness (Fig. 4) and by the apparent failure of athymic nude animals to generate a significant PFC response (Table 1). In contrast to this observation for a protein antigen, the association of haptenic molecules with liposomes has been reported to induce a T independence to the hapten-specific antibody response (Yasuda *et al.*, 1979; Van Houte *et al.*, 1979). This descrepancy may be due to a difference in the location of the antigenic determinants associated with the liposomal vesicles. Whereas haptenic groups were substituted predominantly on the surface of the liposomal membranes, BSA molecules were entrapped primarily within the hydrophilic compartments of the liposomes. Haptenic surface substitution

could conceivably facilitate direct presentation of the antigen to B cells, thus bypassing A-cell processing and T-cell help (Yasuda *et al.*, 1979). On the other hand, internal entrapment of the BSA could prevent the antigen from being directly accessible to B cells. Thus, it is more likely that liposome-entrapped BSA must first be processed by macrophages or other accessory cells before triggering the collaboration between T and B cells.

The application of the PFC technique in evaluating the immunopotentiating capacity of liposome-associated antigen, provides information which may not be readily available from serological analysis. In the latter, extraneous antigen may bind to circulating antibodies and thus mask the actual magnitude of the antibody response. Evaluation of the PFC response, on the other hand, permits a quantitative assessment of the cellular kinetics occurring during the course of antibody formation. In concert with cell separation and reconstitution techniques, this approach should facilitate future studies to understand better the cellular mechanisms underlying the immunopotentiation process mediated by liposome-associated protein antigens.

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