

Efficacy of liposome entrapped allergen in down regulation of IgE response in mice

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Summary

The effect of liposome entrapped allergen on primary, secondary and ongoing IgE response was studied in mice. It was observed that mice primed with liposome entrapped allergen maintained significantly lower levels of serum specific IgE as compared to that of controls primed with allergen. Although alum adsorbed allergen could induce IgE synthesis in mice primed with liposome entrapped allergen the increase in serum specific IgE levels was lower than the animals primed and challenged with alum adsorbed allergen. On the contrary when BALB/c mice were primed with alum adsorbed allergen to induce IgE synthesis subsequent challenge by liposome entrapped allergen could down regulate the serum specific IgE response. Serum specific IgG response did not show any significant difference between the two groups. There was an increase in T suppressor cell subpopulation as measured by immunofluorescence technique in mice injected liposome entrapped allergen as compared to that in controls. The results indicate that liposome entrapped allergens may prove to be safe and effective in immunotherapy by reducing the allergenic response of the allergen at the same time increasing the antigenicity.

Clinical and Experimental Allergy, Vol. 22, pp. 35–42. Submitted 29 June 1990; revised 17 January 1991; accepted 11 July 1991.

Introduction

Numerous studies have shown that liposomes act as an adjuvant for antibody enhancement [1–3]. The advantage of liposomes as a carrier system resides in the compatibility of its constituents with the body. Being made up of naturally occurring lipids, liposomes are biodegradable, non-toxic and very feebly immunogenic [4]. Gregoriadis and Allison reported that the entrapment of diphtheria toxoid in liposomes reduced or eliminated hypersensitive reactions at the site of the injection thus preventing the development of severe or even fatal allergic reactions [5]. Elimination of anaphylactic reactions in ragweed sensitive guinea pigs after injection of liposome entrapped ragweed allergen has also been observed by Wagner *et al.* [6].

Anaphylactic reactions in Type I disorders such as hayfever, extrinsic asthma, drug hypersensitivity etc. are mediated by specific IgE antibodies. Several attempts have been made to suppress or modulate IgE antibody

response by administration of chemically modified allergens [7–11]. Our preliminary studies have shown that injection of liposome entrapped allergen reduced serum specific IgE levels in mice [12]. In the present study, we report the effect of liposome entrapped allergen in reducing primary IgE response and induced ongoing IgE response and discuss its mode of action and scope in relation to immunotherapy of Type I allergic disorders.

Materials and methods

Allergen extract

Artemisia scoparia pollen was extracted and allergenic protein was purified as reported elsewhere [13]. 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 to get a crude allergenic protein extract (CAP). The crude allergenic preparation was fractionated on DEAE Sephadex A-50 and Sephadex G-50 column. The purified, highly potent allergenic protein fraction obtained was denoted as Art VIb (Art S1B as per IUIS).

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Preparation of liposome-entrapped allergen

Liposomes were prepared according to the method of Gregoriadis and Ryman with some modification [14]. Negatively charged liposomes were prepared with phosphatidylcholine:cholesterol:phosphatidic acid (molar ratio 7:2:2). 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 briefly (30 sec + 30 sec). Liposomes formed were sedimented at $105\,000 \times g$ for 45 min in an ultracentrifuge and untrapped protein was removed by washing the liposome pellet twice with PBS. However, some of the protein may associate on the lipid vesicles. Protein was estimated in the vesicles by the procedure of Lowry *et al.* [15] after disrupting the liposomes with equal volume of 0.9% sodium dodecylsulphate taking appropriate blanks.

Immunization protocol

In a preliminary experiment it was observed that 2.5 µg of allergen was sufficient to produce allergen specific IgE (sp.IgE) and specific IgG (sp.IgG) response in BALB/c mice. BALB/c mice were divided randomly into two groups of 14 animals each. Animals of both the groups were primed intraperitoneally (i.p.) with 2.5 µg of allergen adsorbed on 4 mg of alum. Four animals were killed on the seventh day and serum was collected to monitor sp.IgG and sp.IgE levels. Group I and group II animals were challenged subcutaneously on the eighth day with 2.5 µg of alum adsorbed allergen and liposome-entrapped allergen respectively. Four animals of each group were subsequently killed on the 12th, 17th and the 25th day of the experiment and serum was collected to estimate sp.IgG and sp.IgE levels.

