ORIGINAL ARTICLE

Chitosan Microparticles Loaded With Mite Group 2 Allergen Der f 2 Alleviate Asthma in Mice

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Abstract

Background: In recent years, nanoparticle materials have found wide applications in drug transport and release systems. Chitosan is a good carrier for proteins, peptides, and nucleic acids because of its favorable release properties and ability to increase membrane permeability.
Objective: To investigate the effect of chitosan microparticles loaded with the major epitope peptide of mite group 2 allergen Der f 2 from *Dermatophagoides farinae* (Der f 2-47-67) in alleviating asthma in mice.
Methods: Der f 2-47-67 was entrapped in chitosan to obtain Der f 2-47-67-loaded chitosan microparticles, which were injected intraperitoneally into BALB/c mice prior to an intranasal challenge with a Der f extract allergen. Airway hyperreactivity was measured via whole-body plethysmography, and bronchoalveolar lavage (BAL) fluid was collected to calculate total cell and eosinophil counts. Changes in lung histology were assessed after hematoxylin–eosin staining, and serum levels of Der f-specific immunoglobulin (Ig) G2a and IgE were determined by enzyme-linked immunoabsorbent assay.
Results: Mice immunized with Der f 2-47-67-loaded chitosan microparticles displayed decreased airway hyperreactivity, reduced numbers of eosinophils in BAL fluid, alleviated lung inflammation and mucus production, a reduced serum level of Der f-specific IgE and an increased serum level of Der f-specific IgG2a.
Conclusion: Our data showed that Der f 2-47-67-loaded chitosan microparticles inhibited airway allergic inflammation.

Key words: Chitosan. *Dermatophagoides farinae.* Polypeptide. Microparticles. Allergic airway inflammation.

Resumen

Antecedentes: En los últimos años, las nanopartículas han encontrado un gran número de aplicaciones en el transporte de fármacos y sistemas de liberación. El chitosan es un buen transportador de proteínas, péptidos y ácidos nucléicos debido a sus favorables propiedades de liberación y a su habilidad por aumentar la permeabilidad de membrana.
Objetivo: Investigar el efecto de micropartículas de chitosan cargadas con el péptido del epítopo principal del alérgenos del grupo 2 de ácaros Der f 2 de *Dermatophagoides farinae* (Der f 2-47-67) disminuyendo el asma en ratones.
Métodos: Se incluyó Der f 2-47-67 en chitosan para obtener micropartículas de chitosan cargadas de Der f 2-47-67, que se inyectaron intraperitonealmente en ratones BALB/c previo a la provocación nasal con extracto alergénico de Der f.
La hiperreactividad de la vía aérea se midió mediante pletismografía de cuerpo entero, y se tomó el fluido del lavado broncoalveolar (LBA) para calcular el número total de células y de eosinófilos. Los cambios en la histología pulmonar se evaluaron tras teñir con hematoxilina-eosina, y los niveles de IgG2a e IgE específica frente a Der f se determinaron mediante ensayo de enzimoinmunoanálisis.
Resultado: los ratones inmunizados con micropartículas de chitosan cargadas de Der f 2-47-67 mostraban menor hiperreactividad, menor número de eosinófilos en LBA, menor inflamación pulmonar y producción de moco, reducción de la IgE específica frente a Der f y aumento de los niveles séricos de IgG2a específica frente a Der f.
Conclusión: nuestros datos muestran que las micropartículas de chitosan cargadas de Der f 2-47-67 inhiben la inflamación alérgica de la vía aérea.

Palabras clave: Chitosan. *Dermatophagoides farinae*. Polipéptido. Micropartículas. Inflamación alérgica de vías aéreas.

Introduction

Bronchial asthma is a chronic inflammatory process of the airways affecting millions of people worldwide, and particularly children in developed countries. While a large number of studies have indicated that allergen-specific immunotherapies can modify and correct pathologic immune responses in asthma [1, 2], the use of these therapies has been limited by their potential serious adverse effects. It has been suggested that these adverse effects might be related to the sudden withdrawal of the allergen and the high serum levels of immunoglobulin (Ig) E induced by allergen extracts [3]. It is known that short synthetic peptides of the main allergen are capable of lowering allergenicity while retaining immunogenicity [4] but the absorption of peptides is greatly reduced by the high metabolic activity and low permeability of absorbing tissues. Chitosan is a natural biocompatible polysaccharide capable of promoting the transmucosal absorption of peptides and proteins. Furthermore, chitosan microparticles have been demonstrated to be safe and effective in allergen delivery [5].

