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Liposome Entrapped Allergen Reduces Plasma Histamine in Sensitized Mice

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SUMMARY **Immunotherapy of allergic diseases is associated with problems of adverse systemic reactions. We have shown earlier that liposome entrapped allergen (LEA) is effective in inducing IgG response and restricting IgE response in immunized mice. This mode of treatment may be more effective and safer if it can prevent anaphylaxis. To determine this feature, mice were administered allergen preparations repeatedly and later challenged with the same allergen. Mice given liposomal preparation showed lower specific IgE response as compared to the mice given free allergen or alum adsorbed allergen of *Artemisia scoparia*. Specific IgG response was higher in mice immunized with LEA. The mice immunized with liposomal preparation survived whereas others injected with free allergen or alum adsorbed allergen died probably due to anaphylaxis. High levels of histamine were observed in mice injected with free allergen as compared to the mice injected LEA. The increase in plasma histamine level may be the cause of anaphylaxis during allergen challenge. In conclusion, LEA could be used as a safe and effective mode of immunotherapy for allergy diseases, since it reduces plasma histamine levels considerably thereby reducing the chances of anaphylaxis.**

Immunotherapy of type I allergic disorder involves a series of injections of the offending allergens to which the patient is hypersensitive. With this schedule there is an increase in specific IgG (blocking antibody) against the offending allergen. There is also an initial increase in specific IgE, which causes local or adverse systemic reactions.[1-2] Hence, further injections have to cease, and because of this the effective treatment dose is not achieved which outweighs the possible benefit.

Studies with liposome entrapped diphtheria toxoid have shown to enhance IgG response and prevent allergic reaction in preimmunized mice.[3] In ragweed sensitive guinea pigs liposome entrapped AgE have eliminated anaphylactic reactions.[4] Audera and coworkers demonstrated that liposome preparations gave high levels of allergen specific IgG.[5] We have shown earlier that crude or purified allergens of *Artemisia scoparia* entrapped in negatively charged liposomes not only induced specific IgG levels but also diminished specific IgE response.[6-9] Furthermore, we have shown that liposome entrapped Art VIb (a purified allergenic protein from *Artemisia scoparia*) down regulated existing allergen specific IgE response.[9] Studies conducted earlier have indicated the importance of plasma histamine as a potential for severe adverse reactions (anaphylaxis) in allergen immunotherapy. Hence, in the present study plasma histamine levels were measured in preimmunized mice with liposome entrapped allergen (LEA).

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MATERIALS AND METHODS

Preparation of liposome entrapped Art VIb (LEA)

Allergens from *Artemisia scoparia* pollen were extracted and purified as described earlier.[9] Small unilamellar vesicles with negative charge were prepared with phosphatidylcholine:cholesterol:phosphatidic acid (7:2:2) by detergent dialysis method[8] with a Liposomat (Dianorm, Germany). Protein entrapped in the liposomes was estimated by Lowry's method after disrupting the liposomes using appropriate blanks.[11]

Immunization protocol

In the first set of experiments, Balb/C mice (6-8 week old) were divided into four groups consisting of three mice each. Group I mice were primed with 15 μg of free allergen, group II were primed with 15 μg of alum adsorbed allergen, group III were primed with empty liposome+15 μg of free allergen and group IV were primed with 15 μg of LEA on the first day of the experiment. Mice were boosted on the 7th day of the experiment with 15 μg of similar allergen preparation. Mice were bled on the 14th day of the experiment to determine specific IgG and IgE response. Mice were given an i.v. challenge with 100 μg of crude allergenic protein on day 23 of the experiment. The serum was stored at -70°C until used for specific IgG and specific IgE determination by ELISA.[7]

In another set of experiments mice were divided into two groups of six animals each. Group I animals were injected with free allergen and group II animals were injected with LEA on the 0, 7th and the 16th day of the experiment with 25 μg of allergenic protein ip. Mice were bled from retro-orbital plexuses on the 15th day and the 24th day of the experiment for measurement of serum specific IgG and IgE. Animals were challenged with 100 μg of allergen on the 16th day and again on the 25th day. The liberated histamine was estimated in the plasma.

