

# Liposomes as carriers for allergy immunotherapy

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## Summary

With the aim of obtaining an efficient but safer vaccine for allergy immunotherapy, the possibility of using liposomes as adjuvants has been considered given their proven low toxicity, adjuvant properties and ability to maintain the encapsulated substance in their interior for some time *in vivo*. Different methods of encapsulating allergenic extracts into neutral, positively, and negatively charged *DPPC:cholesterol* liposomes have been investigated and the immune response provoked by these in mice was studied and compared to the immune response to free allergen or other adjuvants such as aluminium hydroxide. The results obtained show that allergen encapsulated in all three types of liposomes elicit an increase in specific IgG levels higher than that provoked by free allergen, however, both encapsulation efficiency and specific IgG titre were higher when positively charged (*DPPC:cholesterol stearylamine*) liposomes were used. Specific IgE levels to allergen in positive and neutral liposomes was lower than to allergen in negative liposomes or adsorbed to  $Al(OH)_3$ . No differences were found in the behaviour of liposomes prepared by different methods (the results were obtained from pooled sera from different groups of mice so there is no statistical data). These results confirm the immunoadjuvant effect of liposomes for allergy immunotherapy. Further studies to determine their lack of toxicity, pharmacokinetic studies and human clinical studies are necessary to confirm their adequacy for human use and advantage over current immunotherapy methods.

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## Introduction

Immunotherapy treatment of IgE-mediated allergy disorders has proven to be successful when used correctly [1]. One of the main drawbacks of traditional immunotherapy is the high inherent risk of local and systemic anaphylactic reactions [2] which in many instances outweigh the possible benefits obtained. Some of the newest preparations, such as polymerized allergens [3], partially reduce the risk of allergen interaction with specific IgE by hiding some of the allergenic determinants inside the polymer or changing their structure. However, adverse reactions still occur even with this kind of product.

An attempt has been made to obtain an alternative to the currently available allergy vaccines using liposomes as allergen carriers.

Liposomes are lipid vesicles formed by one or more phospholipid bilayers capable of entrapping water sol-

uble substances in their internal aqueous compartment. Since their discovery [4], the use of liposomes as carriers has been studied in many different fields of medicine. Among other properties, such as being biodegradable, stable, prolonging half-life of many drugs, etc. [5], numerous studies have demonstrated the immunoadjuvant (although not immunogenic) properties of liposomes for a wide range of antigens of different origin [6–8]. Little work, however, has been done on the effect of liposomes as adjuvants for allergy immunotherapy [9,10]. The rationale behind the use of liposomes as allergen carriers is that because allergens are water soluble these would be hidden inside the aqueous space of liposomes thus avoiding local reaction when injected subcutaneously. There is controversy on the type of liposome that will have an optimal adjuvant effect [11]. We have aimed for liposomes in the size range from 100 to 200 nm which are known to have maximum encapsulation efficiency, and in the case of subcutaneous injection, follow a lymphatic route to lymph nodes [12], where macrophages will phagocytose liposomes, process antigens, and present

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them to T lymphocytes, thus initiating the immune response without free allergen available, thus also minimizing the risk of systemic reactions.

In order to assess the possibility of using liposomes as allergen carriers, several allergenic extracts have been encapsulated in liposomes and the *in-vivo* immune response has been studied in animal models and compared to other immunotherapy methods. In addition, different methods of preparation and different types of liposomes have been studied in order to obtain optimal results based on encapsulation efficacy, stability, toxicity and *in-vivo* immune response.

## Materials and methods

### Preparation of allergenic extracts

*Dermatophagoides pteronyssinus*, *Lolium perenne*, *Phleum pratense*, *Parietaria judaica*, *Artemisia vulgaris* and cat dander extract were prepared and standardized in Alergia e Inmunología Abelló [13,14]. In brief, proteins were solubilized from the whole extract in 50 mM ammonium bicarbonate and the suspension was clarified by centrifugation at 22 000 g for 1 hr at 4°C. The supernatant was dialysed against distilled water, filtered through a 0.22 µm membrane filter and lyophilized. Allergenic activity was determined by RAST inhibition assay [15] and protein content was determined by the ninhydrin [16] and Lowry [17] methods.

