

# Liposomes as vehicle for allergen presentation in the immunotherapy of allergic diseases

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Liposomes are non-toxic, biodegradable and weakly immunogenic lipid vesicles which can be used as immunomodulating agents. In the present study, multilamellar vesicles (MLV) and small unilamellar vesicles (SUV) were used to incorporate an allergenic protein from *Artemisia scoparia* pollen. MLV incorporated more allergenic protein than SUV. To assess the immunomodulating effect of allergen entrapped in liposomes, Swiss strain mice (made IgE responders) were injected with either free allergen or liposome-entrapped allergen (LEA) and their immune response was measured in terms of specific IgG and specific IgE levels. Results indicated that specific IgE response was significantly lower in mice injected LEA ( $P < 0.02$ ) than in mice injected free allergenic protein. Although specific IgG response was higher in mice injected LEA, there was no statistically significant difference between the two groups. Potential use of liposomes as non-immunogenic biocompatible vehicle for antigen presentation in immunotherapy will be discussed.

**Key words:** allergen; immune response; immunotherapy; liposome.

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Liposomes are artificial lipid vesicles where the lipids are arranged in a continuous bilayer configuration encapsulating an aqueous space. A wide range of water soluble molecules of varying sizes from small ions to macromolecules can be entrapped in liposomes. Being prepared from naturally occurring lipids, liposomes are non-toxic, biodegradable and weakly immunogenic (9). The advantage of liposomes as a carrier system resides in the compatibility of its constituents with the body.

Allison & Gregoriadis (2) reported that administration of antigen entrapped in liposomes induces a greater amount of antibodies than are elicited by the same dose of free antigen. Further, it was demonstrated that liposomes do not appear to induce anaphylaxis when injected intravenously into the immunized animals (10). Wagner et al. (27) reported that allergen entrapped in liposomes avoids anaphylactic reactions in sensitive animals. These observations

suggest that liposome-entrapped allergen induces an immune response and at the same time reduces allergic reactions.

In immunotherapy of type I disorder there is an initial rise in specific IgE antibody due to the increased dose of antigen causing systemic reactions and, therefore, the required dose for treatment is not achieved. Potential use of liposome-entrapped allergen (LEA) in inducing the antibody formation combined with its anti-anaphylactic property might enhance the efficacy and safety of immunotherapy. Numerous attempts have been made to suppress or diminish the IgE antibody response by administration of chemically modified allergens (8, 19); as yet there is no suitable method without any drawbacks. Thus, newer methods must be developed to improve immunotherapy procedure.

We report the preparation of different types of multilamellar and small unilamellar vesicles by entrapping allergenic protein from the pollen

of *Artemisia scoparia*. The effect of LEA on its immune response in terms of specific IgG and IgE was studied in Swiss strain mice by making them responders with cyclophosphamide treatment on day 7 of the experiment. The use of liposomes in immunotherapy will be discussed.

## MATERIAL AND METHODS

**Material.** Egg phosphatidylcholine (PC), phosphatidic acid (PA) and cholesterol (CHOL) were obtained from CSIR Centre for Biochemicals, India. Dicetyl phosphate (DCP) and stearylamine (SA), mouse IgG and anti-mouse IgG alkaline phosphatase conjugate were obtained from Sigma Chemical Co., USA. Anti-mouse IgE was obtained from Nordic Immunochemicals, Netherlands. All other chemicals used were reagent grade.

**Allergen extract.** *Artemisia scoparia* pollen was extracted and semi-purified according to the method of Jaggi & Gangal (14). Briefly, defatted *Artemisia scoparia* pollen was extracted in phosphate buffered saline overnight and centrifuged. The supernatant was saturated to 90% ammonium sulphate. After centrifugation the pellet was resuspended and dialysed against distilled water until free from ammonium sulphate as checked by chemical methods to obtain a crude allergenic protein extract (CAP).