The prevalence of asthma is associated with exposure to indoor allergens and the house mite *Dermatophagoides farinae* has been described as an important source of indoor allergens in asthma and other allergic conditions [6]. The mite group 2 allergen Der f 2 is one of the main allergens in *Dermatophagoides farinae*, and elevated Der f 2 IgE levels are found in 70% to 80% of allergic patients [7]. Der f 2-47-67 is the crucial epitope peptide of this allergen [8]. When trapped in chitosan, this polypeptide can be released gradually, effectively facilitating uptake by antigen-presenting cells, and making it a very promising candidate for use in immunotherapy.

The aim of this study was to determine the effect of Der f 2-47-67-loaded chitosan microparticles in alleviating asthma in mice and to explore the mechanisms involved.

Methods

Methacholine and chitosan (90% deacetylated) were obtained from Sigma-Aldrich (Madison, USA) and the polypeptide Der f 2-47-67 from Hybio Engineering Limited Company (Shenzen, China), with the following sequence: EALFDANQNTKTAKIEIKASL. Dust mite extract was provided by the Shenzhen University Allergy and Immunology Institute (Shenzen, China), and horseradish peroxidase-conjugated anti-mouse IgG2a and IgE antibodies were obtained from Southern Biotech Associates Inc. (Birmingham, USA). BALB/c mice aged 4 to 6 weeks were obtained from the Guangzhou Experiment Animal Center, (Guangzhou, China) and raised under specific pathogen-free conditions.

Preparation of Der f 2-47-67- Chitosan Microparticles

Sodium tripolyphosphate (STPP) was dissolved in H_2O at 0.84 mg/mL and chitosan in acetic acid (2% v/v, pH 2.3) at 2 mg/mL. A volume of 1.2 mL of the first solution was added to 3 mL of the second, and an opalescent suspension was formed by stirring the mixture at 4ºC. Next, 3 mg of Der f 2-47-67 polypeptide was added to the microparticle solution and centrifuged at 10 000 g for 20 minutes at 4ºC. After removal of the supernatant, the pellet was resuspended in H_2O to form the Der f 2-47-67-loaded chitosan microparticle vaccine [9].

Characterization of Chitosan and Der f 2-47-67-Chitosan Microparticles

The formation of chitosan and Der f 2-47-67-loaded chitosan microparticles was analyzed by scanning electron microscopy (JEOL, Akishima, Japan). Samples were measured by Fourier transform infrared (FT-IR) spectroscopy (360 FT-IR; Nicolet, Madison, USA) and FT-IR spectra recorded using KBr pellets. The amount of Der f 2-47-67 absorbed by the chitosan microparticles was measured using the Biuret method [10].

Immunization

Twenty BALB/c mice were randomly divided into 5 groups of 4 mice: a healthy control group (healthy group), an untreated asthma group (untreated group), an asthma group treated with Der f 2-47-67 (Der f 2-47-67 group), an asthma group treated with Der f 2-47-67-loaded chitosan microparticles (Der f 22-47-67-chitosan group), and an asthma group treated with empty chitosan microparticles (chitosan group). All the groups except the healthy group were sensitized via an intraperitoneal injection of 200 μg of Der f allergen extract in 2 mg of aluminum potassium sulfate on days 0, 7, and 14.

Fourteen days after the final sensitization, the untreated group was intraperitoneally injected with 0.8 mL of phosphate buffered saline (PBS), the Der f 2-47-67 group with 2 mg of Der f 2-47-67 dissolved in PBS, the Der f 2-47-67-chitosan group with 2 mg of Der f 2-47-67-loaded chitosan microparticles, and the chitosan group with 2 mg of empty chitosan microparticles. The injections were administered once a day for 8 days. Seven days after the final injection, the mice in the 4 sensitized groups were intranasally challenged with 100 μg of Der f allergen for 7 days and the healthy group was administered PBS. Airway hyperreactivity was measured 24 hours after the challenge. The mice were then sacrificed, a bronchoalveolar lavage performed, and the lungs collected.