### Preparation of liposomes

Dipalmitoyl-phosphatidyl-choline (DPPC) (Fluka) and cholesterol (Fluka) were dissolved in chloroform:methanol 2:1 (v/v) (Merck) at a final lipid concentration of 30 mg/ml in a round-bottomed flask. In some cases dicetyl phosphate (DPC) (Fluka) or stearylamine (Sigma) were also used. A dry lipid film was formed with a rotary evaporator. The lipid film was rehydrated with 0.9% NaCl containing the allergenic extract to be encapsulated (4–8 mg/ml) and deoxycholate (Fluka) (lipid/detergent molar ratio = 1) at a final lipid concentration of 30 mg/ml. A clear micelle solution was obtained by gently stirring the mixture and this was then dialysed against NaCl by repeated cycles through a dialysis cell (Liposomat) [18] at 45°C with a flow rate of 0.5 ml/min. Alternatively, liposomes were prepared by the French press method [19]. If this was the case, the dry lipid film was rehydrated with NaCl containing only the allergenic extract. The multilamellar liposomes formed after vigorous mixing were placed in a French press and extruded four consecutive times at 20 000 p.s.i. Liposomes obtained with either method were centrifuged at 27 000 g for 30 min in order to eliminate large liposomes. The supernatant was passed

through a Sephacryl 400 (Pharmacia, Uppsala, Sweden) column, thus separating liposomes from non-encapsulated protein. Liposomes were then filtered under sterile conditions through a 0.22 µm Sartorius membrane.

### Characterization of liposomes

The allergenic activity of the encapsulated protein was determined by a modified RAST inhibition assay [15] in which samples and standards were prepared and sonicated for 15 min in PBS–0.5% BSA containing empty liposomes and 2% deoxycholate in order to lyse the bilayer and liberate the encapsulated protein.

Final lipid concentration was measured by commercially available cholesterol and phospholipid kits (Boehringer). Physical characteristics such as size, homology, etc. were evaluated by electron microscopy (E/M) with negative staining using phosphotungstic acid, and/or by light scattering (Coulter). Stability of liposomes was determined by monthly checking of physical properties after storage in suspension at 4°C and also by evaluating the amount of protein that remained encapsulated. This was done after passing a sample through a Sephacryl 400 column and then determining the amount of protein in the liposome fraction as well as in the fraction where the free protein should elute.

### Animal immunization

Male Balb/c mice 12 weeks old and weighing approximately 20 g were used in all immunizations.

Allergenic extract in (a) saline, (b) adsorbed to Al(OH)<sub>3</sub> or (c) encapsulated in liposomes, was subcutaneously injected into different groups of mice (six mice per group) on days 1, 15, 25 and 28. The same dose of allergen was injected each time. As a control, saline solution was injected to a fourth group of mice on the same schedule. The optimal immunizing dose for each allergenic extract was chosen according to previous allergen immunization studies and varied from one allergenic extract to another. Doses ranged from 0.5 to 40 µg/injection. Three days after the last shot, the animals were killed after drawing blood by jugular phlebotomy. When immunizations were prolonged, the same dose was given every 15 days from day 30 to the end, obtaining blood samples, at the same time intervals, from the orbital globe. Blood from each group of mice was pooled and the sera obtained was stored at –20°C in aliquots until used for antibody determinations. Specific IgG and IgE of non-immunized mice were always undetectable, so baseline blood samples were not necessary.