In another experiment BALB/c mice were randomly divided into two groups of 14 animals each. Animals of both the groups were injected i.p. with 2.5 µg of liposome-entrapped allergen. Four animals were killed on the seventh day to monitor serum sp.IgG and sp.IgE levels. Group I and group II animals were challenged subcutaneously on the eighth day of the experiment with 2.5 µg of liposome-entrapped allergen and alum adsorbed allergen respectively. Four animals of each group were sacrificed on the 12th, 17th and the 25th day of the experiment and serum was obtained to estimate sp.IgG and sp.IgE levels.

In the third experiment BALB/c mice of either sex weighing 20 to 25 g were divided randomly into three groups. On day 0 group I was injected i.p. with 2.5 µg of free Art VIb, group II animals were injected i.p. with 2.5 µg of alum adsorbed allergen and group III animals were injected i.p. with the same quantity of liposome entrapped allergen. Four animals were killed from each group and

serum levels of sp.IgG and sp.IgE were monitored and the remaining animals in each group were boosted with the respective type of allergen preparation given before. Four animals were killed on the 14th day to quantitate secondary immune response in terms of sp.IgG and sp.IgE. Group I and III animals were then challenged with 2.5 µg alum adsorbed allergen and group II animals were challenged with an equivalent quantity of liposome-entrapped allergen. Four animals of each group were thereafter killed on the 28th, 35th and 42nd day of the experiment. Serum sp.IgG and sp.IgE levels were estimated.

T cell subset studies

BALB/c animals were divided into two groups of 12 animals each and were primed (i.p.) either with 2.5 µg of liposome-entrapped allergen or of equal quantity of free allergen on the day 0. Animals were given booster injections on the seventh and the 14th day of the experiment with the same type of antigen preparation. Six mice from each group were killed on the 13th and the 20th day of the experiment and the blood was collected in heparin (25 units/ml) for T helper and T suppressor subset assay.

Separation of lymphocytes from the blood

The lymphocytes were separated from the heparinized blood using Isopaque (Sigma Chemical Co., St Louis, Missouri, U.S.A.), gradient centrifugation as described by Boyum [16]. Blood (2.5 ml) was diluted with an equal volume of PBS. The diluted blood was layered on 5 ml isopaque solution and centrifuged at $400 \times g$ r.p.m. for 45 min at room temperature. The white opaque ring of lymphocytes was carefully transferred into Hanks' buffered salt solution (HBSS), mixed gently and centrifuged. The cell pellet so obtained was washed twice with HBSS and resuspended in HBSS containing 5% fetal calf serum to check viability with 0.2% Trypan Blue. It was observed that more than 90% of the cells were viable.

T cell subsets were identified by the two step immunofluorescence technique [17]. Approximately 10^5 lymphocytes were taken and monoclonal antibodies against helper T cell (Rat anti-mouse IgG2b L3/T4, 1:50 dilution, Sera Lab., Sussex, U.K.) or suppressor T cell (Rat anti-mouse IgG2b Lyt 2+, 1:50 dilution, Sera Lab., Sussex, U.K.) were added. The tubes were incubated at 4°C for 1 hr. Cells were washed three times with HBSS and 20 µl of FITC anti-rat antibody was added (1:10 dil, Sera Lab., Sussex, U.K.). The tubes were incubated at 4°C for 1 hr and cells were washed three times with HBSS. The preparation was then examined under immunofluor-

escence microscope (Olympus, Japan). Different fields in the slide were observed for total and fluorescing lymphocytes. A minimum of 200 cells were counted and the percentage of fluorescing lymphocytes was calculated. Statistical analysis was not carried out in this experiment because the blood was pooled for isolating lymphocyte population.