Airway Hyperreactivity

Airway hyperreactivity was measured using unrestrained whole-body double-chamber plethysmography with an aerosol delivery system (Buxco, Wilmington, USA) at 24 hours post challenge. Specifically, the mice were placed in the chamber and their breathing monitored for 10 minutes. Once acclimated, the mice had their baseline responses measured for 5 minutes and were then administered aerosolized PBS for 1.5 minutes, followed by incremental doses of methacholine (1, 5, 10, 30, 50, 100 mg/mL of PBS). Responses were recorded for 5 minutes [11].

Bronchoalveolar Lavage

Twelve hours after airway hyperreactivity was measured, blood was collected from the retro-orbital venous plexus of all the mice, which were then sacrificed. The lungs were immediately lavaged via the trachea cannula 3 times with

1 mL of PBS. Total cell counts were determined using a hemocytometer. Cytocentrifuged preparations were stained with Liu's stain for differential leukocyte counts, and a minimum of 200 cells were counted; these were classified into macrophages, lymphocytes, neutrophils, and eosinophils on the basis of their morphology.

Lung Histology and Inflammation Scoring

Once lavage was complete, the lungs were immediately removed, fixed in cold formalin, embedded in paraffin, and cut into 5-µm-thick sections, which were stained with hematoxylin–eosin and examined under light microscopy. Pathologic changes were scored in a blinded manner using a system previously described [12]. This scoring system discriminates between edema and epithelium impairment (score, 0-5) and between perivascular and peribronchiolar infiltration of eosinophils (score, 0-5).

Measurement of Der f-Specific Antibodies by ELISA

Blood was collected from the 5 groups of mice after the last 36-hour challenge. Der f-specific IgE and IgG2a levels were determined by enzyme-linked immunosorbent assay (ELISA) as follows: 96-well plates were coated overnight at 4°C with 100 µL of Der f (10 µg/mL in 0.1 mol/L of carbonate buffer, pH 9.6). The antigen-coated plates were washed 5 times with 0.05% Tween 20 in PBS (PBST). Mouse sera were added to the antigen-coated wells, and the plates were incubated with peroxidase-conjugated anti-mouse IgE and IgG2a antibody (Southern Biotech Associates) for 2 hours at 37°C, and washed 5 times with PBST before adding 100 µL of trimethyl benzene. Color was developed at 37°C in the dark; the reaction was stopped with 2 mol/L of sulfuric acid, and absorbance values were measured at 450 nm.

Statistical Analysis

Data were expressed as means (SD) and analyzed using analysis of variance. A P value of less than .05 was considered significant.

Results

Characterization of Chitosan and Der f 2-47-67-Chitosan Microparticles

Chitosan dissolved in acetate buffer presented a -NH_3^{+}-AC group and STPP a structural unit of -$PO^{-}Na^{+}$. When the STPP solution was added to the chitosan solution, chitosan microparticles were formed according to the following reaction: Chitosan-NH_3^{+}-AC+TPP-$PO^{-}Na^{+}$—Chitosan-NH_3^{+}-OP-TPP.

The existence of chitosan microparticles was confirmed by analysis with infrared absorption spectrum. In the FT-IR spectrum of Der f 2-47-67-loaded chitosan microparticles (Table 1), the band at 3421 cm^{-1} corresponded to the O-H stretching vibration, that at 1081 cm^{-1} to the C-O-C stretching vibration, and that at 1573 cm^{-1} to the bending vibration of N-H. A characteristic increase in absorption was observed at 1630 cm^{-1} and 1548 cm^{-1}, indicating the presence of chitosan and STTP phosphate bonds.

Table 1. Characterization of Chitosan and Der f 2-47-67-Loaded Chitosan. Microparticle Analysis by Fourier Transform Infrared Spectroscopy

Chitosan	Der f 2-47-67-Chitosan, cm^{-1}
3427	3421.2
2873.6	2871.2
1643	1656.1
1380.4	1377.0
1155.0	1151.2
1078.0	1081.4
596.17	588.0

Abbreviation: Der f 2-47-67, the epitope peptide of the major allergen *Dermatophagoides farinae* 2.