### Determination of specific immunoglobulins

**Specific IgE.** Specific IgE production to different allergens was determined in the pool of sera from each group of

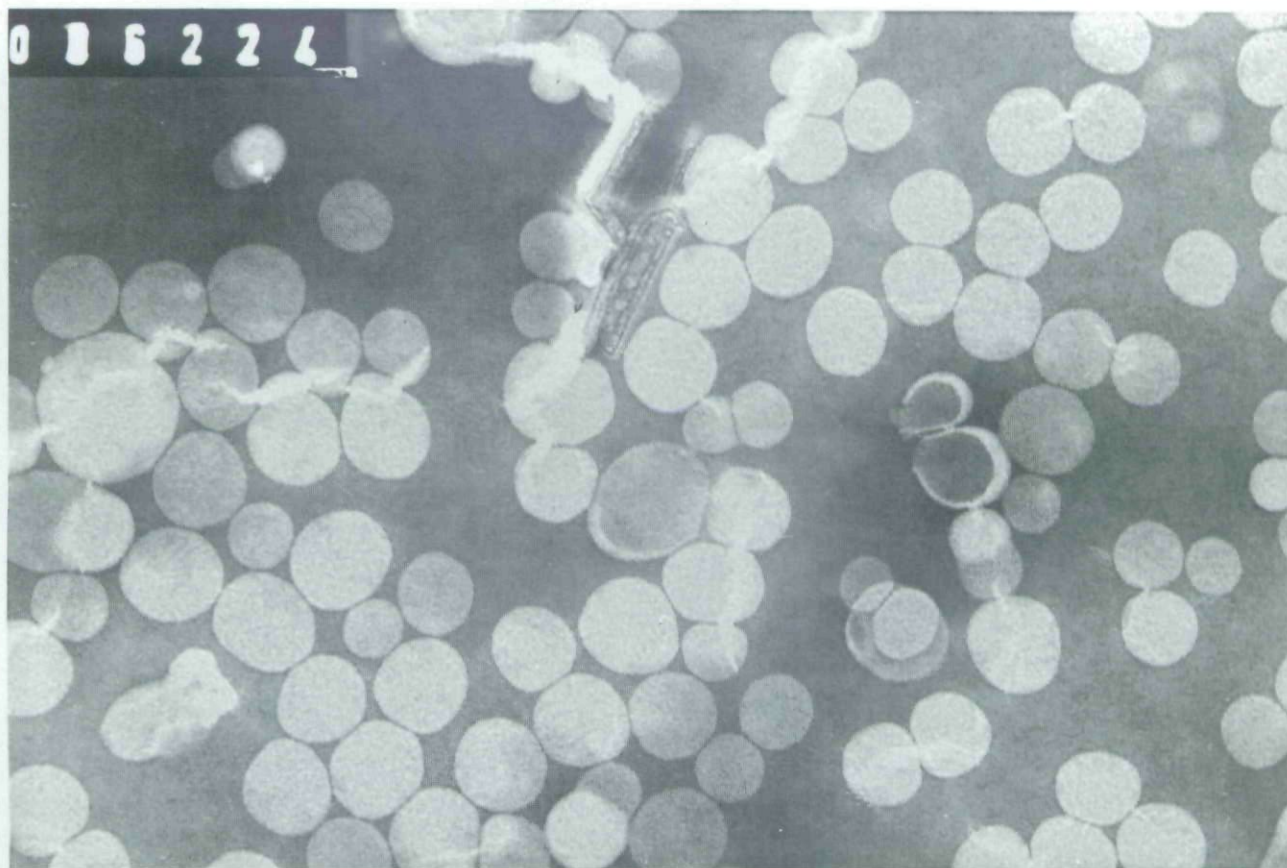


Fig. 1. Electron micrograph of negatively stained DPPC:cholesterol (60:40) liposomes prepared by the French press method ( $\times 120\,000$ ).

mice by passive cutaneous anaphylaxis (PCA) in rats [21]. In brief, 50  $\mu$ l of the appropriate serum dilution were intradermally injected into the shaved back of a rat. Twenty-four hours later, 200  $\mu$ l of saline solution containing allergen (10–70  $\mu$ g/ml) and 0.5% Evans blue dye were injected intravenously. After 1 hr, the rats were killed, the skin removed and the macules formed evaluated. IgE titres were calculated as the reciprocal of the highest serum dilution that produced a macule 10 mm in diameter. Three dilutions of each pool of sera from the four groups of mice (total of 12 injection sites/rat) were injected on the same rat in order to avoid differences due to the recipient animal. The assay was always carried out in duplicate.

**Specific IgG.** Specific IgG titres were determined by ELISA. Different allergens were bound to a solid phase on plastic microtitre ELISA plates (Dynatech). The antigen was incubated, first with different dilutions of sera, and after washing off the excess sera, it was incubated with horse radish-peroxidase labelled goat anti-mouse IgG (Nordic). Subsequently the colour change after 30 min incubation at room temperature with

substrate (*o*-phenylenediamine) (Dakopatts) was measured spectrophotometrically at 492 nm in an automatic ELISA reader. Each serum dilution was assayed in duplicate and four ELISAs were done on different days. In order to avoid day-to-day differences, the results were expressed as the ratio of the mean absorbance of a sample over the absorbance of the control serum from each immunization for a serum dilution on the linear portion of the curve.