**Preparation of liposome-entrapped allergen.** Liposomes were prepared according to the method of Gregoriadis & Ryman (12) with some modification. Negatively charged, positively charged and neutral liposomes were prepared with phosphatidylcholine: cholesterol: phosphatidic acid (molar ratio 7:2:2); phosphatidylcholine: cholesterol: stearylamine (molar ratio 7:2:2) and phosphatidylcholine: cholesterol (molar ratio 7:2) respectively. Briefly, a thin dry film consisting of lipid mixture was dispersed in PBS (phosphate buffer 50 mM, saline 0.15 M, pH 7.4) containing 5 mg of allergen. The lipid suspension was sonicated for 1 min (30 s + 30 s) with an interval of 1 min. Liposomes were sedimented at  $105,000 \times g$  for 45 min in an ultracentrifuge and untrapped protein was removed by wash-

ing the liposome pellet twice with PBS. However, some of the protein may associate with the lipid bilayer. Small unilamellar vesicles with different charges were prepared with the same lipids with the help of liposomat (Dianorm, München, Germany). The lipid films were dissolved in the deoxy cholate in the ratio of lipid: detergent at 0.6 and then the same amount of lyophilised protein was added. Detergent was dialysed against PBS for 2½ h and the material was loaded on the Sephacryl S-300 column (1.5 × 40 cm). The void volume peak was collected and the protein estimated as given below.

**Protein estimation.** The amount of protein entrapped in the vesicle was determined by the procedure of Lowry et al. (15) after disrupting the liposome pellet using 0.9% sodium dodecyl sulphate (SDS) and taking liposome with buffer as control. The control contains the same amount of lipid and SDS as that of the test.

**Immunization protocol.** Depending on the degree of magnitude of IgE antibody production against an allergen, inbred mice strains were classified into low and high responders. In the present study this was carried out in Swiss strain mice (low responders to IgE) and these were given cyclophosphamide to make them high responders. For treatment with cyclophosphamide Swiss strain mice of both sexes weighing 20–25 g were divided randomly into three groups of 12 mice each. Animals were injected with different quantities of cyclophosphamide, i.e. 75 mg/kg body weight (Group I), 100 mg/kg body weight (Group II) and 125 mg/kg body weight (Group III). The priming of the animals was done ip on day 0 with 50 µg of CAP, and cyclophosphamide was injected on day 7 of the experiment. Mice were boosted with the same amount of CAP subcutaneously on day 9. Mice were killed on days 15, 23 and 30 and blood was collected by heart puncture. Serum was separated from blood and stored at -70°C until used for specific IgG and specific IgE assays.

In another experiment Swiss mice of both sexes weighing 20–25 g were divided randomly into two groups of 16 animals each. Group I animals were injected with free CAP and Group

II animals with liposome-entrapped CAP. Mice were primed with 50 µg of CAP ip on day 0. Cyclophosphamide was injected on day 7 and mice were boosted on days 9 and 22 with the same amount of CAP. Four animals at a time were killed on days 15, 21, 29 and 35. Blood was collected by heart puncture. Serum was separated from blood and stored at -70°C until used for measuring specific IgG and specific IgE by ELISA.

*Serum antibody analysis.* Serum-specific IgG and specific IgE antibodies against CAP were determined by the method of Malley et al. (16). Briefly, microtitre plates (Dynatech, polyvinyl coated) were coated with 50 µg of antigen in carbonate buffer (pH 9.6). The plates were washed with PBS. The remaining sites were blocked by 1% bovine serum albumin in PBS for 2 h at room temperature. The plates were again washed and incubated overnight with serum dilutions. After additional washing, microtitre plates were incubated for 3 h at room temperature with purified antibody-enzyme conjugate (rabbit anti-mouse IgG alkaline phosphatase Sigma Chemical Co. USA, or goat anti-mouse IgE peroxidase conjugate, Nordic Immunologicals, Netherlands; dilution 1:600). The suitable substrates were added and the reaction stopped after the appropriate time. The colour developed was read on a Nunc ELISA reader. All serum samples were tested in triplicate and read against control (sera from unimmunized mouse). Serum-specific IgG concentration was calculated by the standard curve

using mouse IgG (Sigma Chemical Co., USA). However, the concentration of IgE could not be calculated due to the non-availability of mouse IgE, thus, optical density has been taken as a measure of IgE concentration. The anti-mouse IgE used, however, did not show any cross-reactivity with mouse IgG. As the measurement of specific IgE and specific IgG was determined at the same time for the entire experiment the time required for incubation and colour development remains the same.

#### Statistical analysis

As the size of the sample was small Student's *t* distribution was applied. Student's *t* distribution in which the difference between the means of two independent samples was taken for statistical analysis.

## RESULTS

Tables 1 and 2 show the capacity of different types of liposomes, i.e. multilamellar and small unilamellar vesicles, with different charges for entrapping allergenic protein. It was observed that negatively charged liposomes entrapped maximum amount of protein compared with neutral or positively charged liposomes. Negatively charged liposomes with DCP incorporate more protein than liposomes prepared from PA. It was also observed that MLV entrapped more protein than SUV. In the present study MLV prepared from PC:CHOL:PA were used for immunization.