We analyzed the Der f 2-47-67-chitosan microparticles by scanning microscopic analysis to confirm the integration of Der f 2-47-67. As can be seen in Figure 1, the loaded chitosan microparticles are larger than the empty ones, with a mean (SD) size of 1160 (42) nm and 956 (23) nm, respectively ($P < .05$), demonstrating that the Der f 2-47-67 polypeptide had been absorbed by the chitosan.

According to protein determinations made using the Biuret method, 1 mg of Der f 2-47-67-loaded chitosan microparticles contained 0.3 mg of Der f 2-47-67 peptides.

Der f 2-47-67-Chitosan Microparticles Reduce Airway Hyperreactivity

To evaluate the efficacy of the Der f 2-47-67-chitosan vaccine, we examined airway hyperreactivity 24 hours post challenge. As can be seen in Figure 2, treatment with the loaded chitosan microparticles prior to the challenge with the Der f extract led to significantly reduced airway hyperreactivity in sensitized mice (n = 4, $P < .05$), suggesting an alleviation of bronchial asthma.

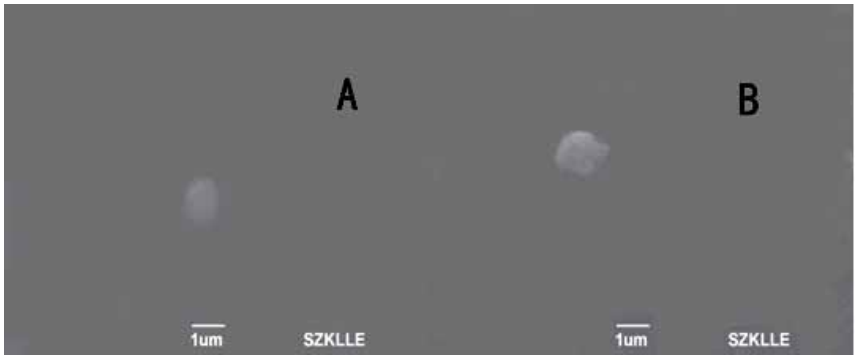

Figure 1. Scanning electron microscopic images of empty chitosan microparticles (A) and Der f 2-47-67-loaded chitosan microparticles (B). Der f 2-47-67 indicates the epitope peptide of the major allergen *Dermatophagoides farinae* 2.

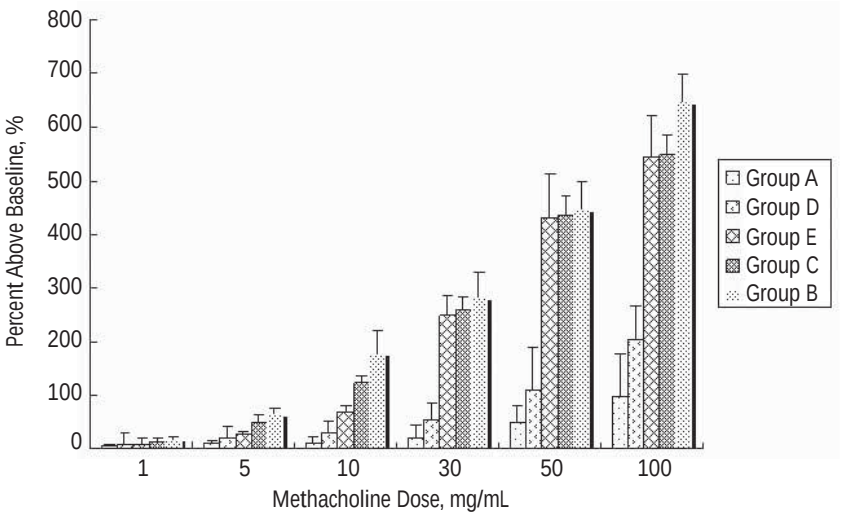

Figure 2. Effect of Der f 2-47-67-loaded chitosan microparticles on airway hyperreactivity. Group A, healthy control mice. Group B, untreated asthmatic mice. Group C, asthmatic mice treated with Der f 2-47-67. Group D, asthmatic mice treated with Der f 2-47-67-loaded chitosan microparticles. Group E, asthmatic mice treated with empty chitosan microparticles. Der f 2-47-67 indicates the epitope peptide of the major allergen *Dermatophagoides farinae* 2.