As immunoglobulin levels were determined in pools of sera from the different groups of mice, statistical analysis was not performed.

## Results

### *Characteristics of liposomes*

Liposomes obtained either by dialysis or the French press method were unilamellar with a mean diameter of approximately 80 nm assessed by light scattering. The E/M images confirmed these findings (Fig. 1). Final protein/lipid ratios varied depending on the initial protein concentration and ranged from 0.4 to 4.1  $\mu$ g of protein/

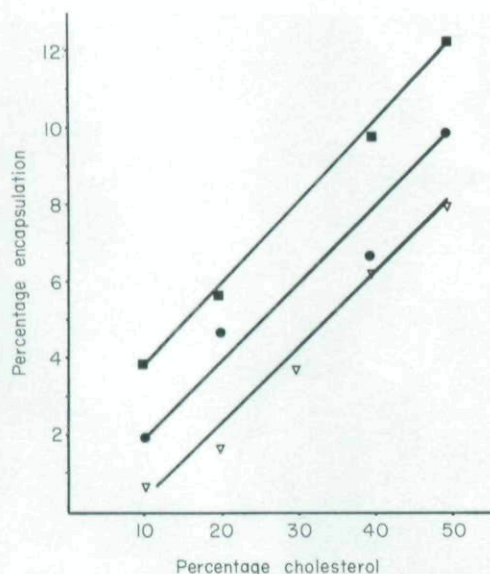


Fig. 2. Percent encapsulation of *Dermatophagoides pteronyssinus* in DPPC:cholesterol (▽), DPPC:cholesterol:10% stearylamine (■), and DPPC:cholesterol:10% DCP (●) liposomes with increasing molar percentages of cholesterol. Liposomes were prepared by dialysis as described.

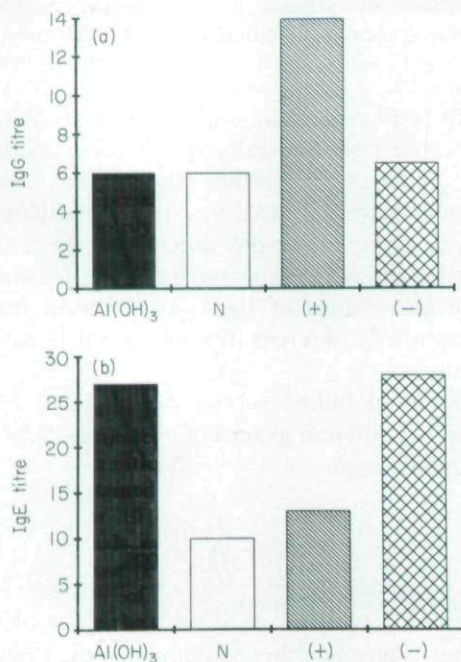


Fig. 3. Mean IgG (a) and IgE (b) titre in pool of sera from mice immunized with *Parietaria judaica* adsorbed to Al(OH)<sub>3</sub> (■) or encapsulated in neutral DPPC:cholesterol (60:40) (□), positive DPPC:cholesterol stearylamine (50:40:10) (▨) or negative DPPC:cholesterol:DCP (50:40:10) (▩) liposomes. Mean value of four ELISA determinations and two PCA, respectively.

μmol of lipid. The encapsulation efficiency depended on the lipid composition of liposomes, thus increasing percentages of cholesterol resulted in higher encapsulation percentage (Fig. 2). Similarly, when DPPC:cholesterol:stearylamine (50:40:10 molar ratio) or DPPC:cholesterol:DCP (50:40:10) liposomes were used, an increase in encapsulation efficiency was observed (Fig. 2). The formulation chosen finally DPPC:cholesterol (60:40), had an encapsulation efficiency of approximately 5% of the initial protein. No differences were observed, however, in the encapsulation efficiency of different allergens. Empty liposomes maintained their physical characteristics for at least 2 y, stored at 4°C in sterile conditions. Protein loss in DPPC:cholesterol (60:40) liposomes containing *L. perenne* at 4°C was determined over a period of 10 months at 1 month intervals. There was no protein loss during the first 6 months. From this time on there was a gradual loss of approximately 2% at each monthly determination.