Of the three concentrations of cyclophosphamide used to make mice high IgE responders, it was observed that at the concentration of 75

Table 1.  
Capacity of multilamellar vesicles to entrap allergenic protein of *Artemisia scoparia* pollen

| Lipids      | Molar ratio | Charge of vesicles | µg Protein/mg lipid |
|-------------|-------------|--------------------|---------------------|
| PC:CHOL     | 7:2         | neutral            | 9.80                |
| PC:CHOL:SA  | 7:2:2       | pos.               | 10.03               |
| PC:CHOL:PA  | 7:2:2       | neg.               | 12.60               |
| PC:CHOL:DCP | 7:2:2       | neg.               | 20.05               |

PC: Egg phosphatidylcholine (natural lipid)  
CHOL: Cholesterol (natural lipid)  
PA: Phosphatidic acid (natural lipid)  
DCP: Dicetyl phosphate (synthetic lipid)  
SA: Stearylamine (synthetic lipid)

Table 2.  
Capacity of small unilamellar vesicles to entrap allergenic protein of *Artemisia scoparia* pollen

| Lipids     | Molar ratio | Charge of vesicles | µg Protein/mg lipid |
|------------|-------------|--------------------|---------------------|
| PC:CHOL    | 7:2         | neutral            | 7.60                |
| PC:CHOL:SA | 7:2:2       | pos.               | 6.80                |
| PC:CHOL:PA | 7:2:2       | neg.               | 8.00                |

For abbreviations see Table 1

Table 3.

Mean specific IgG titre with different quantities of cyclophosphamide injected ip

| Day of exp. | G I              | G II              | G III             |
|-------------|------------------|-------------------|-------------------|
|             | 75 mg/kg body wt | 100 mg/kg body wt | 125 mg/kg body wt |
| 15          | 0.684±.085       | 0.628±.067        | 0.472±.041        |
| 23          | 0.418±.018       | 0.241±.046        | 0.198±.026        |
| 30          | 0.299±.032       | 0.208±.009        | 0.105±.005        |

Swiss strain mice were divided into three groups of 12 animals each. Mice were primed on day 0 and boosted on day 9 with *Artemisia scoparia* pollen allergenic protein. Cyclophosphamide was injected ip on day 7 of the experiment and four mice were killed on days 15, 23 and 30 to estimate specific IgG in serum. Data is expressed as mean±SEM.

mg/kg body weight, cyclophosphamide was capable of inducing maximum amount of mean specific IgE against the allergen compared with concentrations of 100 and 125 mg/kg body weight (Table 4). It was also observed that specific IgG response was higher in animals injected with 75 mg/kg body weight of cyclophosphamide and the response persisted for a longer time (Table 3). In this study, Swiss mice were made IgE responders by injecting cyclophosphamide at a concentration of 75 mg/kg body weight on day 7 of the experiment, which gives a reasonable response for both specific IgE and specific IgG antibodies.

Fig. 1 shows the calibration curve for specific IgG in which mouse IgG was taken as standard. It shows the linearity between the 50–200 ng of mouse IgG. Fig. 2 shows the mean specific IgE response of mice injected either LEA or free allergen. It was observed that serum specific IgE levels were lower in animals given LEA than in animals given free allergenic protein. The data shows statistically significant difference at  $P < 0.02$  on all days at which response was measured. It was also observed that mean specific IgG levels were higher in mice given LEA than in mice given free allergenic protein

(Fig. 3). However, the difference was not statistically significant. The ratio of serum specific IgG to specific IgE was found to be significantly higher in mice injected allergen entrapped in liposomes than in mice injected free allergenic protein (Fig. 4).

## DISCUSSION

We report that allergenic protein from the pollen of *Artemisia scoparia* can be entrapped in liposomes. LEA are significantly less allergenic ( $P < 0.02$ ) and more immunogenic than free allergen in Swiss mice made responders with cyclophosphamide treatment on day 7 of the experiment.

The entrapment of *Artemisia scoparia* pollen allergenic protein showed that negatively charged liposomes entrap maximum amount of allergen compared with positively charged or neutral liposomes (Table 1). Negatively charged liposomes prepared with DCP entrapped more allergen than negatively charged liposomes prepared with phosphatidic acid (Table 1). In the present study, however, PA liposomes were used for the animal experiments because DCP and SA (synthetic lipids) liposomes have been

Table 4.