Serum antibody analysis

Serum specific IgG and sp.IgE antibodies against Art VIb were determined by ELISA [18]. Briefly, microtitre plates were coated with 10 µg/well of antigen (Art VIb) in carbonate buffer (pH 9.6). The plates were washed with PBS. The remaining sites were blocked by 1% bovine serum albumin in PBS. The plates were again washed and incubated overnight with serum dilutions (1:1 for IgE and 1:10 for IgG) arrived after trial with various dilutions of the sera. After additional washing microtitre plates were incubated for 3 hr at room temperature with respective purified antibody-enzyme conjugate (rabbit anti-mouse IgG alkaline phosphatase, Sigma Chemical Co., St Louis, Missouri, U.S.A. or goat antimouse IgE peroxidase conjugate, Nordic Immunologicals, Netherlands; dilution 1:600). The appropriate substrates were added and the reaction was stopped after appropriate time. The colour developed was read on Nunc ELISA reader. All serum samples were tested in triplicate. Serum sp.IgG concentration was calculated by the help of standard curve using mouse IgG (Sigma Chemical Co., St Louis, Missouri, U.S.A.). A known amount of mouse IgG was applied on the plate. However, the absolute IgE concentration could not be calculated due to the non-availability of mouse IgE to us to construct the calibration curve and hence optical density has been taken as a measure of concentration of IgE. The anti-mouse IgE used however, did not show any cross-reactivity with mouse IgG. All the IgE measurements in each experiment were done simultaneously on the same plate for both groups to avoid any variation from plate to plate or due to other conditions. The control was a mouse serum of the same dilution against which absorbance was measured for both IgG and IgE.

Statistical analysis

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

Results

IgE measurements have earlier been reported on the basis

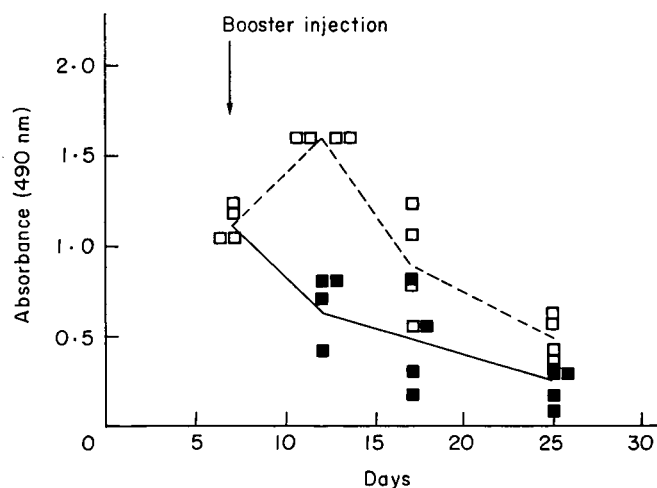


Fig. 1. BALB/c mice were primed with 2.5 µg of alum adsorbed allergen and subsequently challenged on the 8th day with either alum adsorbed allergen or liposome entrapped allergen. Specific IgE levels were measured by ELISA on the 7th, 12th, 17th and 25th day of the experiment. □, —, Al (OH)₃ adsorbed ART VIb; ■, —, AL (OH)₃ adsorbed ART VIb.

of PCA reaction which is difficult to quantitate. Recently, quantitative sp.IgE and sp.IgG measurements based on ELISA have been reported [19]. The allergenic response in terms of sp.IgE was therefore measured in terms of serum sp.IgE and that of antigenic response in terms of serum sp.IgG by ELISA.

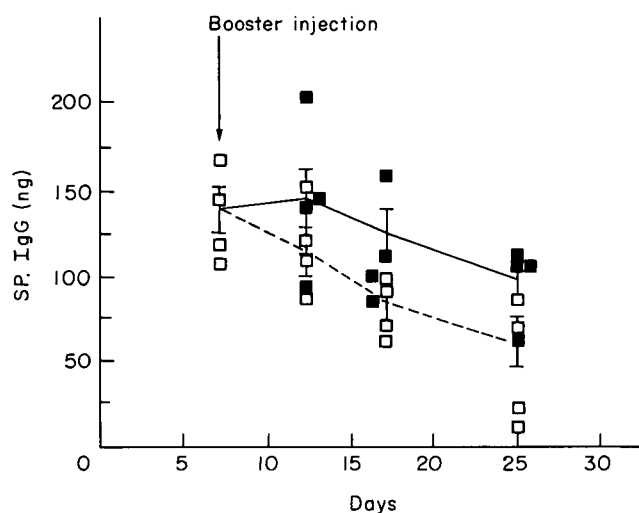


Fig. 2. BALB/c mice were primed with 2.5 µg of alum adsorbed allergen and subsequently challenged on the 8th day with either alum adsorbed allergen or liposome entrapped allergen. Specific IgG levels were measured by ELISA on the 7th, 12th, 17th and 25th day of the experiment. For key, see Fig. 1.