Der f 2-47-67-Chitosan Microparticles Reduce Eosinophils in Bronchoalveolar Lavage Fluid

To examine whether the Der f2-47-67-chitosan microparticle vaccine had an effect on eosinophil infiltration, we counted total cells and eosinophils in bronchoalveolar lavage (BAL) fluid, and found that both were significantly lower in the group treated with the vaccine (n = 4) than in the untreated group (n = 4) ($P < .05$). A modest reduction in the number of eosinophils was found in mice treated with chitosan alone but no significant difference was found for total cell numbers (Figure 3, Table 2). There was very little difference between the untreated group and either the chitosan group or the Der f 2-47-67 group in terms of total cell or eosinophil count (Table 2). Finally, treatment with Der f 2-47-67 alone did not exert any evident effect on the numbers of total cells or eosinophils (Table 2).

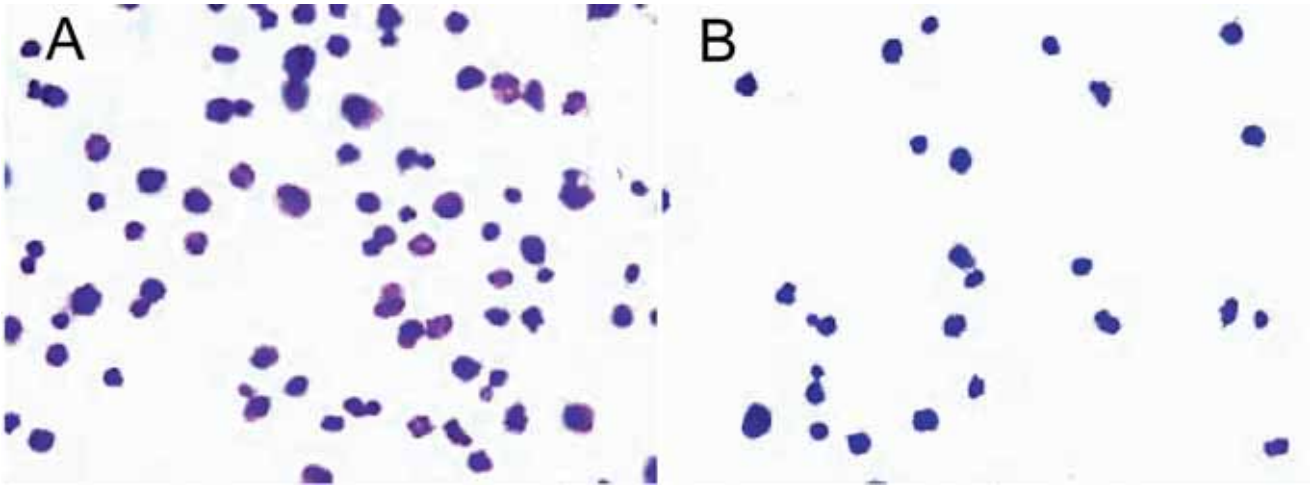

Figure 3. Representative images of cells in bronchoalveolar lavage fluid after Liu's stain. A, cells from untreated asthmatic mice. B, cells from asthmatic mice treated with Der f 2-47-67-loaded chitosan microparticles. (Original magnification, ×600). Der f 2-47-67 indicates the epitope peptide of the major allergen *Dermatophagoides farinae* 2.

Table 2. Numbers of Total Cells and Eosinophils in Bronchoalveolar Lung Fluid by Group[a]

No of Cells $\times 10^4$/mL	Healthy controls	Untreated Asthma	Der f 2	Der f 2-Chitosan	Chitosan
Total Cells	38.75 (1.31)	179.50 (1.55)	175.75 (1.8)	128.25 (3.04)[b]	151.75 (6.97)
Eosinophils	0	35.75 (1.38)	30.50 (0.65)	12.25 (1.25)[b]	27.00 (0.91)

Abbreviation: Der f 2, major allergen *Dermatophagoides farinae* 2.
[a] Values are presented as means (SD); number of mice in each group, 4
[b] Statistically significant difference with respect to untreated asthma group.