Histamine analysis

For histamine analysis the method of Shaff and Beaven (1979) was used.[12] Briefly, for the enzymatic analysis of histamine, histamine-n-methyl transferase was prepared from rat kidney. Kidneys from 3 rats were homogenized in 9 volumes of 0.25 M ice-cold sucrose solution. The particulate matter was removed by centrifugation at 40,000 x *g* for 1 hour at 4°C. The supernatant was precipitated with ammonium sulfate and the protein fraction between 45% (258 g/l) and 70% (additional 156 g/l) saturation was collected and dissolved in 100 mM phosphate buffer, pH 7.4. This fraction was dialyzed against three changes of 2 liters of 10 mM phosphate buffer, pH 7.4. The final preparation 10-12 ml was distributed in 100 μl aliquots and stored at -20°C. The principle of the assay is that sample or standard was incubated with a methyl donor, S-adenosyl-L-[methyl-^{3}H] methionine. The enzyme histamine-N-methyltransferase converts histamine to labeled N-methyl histamine. The labeled N-methylhistamine is then separated from S-adenosyl-L-methionine by extraction into chloroform, which is dried and counted in a counter. For the single isotope assay, the incubation mixture consists of 10 μl of 1:10 diluted rat kidney preparation with 10 μl of plasma sample or histamine standard (0.1 ng, 0.25 ng, 0.5 ng and 1 ng) and 1 μCi of S-Adenosyl-L-[methyl-^{3}H] methionine (66 Ci/mmol, Amersham) in 100 mM phosphate buffer, pH 7.9 in a total volume of 40 μl. The samples were incubated at 37°C for 90 minutes and the reaction was stopped by the addition of 200 μl of N-methyl histamine (50 μg/200μl) in 0.8 M perchloric acid. After adding 200 μl of 10 N NaOH, the labeled and the unlabeled methylhistamine were extracted into 4 ml chloroform. The chloroform solution was extracted again with 0.5 ml of 3.3 N NaOH, transferred to the counting vials, dried under nitrogen and assayed for radioactivity. Each sample was run in triplicates and counted individually.

RESULTS AND DISCUSSION

Many workers have shown that antigen entrapment in liposomes induces specific IgG response.[1,3,5,10] We have reported earlier that the increase in specific IgG response is also coupled with diminished specific IgE response with LEA.[6-9] Thus, LEA have prospects to be used in immunotherapy of allergy diseases. However, the present study was essential to evaluate the safety of LEA for immunotherapy in terms of anaphylaxis which is compared to free allergen.

Alum has been the only approved adjuvant which is used in human vaccines. Alum adsorbed allergens gave higher specific IgG response and have shown increased IgE response against the offending allergens.[6] Besides this, alum injection produces severe local reaction at the site of injection. Hence, it cannot be used for immunotherapy of type I allergic disorders. To de-

termine safety and efficacy of LEA, mice were immunized repeatedly with different allergen preparations as mentioned earlier. Specific IgG response was similar initially in all four groups and became higher for mice injected with LEA (Fig. 1). Specific IgE response was significantly lower in mice injected either with liposome+allergen or LEA as compared to that of mice injected with free allergen or alum adsorbed allergen (Fig. 2). On iv. challenge with 100 µg of free allergen 2 out of 3 animals primed and boosted with alum adsorbed allergen and 1 out of 3 animals injected with free allergen died. However, there was no death in the animals receiving liposomal preparation. These results demonstrate that mice primed and boosted with liposomal preparaion could eliminate systemic reactions on challenge. It was also noted that both the preparation of liposomes (liposome+allergen and liposome entrapped allergen) could reduce the specific IgE response. This shows that the lipid environment has the potential to change the type of response ie. from allergenic to antigenic.