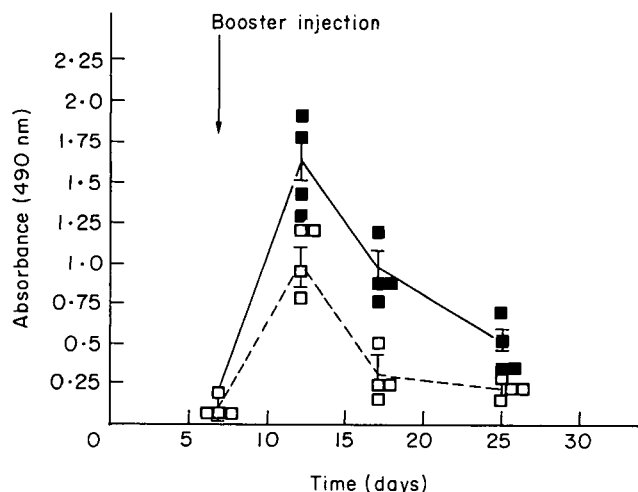


Fig. 3. BALB/c mice were primed with 2.5 µg of liposome entrapped allergen and subsequently challenged with the same quantity of protein on the 8th day either as liposome entrapped allergen or alum adsorbed allergen. Specific IgE levels in serum were measured by ELISA on the 7th, 12th, 17th and 25th day of the experiment. □, —, liposome entrapped ART VIb; ■ —, AL (OH)₃ adsorbed ART VIb.

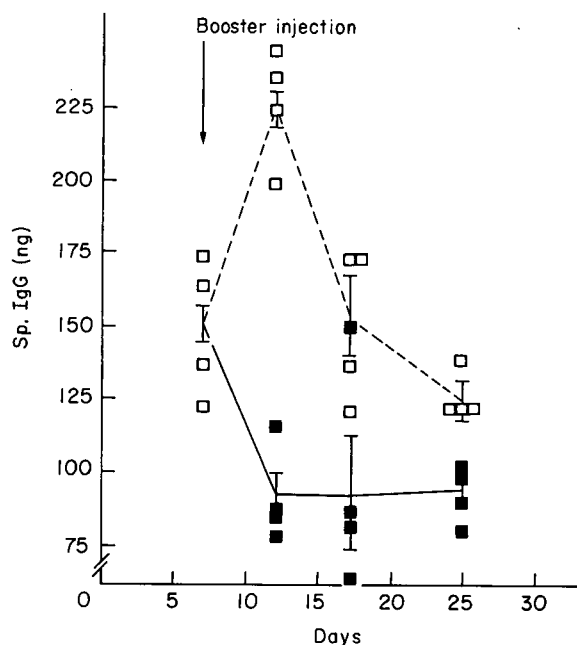


Fig. 4. BALB/c mice were primed with 2.5 µg of liposome entrapped allergen and subsequently challenged with the same quantity of protein on the 8th day either as liposome entrapped allergen or alum adsorbed allergen. Specific IgG levels in serum were measured by ELISA on the 7th, 12th, 17th and 25th day of the experiment. For key, see Fig. 3.

Down-regulation of ongoing IgE response by liposome entrapped allergen

Figures 1 and 2 show serum sp.IgE and serum sp.IgG in mice primed with alum adsorbed allergen and challenged with either liposome entrapped allergen or alum adsorbed allergen. Animals primed and challenged with alum adsorbed allergen showed a significant increase ($P < 0.01$) in the serum sp.IgE antibody levels on the 12th day as compared to those on the 7th day. It was observed that animals primed with alum adsorbed allergen and challenged with liposome-entrapped allergen showed a statistically significant decrease ($P < 0.01$) in the serum sp.IgE antibody levels as compared to those before challenge. On the contrary serum sp.IgG titre in this group showed an increase (Fig. 2). However, this increase was not statistically significant but it revealed that the antigenicity was not affected as measured in terms of serum specific IgG.

The ratio of optical density of serum sp.IgG to sp.IgE was significantly higher in the animals challenged with liposome entrapped allergen as compared to those in animals challenged with equal quantity of alum adsorbed allergen ($P < 0.05$).

Induction of IgE response by alum adsorbed allergen in animals sensitized with liposome entrapped allergen

It was observed that priming of mice with liposome-entrapped allergen did not prevent induction of IgE by alum adsorbed allergen (Fig. 3). Serum sp.IgE levels were lower in the animals primed and challenged with liposome entrapped allergen as compared to that in animals primed with liposome entrapped allergen and challenged with alum adsorbed allergen. Statistically significant difference was observed in the sp.IgE levels in the two groups on subsequent days of the experiment ($P < 0.02$).