Table 3. Histologic Peribronchial and Perivascular Inflammation Scores[12] by Group

Group	Cellular Infiltration, Score (Range)	Epithelial Edema, Score (Range)	Impairment of Epithelium, Score (Range)	Total Score
Healthy controls	0	0	0	0
Untreated asthma4	(3-5)	4 (3-5)	5 (5-5)	13
Der f 2	3 (2-5)	3 (2-5)	3 (2-5)	9
Der f 2-chitosan	1 (1-2)	1 (1-3)	1 (1-2)	3
Chitosan	3 (2-5)	3 (2-5)	3 (2-5)	9

Abbreviation: Der f 2, major allergen *Dermatophagoides farinae* 2.

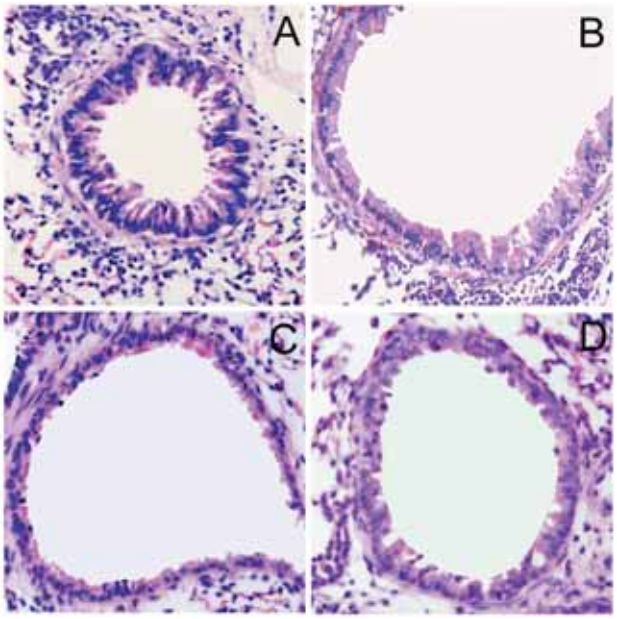

Figure 4. Histologic analysis of lung tissue from different treatment groups 36 hours after the allergen challenge. Representative images are shown (hematoxylin-eoxin stain, original magnification ×400). Note the perivascular and peribronchiolar infiltration of eosinophils and lymphocytes. A, healthy control mice. B, untreated asthmatic mice, C, asthmatic mice treated with Der f 2-47-67. D, asthmatic mice treated with Der f 2-47-67-loaded chitosan microparticles. Der f 2-47-67 indicates the epitope peptide of the major allergen *Dermatophagoides farinae* 2.

Table 4. Serum Levels of Der f-Specific Immunoglobulin (Ig) E and IgG2a in Mice by Group[a]

	Healthy controls	Untreated Asthma	Der f 2	Der f 2-Chitosan	Chitosan
IgG2a	0.12 (0.02)	0.83 (0.03)	0.84 (0.02)	0.94 (0.01)[b]	0.86 (0.02)
IgE	0	0.33 (0.02)	0.32 (0.02)	0.21 (0.04)[b]	0.29 (0.01)

Abbreviation: Der f 2, major allergen *Dermatophagoides farinae* 2.
[a] Values are presented as means (SD) and correspond to optical density readings at 450 nm; number of mice in each group, 4.
[b] Statistically significant difference with respect to untreated asthma group.

Der f 2-47-67-Chitosan Microparticles Inhibit Allergen-Induced Airway Inflammation

To examine whether the Der f 2-47-67-chitosan microparticle vaccine was capable of alleviating airway inflammation, we performed a histologic examination of lung specimens. As shown in Figure 4, there was pronounced perivascular and peribronchiolar infiltration of eosinophils and lymphocytes in the untreated asthma group but dramatically reduced inflammation

in the Der f 2-47-67-chitosan group. We scored the degree of cellular infiltration around the central bronchi, alveoli, and blood vessels. As can be seen in Table 3, infiltration by cells such as eosinophils around the central bronchi, alveoli and blood vessels, goblet cell hyperplasia, and mucin production was significantly decreased in the Der f 2-47-67-chitosan group compared to the untreated group, the Der f 2-47-67 group, and the chitosan group, as was epithelial impairment and edema.