Specific IgE titre with different quantities of cyclophosphamide injected ip

| Day of exp. | G I              | G II              | G III             |
|-------------|------------------|-------------------|-------------------|
|             | 75 mg/kg body wt | 100 mg/kg body wt | 125 mg/kg body wt |
| 15          | 0.422±.019       | 0.291±.025        | 0.248±.008        |
| 23          | 0.153±.030       | 0.087±.018        | 0.065±.001        |
| 30          | 0.095±.012       | 0.000             | 0.000             |

For estimating specific IgE in serum, see procedure in Table 3.

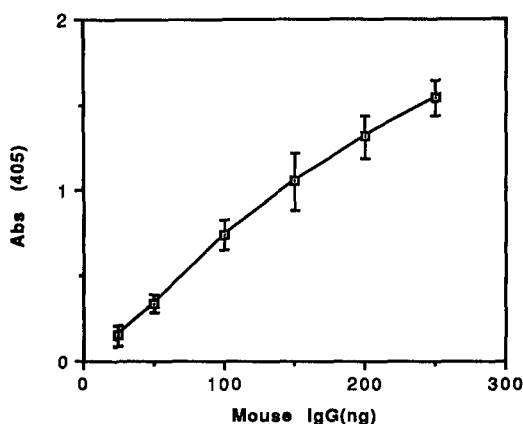


Fig. 1. Calibration curve of mouse IgG showing a response between 25-250 ng. Each value represents a mean of three wells. Values shown in graph represent mean  $\pm$  SEM.

found to be toxic at higher concentrations (17). Antibodies against DCP or SA might also be produced. As liposomes are to be widely administered clinically, the liposomes used must be those with the least potential for direct cellular toxicity. It was also observed that MLV entrapped 2-3 times more protein than SUV (Tables 1 and 2). In contrast McWilliam & Stewart (18) reported that SUV liposomes en-

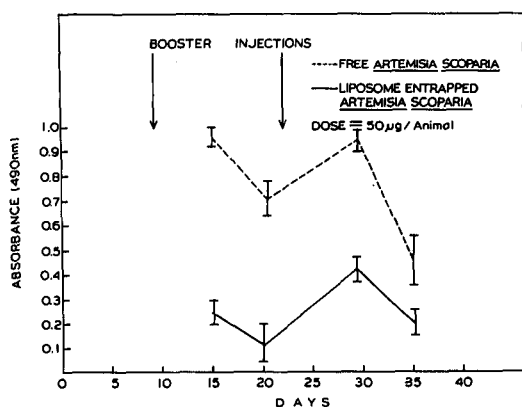


Fig. 2. Mean specific IgE response in Swiss strain mice on days 15, 21, 29 and 35 of experiment. Mice were injected with either free allergen or liposome-entrapped allergen on days 0, 9 and 22. Mice were injected 75 mg/kg body weight of cyclophosphamide on day 7 to make them IgE responders. Data is expressed as mean  $\pm$  SEM.

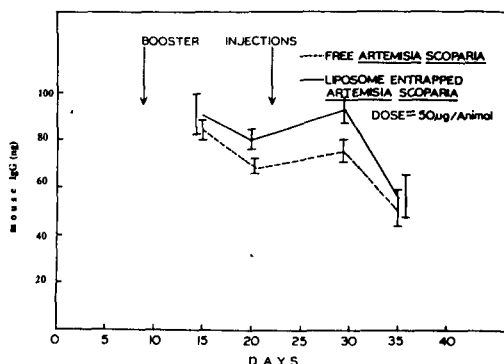


Fig. 3. Mean specific IgG response in Swiss strain mice on days 15, 21, 29 and 35 of experiment. Mice were injected with either free allergen or liposome-entrapped allergen on days 0, 9 and 22. Mice were injected 75 mg/kg body weight of cyclophosphamide on day 7 to make them high IgE responders. Data is expressed as mean  $\pm$  SEM.

trap more protein. Other investigators have also shown that MLV entrap more protein than SUV (22, 26).