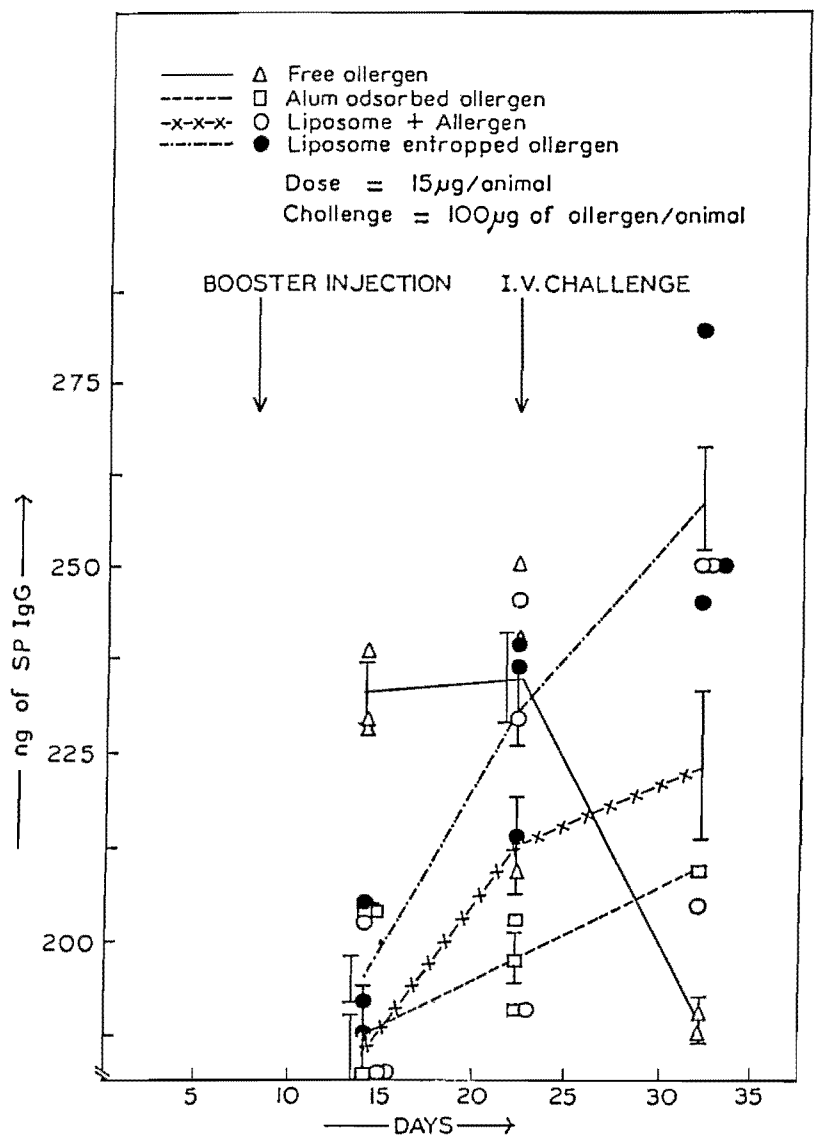

Fig. 1 Specific IgG response in mice as determined on 14th, 22nd and 32nd day of the experiment. Balb/C mice were primed with 15 µg of different allergen preparation and boosted on the 7th day with the same allergen preparation. Mice were given intravenous challenge with 100 µg of crude allergen.

Levels of plasma histamine could be correlated with severity and duration of cardio-pulmonary changes observed during anaphylactic shock.[13] Anaphylactic reactions occur in sensitized individuals exposed to foreign antigen or low molecular weight substances. The consequent release of mediators from mast cell and basophil resulted in varied manifestations. The sudden, usually unexpected, onset of systemic anaphylaxis with its rapid clinical course leads to immediate death. Several deaths have been reported during immunotherapy of allergic disorders.[2]

Accordingly, an experiment was carried out in the present study to quantitate histamine, an important mediator of anaphylaxis, in mice after challenge with free allergen. Table 1 shows the specific IgG, specific IgE and histamine release in mice injected either with free allergen or LEA. Specific IgG response was similar in both the groups on the 15th day but were higher in the LEA group on the 24th day of the experiment. Specific IgE response was significantly higher on day 24 in mice injected with free allergen as compared to that of mice given LEA ($p < 0.001$). IgE levels rose three times from 15-24th day in animals