It was observed that serum sp.IgG levels were higher in mice primed and challenged with liposome-entrapped allergen as compared to those of animals primed with liposome entrapped allergen and subsequently challenged with alum adsorbed allergen (Fig. 4). This difference was statistically significant on the 12th day of the experiment ($P < 0.001$).

The ratio of sp.IgG to sp.IgE in animals primed and challenged with liposome entrapped allergen was four to six times higher than those in animals challenged with alum adsorbed allergen ($P < 0.01$). IgG/IgE ratio has been reported to be a better indicator of clinical improvement than IgG or IgE measured separately [20].

Effect of priming and challenge of different allergen preparations on secondary immune response in mice

Table 1 shows the serum sp.IgE response in animals primed and boosted with free allergen, alum adsorbed

Table 1. Mean serum specific IgE levels in BALB/c mice before and after challenge

Day of experiment	GI free allergen	GII alum adsorbed allergen	GIII liposome entrapped allergen
7	0.267 ± 0.005	0.641 ± 0.038	0.214 ± 0.016
14	0.546 ± 0.009	0.780 ± 0.034	0.229 ± 0.031
Challenged the mice on 21	Alum adsorbed allergen	Liposome entrapped allergen	Alum adsorbed allergen
28	0.813 ± 0.101	0.334 ± 0.052	0.712 ± 0.029
35	0.512 ± 0.063	0.252 ± 0.022	0.442 ± 0.028
42	0.210 ± 0.016	0.131 ± 0.014	0.212 ± 0.049

Serum dilution 1:1.

IgE values are expressed as optical density at 490 nm which is the lambda-max for the substrate used. Animals were injected on 0 day of the experiment and boosted on 8th day of the experiment. Mice were sacrificed on 7th and 14th day of the experiment and the remaining animals were challenged on 21st day of the experiment. Values are expressed as ± s.e.m.

Table 2. Mean serum specific IgG levels in BALB/c mice before and after challenge

Day of experiment	GI free allergen	GII alum adsorbed allergen	GIII liposome entrapped allergen
7	95.3 ± 2.67	145.3 ± 11.7	105.8 ± 9.46
14	120.1 ± 1.46	158.67 ± 9.59	163.9 ± 6.26
Challenged the mice on 21	Alum adsorbed allergen	Liposome entrapped allergen	Alum adsorbed allergen
28	154.2 ± 7.19	152.9 ± 3.99	126.6 ± 3.86
35	127.4 ± 10.9	105.3 ± 9.33	112.2 ± 12.1
42	67.9 ± 4.39	79.90 ± 12.1	106.6 ± 1.46

Serum dilution 1:10.

IgG values are expressed in nanograms of mouse IgG. Animals were injected on 0 day of the experiment and boosted on 8th day of the experiment. Mice were sacrificed on 7th and 14th day of the experiment and the remaining animals were challenged on 21st day of the experiment. Values are expressed as ± s.e.m.

Table 3. Effect of repeated injection of free allergen and allergen entrapped in liposome on T cell subsets in mice

Positive cells (%)	Controls	Free allergen		Liposome entrapped allergen	
		13th day	21st day	13th day	21st day
L3/T4 cells T helper	30.8	33.3	34.69	28.0	33.60
Lyt 2+ cells T suppressor	11.9	9.43	11.8	14.3	14.7
Th/Ts ratio	2.58	3.53	2.93	2.02	2.26

allergen and liposome-entrapped allergen respectively. It was observed that alum adsorbed allergen induced increased secondary IgE response in groups given initially free allergen or liposome-entrapped allergen. However, liposome-entrapped allergen elicited down-regulation of IgE response in animals primed and boosted with alum adsorbed allergen. The IgE levels in these animals were decreased. However, specific IgG response did not differ significantly in these groups although animals primed and boosted with liposome entrapped allergen have higher levels of specific IgG response (Table 2).

Effect of repeated injection of free and liposome entrapped allergen on T cell subsets in BALB/c mice

Table 3 gives the levels of the T helper and T suppressor lymphocytes expressed as % of total lymphocyte population in normal mice (non-immunized), mice immunized with free allergenic protein (G I) or with allergen entrapped in liposomes (G II). It was observed that there was an increase in the T helper cell population in mice immunized with free allergenic protein, while an increase in the T suppressor/cytotoxic cell population was observed in the group given liposome entrapped allergen.