Of the three concentrations of cyclophosphamide used to make Swiss mice responders to IgE, 75 mg/kg body weight was found to be optimum for persistent IgE response in mice. With higher doses of cyclophosphamide, specific IgE response was comparatively lower and at the same time the immunogenic response (specific IgG) was also reduced. Therefore, to make

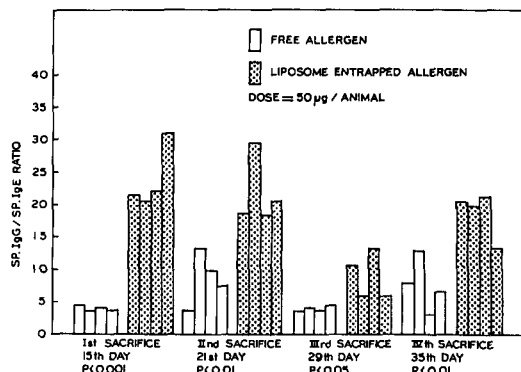


Fig. 4. Ratio of serum specific IgG to specific IgE of each mouse on days 15, 21, 29 and 35 of the experiment. Mice were given injection on days 0, 9 and 22.

mice IgE responders the lowest concentration of cyclophosphamide was used in this study (Tables 3 and 4). The results were in agreement with Chirrozi et al. (5) who showed that higher exposure to X irradiation resulted in significant suppression of both IgG and IgE responses. Murine IgE antibody responses differ among inbred strains (6). Low IgE responder phenotype mice produce low levels of IgE and normal levels of IgG, while high IgE responder phenotype mice produce high persistent levels of IgE to a variety of antigens (23). The removal of the suppressive mechanism by exposure to moderate doses of cyclophosphamide or X irradiation resulted in increased IgE biosynthesis (6, 20). The procedure preferentially removes the suppressive effect produced by suppressor T cells which are involved in IgE synthesis (5). Akaski & Ishizaka (1) reported that cyclophosphamide treatment switched Lyt 1 + Fc gamma R + T cells from forming glycosylation inhibiting factor to forming glycosylation enhancing factor.

The present study has investigated the role of repeated injections of LEA in modulating the effect of allergen in immunotherapy. It was observed that in Swiss mice (made IgE responders) serum specific IgE response was significantly lower in mice injected allergen entrapped in liposomes than in mice injected free allergenic protein ( $P < 0.02$ ). Earlier experiments in Swiss mice, made IgE responders by injecting cyclophosphamide 2 days before starting the immunization schedule, have shown that IgE levels were not statistically significant but were lower in the group injected with LEA than in the group injected with free allergen (3, 4). PCA results have shown that LEA allergen was 10 times less allergenic than the free allergenic protein (3). The decrease in specific IgE levels may be due to the sequestration of allergen from the immune system until it reaches the macrophages (27), or to the activation of T suppressor cells. It has been suggested that the suppression of IgE antibody formation in the hydrophobic environment is due to the generation of suppressor T cells (21).

The immunogenicity as measured in terms of serum specific IgG was higher in animals injected allergen entrapped in liposomes than in

mice injected with free allergenic protein, but it is not statistically significant which may be due to the effect of cyclophosphamide. High levels of specific IgG response might be due to the slow release of allergen from the site of injection, or the way in which allergen is presented to the antigen presenting cells, which preferentially synthesize IgG antibody. It also might be due to the adjuvant effect of liposomes. Numerous studies have reported that liposome-entrapped antigen increases specific IgG antibody response, but its mechanism is not clear (10, 23). Liposomes have been shown to possess adjuvant properties (2, 13). Adjuvant activity of liposomes depends on phospholipid content, liposome size and structure, and the way in which antigen is incorporated (25). Davis & Gregoriadis (7) showed that liposomal adjuvanticity can be influenced by the Tc of the phospholipid component. Gregoriadis & Allison (10) reported that entrapment of diphtheria toxoid in liposomes did not prevent an immune response; however, it inhibited the interaction of the antigen with its antibodies *in vivo* upon repeated challenge and prevented severe or fatal allergic reactions. Thus, liposomes somehow modulate the immune response. The results support those of Allison & Gregoriadis (2) who showed that administration of antigen entrapped in liposomes elicited higher antibody titre than was elicited by the same dose of free allergen.

In summary this study has shown that it is possible to entrap allergen from the pollen of *Artemisia scoparia*. Immune response is modulated by LEA, which showed an increase in specific IgG and diminished specific IgE in mice injected with cyclophosphamide on day 7 of the experiment. Therefore, LEA may prove to be safe and effective for reducing systemic reactions and thereby increasing the efficacy of immunotherapy. Thus, the use of liposomes may be a novel approach to antigen presentation in immunotherapy of allergic diseases.

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