#### In-vivo immune response to different types of liposomes

Specific IgG and IgE production in mice immunized with *P. judaica* extract encapsulated in positive DPPC:cholesterol:stearylamine (50:40:10), negative DPPC:cholesterol:DCP (50:40:10), and neutral DPPC:cholesterol (60:40) liposomes or *P. judaica* extract adsorbed to Al(OH)<sub>3</sub> is shown in Fig. 3. Specific IgG production was optimal with positively charged liposomes (mean IgG titre=14 versus 6 and 6.5 for neutral and negative liposomes, respectively). IgE response, however, was lowest when mice were immunized with neutral or positively charged liposomes giving a mean IgE titre of 10 and 13, respectively, versus 27 and 30 for Al(OH)<sub>3</sub> and negatively charged liposomes.

The immune response to liposomes prepared by different methods was comparable. The immune response to *L. perenne* extract encapsulated in DPPC:cholesterol (60:40) liposomes, prepared by dialysis, was similar to the response of *L. perenne* extract encapsulated in liposomes prepared in a French press, possibly because these two types of liposomes are very similar in size, although the recovery of a homogeneous population of small liposomes was better in the latter.

#### Immune response to different allergens

Specific IgG and IgE response to six different allergenic extracts in saline solution, adsorbed to Al(OH)<sub>3</sub> or encapsulated in DPPC:cholesterol liposomes are shown in Fig. 4. These extracts were *P. pratense*, *L. perenne*, *A. vulgaris*, *P. judaica*, cat dander extract and *D. pteronyssinus*. Specific IgG titre to liposome-encapsulated allergen was similar to the response to allergen adsorbed to

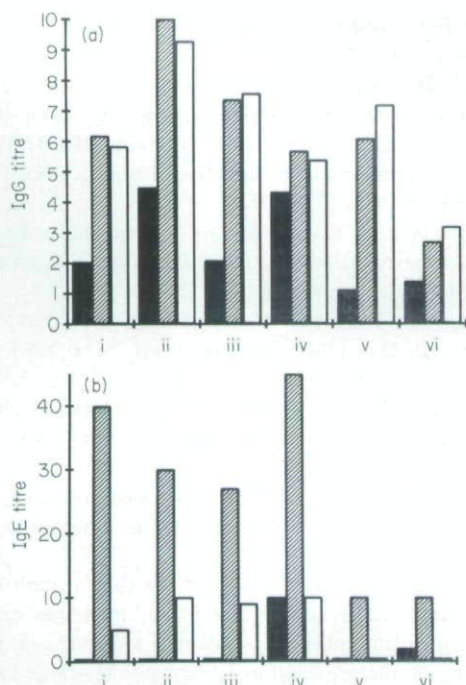


Fig. 4. Specific IgG (a) and IgE (b) in pool of sera from mice immunized with (i) *Lolium perenne*, 5 µg/dose; (ii) *Phleum pratense*, 5 µg/dose; (iii) *Parietaria judaica*, 40 µg/dose; (iv) *Artemisia vulgaris*, 5 µg/dose; (v) Cat dander extract, 0.5 µg/dose and (vi) *Dermatophagoides pteronyssinus*, 10 µg/dose in saline solution (■), adsorbed to Al(OH)<sub>3</sub> (▨) or encapsulated in DPPC:cholesterol liposomes (□). Mean value of four ELISA determinations and two PCA, respectively.

Al(OH)<sub>3</sub> for all six allergens studied and in both cases it was higher than the response to allergen in saline solution. IgE response, however, was always much higher in groups of mice treated with allergen adsorbed to Al(OH)<sub>3</sub> than those treated with allergen incorporated in neutral liposomes.

#### Time-dependent immune response

Immunizations were usually carried out for 30 days but on one occasion it was prolonged to 90 days. The increase in specific IgG titre in response to *L. perenne* encapsulated in liposomes was comparable to the response to the allergen adsorbed to Al(OH)<sub>3</sub>. IgG titre in response to *L. perenne* in saline solution was always lower (Fig. 5a). Although specific IgE titre also increased with time in case of Al(OH)<sub>3</sub>-treated mice, it remained low and close to baseline levels both in liposome-encapsulated allergen and allergen in saline solution throughout the 90 days (Fig. 5b).