Changes in Serum Der f-Specific IgG2a and IgE Levels

To evaluate the efficacy of the Der f 2-47-67-chitosan microparticle vaccine in modulating immune response, we measured serum levels of specific Der f antibodies by ELISA at 36 hours post challenge (Table 4). The mice in the untreated group showed significantly elevated levels of IgE compared to the healthy group ($P < .05$). The level of Der f-specific IgG2a was higher in the Der f 2-47-67-chitosan group than in the untreated group, the chitosan group, and the Der f 2-47-67 group in particular. In contrast, the level of Der f-specific IgE was significantly reduced ($P < .05$).

Discussion

Allergic asthma is a chronic inflammatory process that affects the airways. In recent years, increasing understanding of molecular and cellular mechanisms of allergic disease has led to the development of novel therapies for asthma. A DNA vaccine encoding the major dust mite allergen Der p 2, for example, was found to be effective for allergic diseases in animals [13], while a recombinant house dust mite group 2 allergen vaccine appeared to alleviate allergic airway inflammation in mice [14].

We chose chitosan as a vehicle for Der f 2-47-67 for several reasons. First, chitosan is a cationic polysaccharide capable of binding to negatively charged materials such as cell surfaces and mucus and possibly opening intercellular tight junctions and facilitating paracellular transport across the epithelium [15]; secondly, chitosan delays clearance of transported peptides, thus prolonging their antigenic exposure in vivo [16]; and finally, chitosan easily forms microparticles or nanoparticles while encapsulating antigens such as ovalbumin and tetanus toxoid [17]. These characteristics have made chitosan an ideal oral and nasal carrier for a variety of pharmacologic agents such as proteins [18], peptide drugs [19], and low-molecular weight drugs and compounds [20]. It has also been shown that chitosan might slow down mucociliary clearance of loaded drugs and transiently increase paracellular absorption [21]. Consistently, chitosan has been reported to enhance immune-stimulating activity, namely by increasing the accumulation and activation of macrophages and polymorphonuclear cells, and enhancing cytotoxic T lymphocyte response [22-24]. By enhancing mucosal absorption and immune responses, chitosan is considered to be an ideal mucosal delivering vehicle for DNA and protein vaccines**.** [25].

In this study, we found that airway hyperreactivity decreased significantly in asthmatic mice treated with Der f 2-47-67-loaded chitosan microparticles. Our histologic analysis showed that the Der f extract induced extensive infiltration of inflammatory cells with eosinophils in particular around the central bronchi, alveoli and blood vessels, as well as marked goblet cell metaplasia and mucin production in epithelial cells. The use of the Der f 2-47-67-loaded chitosan microparticle vaccine induced decreased inflammatory cell infiltration, significantly reduced peribronchial and perivascular inflammation, decreased epithelium impairment, and reduced numbers of total cells and eosinophils in BAL fluid. Moreover, our results showed that the microparticles were capable of generating an immune response which inhibited the production of Der f 2-specific IgE antibodies and induced Der f 2-specific IgG2a antibodies in allergen-induced asthmatic mice; this suggests that the vaccine might be capable of modulating an IgE immune response. Previous reports have indicated that allergens initiate allergic responses by triggering CD4+ T cell activation and IgE synthesis; such responses are closely linked to airway inflammation and bronchoconstriction [26]. In mouse models of allergic disease, it is known that IgG2a and IgE antibody isotypes reflect a type 1 helper T cell (Th1)/Th2 response. Concomitant increases in IgG2a levels and reductions in IgE levels may suggest a shift in antigen-specific lymphocytes from a Th2-type to a Th1-type response [27]. The Der f 2-47-67-loaded chitosan microparticle vaccine might have induced a Th1 immune response, suppressing allergen-specific IgE formation and decreasing lung inflammation.

Taken together, our data suggest that the Der f 2-47-67-loaded chitosan microparticle vaccine might be a novel candidate for allergic disease. Further studies on the effect of orally or nasally delivered Der f 2-47-67-loaded chitosan microparticles on asthma to evaluate the feasibility of clinical use of this vaccine in humans are undergoing in our laboratory and the preliminary results are encouraging (data not shown).

Acknowledgments

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