The ratio of T helper to T suppressor/cytotoxic cell was higher in the animals given free allergenic protein as compared to those animals given allergen entrapped in liposomes. The results indicate that allergen entrapped in liposomes could have a direct effect in reducing specific IgE response through induction of T suppressor/cytotoxic cells.

Discussion

Regulation of IgE synthesis is a new approach to control IgE mediated allergic disorders. IgE immunoglobulin isotype specific regulatory factors have been identified [21]. CFA is known to induce T cell population that produced IgE specific regulatory factors [22,23]. Earlier we have shown that repeated injections of allergen entrapped in liposomes did not induce specific IgE response in BALB/c mice (high responders to IgE) and induced specific IgG response to the allergen [12]. However, the mechanism of this isotype shift is not known clearly. In order for the liposome entrapped allergen to be useful in immunotherapy it should be able to down regulate an ongoing IgE response in sensitized animals. Stimulation of suppression of the murine *in vivo* IgE response by pretreatment with CFA and related compounds has also been reported [24]. CFA administered before sensitization was found to be effective in inducing prolonged down regulation of normal and cyclohexamide

enhanced IgE response. However, it was ineffective in presensitized mice [25].

In the present study it was observed that ongoing IgE response induced by alum adsorbed allergen in BALB/c mice could be down regulated by subsequent administration of liposome entrapped allergen (Fig. 1). However, priming of mice with liposome entrapped allergen and later challenge with alum adsorbed allergen could not arrest alum induced IgE response as has been reported in the case of CFA (Fig. 3). Mice primed and boosted with alum adsorbed allergen too gave an increased secondary IgE response and on subsequent injection of liposome entrapped allergen showed a down regulation of IgE response. In this respect injection of liposome entrapped allergen has an advantage in immunotherapy in that it does not cause immediate rise in specific IgE. On the contrary it regulates the ongoing response. The down regulation could be due to the inability of the allergen entrapped in liposome to induce IgE response due to its sequestration from the immuno-apparatus and progressive natural decay of the IgE formed. Unlike other potent immunoadjuvants, i.e. CFA, IFA, alum, etc. which are unacceptable for human use because of the irritable necrotic reactions at the site of injection, the injection of liposome entrapped allergen does not induce granuloma formation [26].

The allergenic response was not observed when liposome entrapped allergen was injected in sensitized mice probably because the lipid bilayer offered some protection to the entrapped allergen and prevented antigen antibody interaction. It could prevent direct interaction of the antigen and its antibody on the circulating basophil by sequestration of the antigen/allergen from the immune system until it reached the macrophages [6]. It could also prevent direct antigen recognition by circulating B cells and thus avoid spontaneous generation of specific IgE in sensitized animals. Absence or reduced anaphylaxis after injection by liposome entrapped antigen to pre-immunized animals has earlier been reported [5].

The study of the non-specific T helper/suppressor cell population reveals a difference in the number of T suppressor cell population in BALB/c mice injected liposome entrapped allergen and those given a similar quantity of free allergen. The results of the present study showed that there was a 22% increase in T suppressor cell population in mice injected liposome entrapped allergen over that of the control (injected equal quantity of untrapped allergen) which may be responsible for the sustained down regulation of IgE response. De-novo generation of suppressor T cells capable of suppressing IgE synthesis after immunotherapy has been reported [27–29]. Thus, liposome entrapped allergen could be useful in preventing spontaneous synthesis of specific IgE

and allergenic response observed in sensitized animals after repeated injections of the allergen.

The immuno-potentiating effect of liposome has been well recognized [30]. Immunoadjuvant action of liposomes with diphtheria toxoid reported earlier has been confirmed with a variety of other antigens [26,31–33]. In the present study it was also observed that allergens entrapped by liposomes induced comparable or slightly enhanced IgG response compared to that given by the animals administered with the same quantity of untrapped allergen or alum adsorbed allergen. Increasing the allergenic protein could also induce greater IgG response (data not shown). This might have been due to the targeting of the antigen/allergen to those cells which endocytose liposomes, such as macrophages [34] where in the presence of lipid environment immunogenic peptides might be formed [35]. These results indicate that administration of liposome entrapped allergens could down regulate allergenic response without affecting the immunogenic response. This ability of liposome entrapped allergen to down regulate on-going IgE response has a special significance in immunotherapy in type I allergic disorders.

Acknowledgments

The authors want to thank Dr Susheela Sridhara for helping in preparing the manuscript and Dr. P. K. Bhatnagar for his help in carrying out T cell subset assay.

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