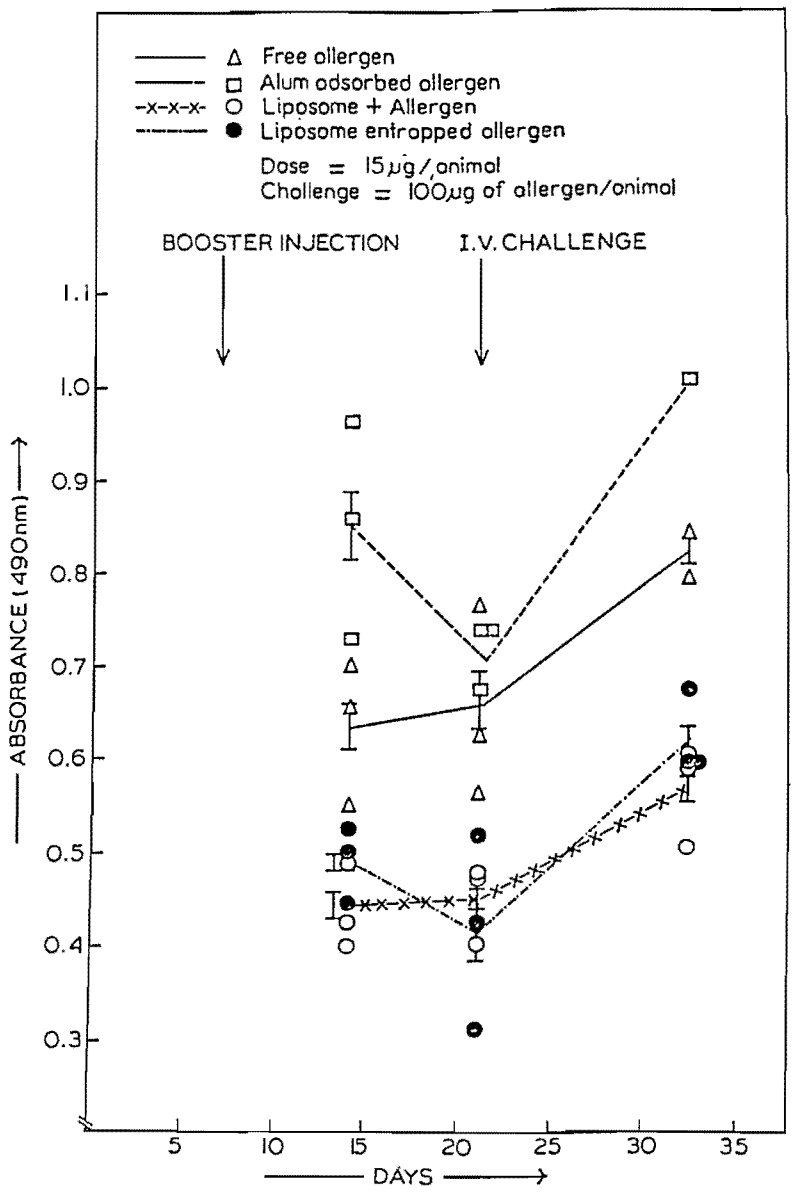

Fig. 2 Specific IgE response in mice as determined on 14th, 22nd and 32nd day of the experiment. Balb/C mice were primed with 15 μg of different allergen preparation and boosted on the 7th day with the same allergen preparation. Mice were given intravenous challenge with 100 μg of crude allergen.

given free allergen but the rise was small in animals injected with LEA. Intravenous challenge of both the groups with 100 μg of crude allergen on the 16th day resulted in increased histamine levels viz, 22 ng/ml in free allergen group and 10 ng/ml in LEA. The control mice showed 4.5 ng/ml of plasma histamine. Rechallenge of animals on the 25th day resulted in increased histamine levels. A 16-fold increase was observed in the free allergen group (70 ng/ml) and 7 fold in LEA group (33 ng/ml). These results indicated that allergen challenge increases histamine release in mice injected with free allergen which could be the cause of mortality in animals. The absence of anaphylaxis in animals given LEA might have been due to a down regulation of specific IgE response[9] which in turn reduces histamine release from mast cells and basophils.

Cytokine production by CD4+ helper T lymphocytes during immune response plays an important role in regulating the immune response. IL-4 secreted by mast cells, basophils and other accessory cells promotes IgE production. IL-2 and IFN-γ antagonize IL-4 in the regulation of IgE response.[14,15] Many reports have shown that IgE synthesis is dependent on the balance in production of IL-4 and IFN-γ.[14-16] Recently, it has been reported that liposomes induce IFN-γ production but not IL-4.[17] This high production of IFN-γ with low production of IL-4 coupled with low histamine release will be advantageous to allergy patients.

In conclusion LEA diminishes plasma histamine release and thus reduces the risk of systemic reactions (anaphylaxis) during immunotherapy. Interestingly, not only LEA but liposome+allergen are also effective in reducing IgE levels. Liposomes modulate the immune response by creating a hydrophobic environment at the level of antigen presentation. The observed increase in IgG response is beneficial and is required in vaccines.

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Table 1 Mean specific IgG and specific IgE in mice before challenge and histamine release after challenge

Experimental day	Free allergen (G I)	Liposome entrapped allergen (G II)
Plasma Histamine (ng/ml)		
day 16th**	21.99 ± 2.46	9.841 ± 2.05
day 25th***	70.00 ± 2.30	32.70 ± 4.45
Specific IgE (Abs)		
day 15th	0.435 ± 0.057	0.320 ± 0.060
day 24th***	1.340 ± 0.031	0.497 ± 0.050
Specific IgG (ng)		
day 15th**	220 ± 22	235 ± 18
day 24th	270 ± 9	330 ± 14

Normal histamine in mouse injected with saline 4.46 ng/ml.
Mice were injected on 0, 7th and 16th day of the experiment with purified allergenic protein ip. and were bled on 15th and 24th day of experiment. Animals were challenged with 100 µg of crude allergen on the 16th day and again on 25th day of the experiment.
Data is expressed as ± SE, $p < 0.05$** and $p < 0.001$***.

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