#### Discussion

The adjuvant effect of liposomes for a number of allergenic extracts has been confirmed in the mouse

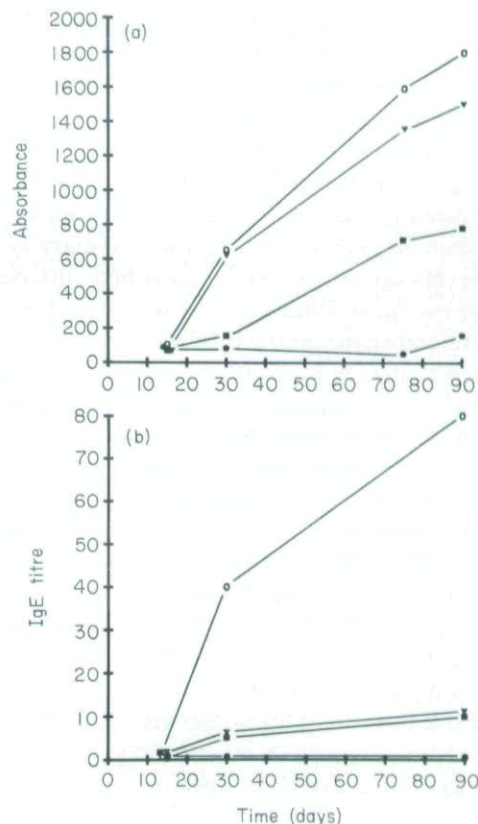


Fig. 5. Specific IgG (a) and IgE (b) response in mice immunized with *Lolium perenne* extract (5 µg/dose) in saline solution (■), adsorbed to Al(OH)<sub>3</sub> (○) or encapsulated in DPPC:cholesterol liposomes (▼). Saline solution alone was injected as control (●).

model. DPPC was chosen because it is a saturated phospholipid with no risk of oxidation, and can be obtained in pure form. Cholesterol was added to the formulation because it is known to increase membrane stability. We also observed that it increased the encapsulation efficiency. Three different methods of liposome production were assayed and although we could obtain liposomes with the chosen characteristics with all three methods, liposomes prepared in the French press were preferred to dialysed liposomes because the recovery of a population of small homogeneous liposomes was poorer in the latter. Furthermore, the presence of detergent traces in liposome preparations could imply some kind of inconvenience for human use. When a positive (stearylamine) or negative (DCP) charge was added to the formulation of liposomes, protein encapsulation increased. This apparent increase was due to non-specific interactions of protein with the liposome surface as was demonstrated later with radiolabelled monoclonal antibodies to some of the extract components (data not shown). Therefore, although the immune response to positively charged liposomes was also optimal, this type of liposome was not considered for human immuno-

therapy for two reasons. First, there was a theoretical possibility of interaction between allergen on the liposome surface and local IgE although this has not been tested, but more important is the fact that stearylamine is toxic for human use.

IgG response to different allergens was 4–5 times greater when it was administered incorporated in liposomes than in free form and was comparable to the response elicited by Al(OH)<sub>3</sub>-adsorbed allergen (currently in use as an immunoadjuvant). There was, however, a difference in specific IgE levels which increased in response to allergen adsorbed to Al(OH)<sub>3</sub>. IgE response to allergen incorporated in positive DPPC:cholesterol:stearylamine or neutral, DPPC:cholesterol liposomes was lower and in some cases it was not detectable. These results, however, are in the mouse model and, although it has been shown that the clinical efficacy of immunotherapy is not related to IgG and IgE levels during immunotherapy, whether this different antibody pattern in response to allergens encapsulated in liposomes has any clinical relevance will only be determined once clinical studies are performed.

Lipid components of liposomes are naturally occurring lipids and are therefore easily metabolized. Nevertheless, toxicology studies are being currently performed in two animal models to ensure their safety for human use. Similarly, pharmacokinetic studies will be performed in rats with radiolabelled material studying absorption, elimination and accumulation in different organs and tissues.

The results obtained so far show the possibility of encapsulating and evaluating allergenic extracts in liposomes and confirm the immunoadjuvant effect of liposomes. These results together with other theoretical properties of this type of preparation such as low toxicity, sustained release and minimum risk of interactions between allergen and specific IgE make liposomes good candidates for allergy immunotherapy and make further investigations to optimize this type of vaccine and try to understand the mechanisms involved in the immune response to allergens encapsulated in different types of liposomes worthwhile.

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