Article

Induction of Th1-Type Immune Response by Chitosan Nanoparticles Containing Plasmid DNA Encoding House Dust Mite Allergen Der p 2 for Oral Vaccination in Mice

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This study was to prepare the chitosan-pDer p 2 nanoparticles and to investigate the effect of chitosan-DNA nanoparticles on immune response in mice by oral delivery of chitosan-DNA nanoparticles. The nanoparticles were synthesized by complexing chitosan with plasmid DNA. The DNA was fully complexed into chitosan-DNA nanoparticles, suggesting a 100% encapsulation efficiency. Chitosan-DNA complex renders a significant protection of the plasmid. No effect on cell viability was observed in both cell types and average cell viability over 100% was obtained. Oral gene delivery with chitosan-DNA nanoparticles can generate a higher level expression of gene *in vivo*. Oral chitosan-pDer p 2 nanoparticles in BALB/c mice can induce IFN-γ in serum and prevent subsequent sensitization of Th2 cell-regulated specific IgE responses. The data indicate that the oral administration of chitosan-pDer p 2 nanoparticles results in the expression of Der p 2 in the epithelial cells of both stomach and small intestine and the induction of Th1-type immune response. *Cellular & Molecular Immunology*. 2009;6(1):45-50.

Key Words: dermatophagoides pteronyssinus, DNA vaccine, chitosan nanoparticle

Introduction

Allergens derived from house dust mites dermatophagoides pteronyssinus (Der p) have been recognized as common allergens of asthma, perennial rhinitis and atopic dermatitis (1). Mite allergy affects about 10-20% of world population. Allergic diseases are characterized by sensitization of allergen-specific Th2 cells and Th2 cytokine-dependent IgE production. DNA vaccines encoding allergen such as Der p 5 and Der p 1 have been used to prevent sensitization of IgE reactions in mice (2, 3). The dominance of the Th1 response generated by DNA vaccination may be used to modify an ongoing Th2-type immune response in allergic diseases. This property should be further explored as therapy for allergic disease such as asthma (4).

To date, most gene delivery strategies have concentrated on the parenteral route of delivery and oral administration has been largely ignored. The main advantages presented by oral gene delivery are the ease of target accessibility, and enhanced patient compliance owing to the non-invasive delivery method. But oral gene delivery must overcome the large hurdles that DNA is degraded in the stomach and intestinal tract, and the poor permeability of both genes and gene vectors across the intestinal epithelium owing to the size and charge of the gene delivery vehicles (5). It has been observed that the number of nanoparticles can cross the intestinal epithelium. Encapsulation of plasmid DNA (pDNA) in biodegradable polymer to form nanoparticles offers a way to protect pDNA from degradation. Chitosan has been an attractive gene carrier because of its high positive charges and non-toxicity to cells. In addition, chitosan has mucoadhesivity and ability to enhance the penetration of large molecules across mucosal surface (6). Chitosan particles have also been demonstrated to be taken up by the Payer's patches (7). In the present study, DNA vaccine encoding mite dust allergen Der p 2 was constructed, and chitosan Der p 2-pDNA nanoparticles were prepared. We determined the antigen expression of a vaccine encoding the mite dust allergen Der p 2 by oral chitosan-pDer p 2 nanoparticles delivery, and the potential utility of oral nanoparticle-mediated gene immunization in modulating the immune response.

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Materials and Methods

Reagents and mice

pBC167 containing Der p 2 coding region gene segment was obtained from HESKA (USA). pcDNA3.1 plasmid was obtained from Invitrogen (USA). Chitosan (molecular weight, about 390,000 Da) was obtained from Sigma (USA). MAXIPREP GFII™ Endo-Free Kit was obtained from QBIOgene (USA). Peroxidase-conjugated anti-mouse IgE and IgG2a antibody were obtained from SouthernBiotech (USA). Anti-Der p 2 mAb was a gift from Department of Parasitology and Institute of Tropical Medicine, Yonsei University College of Medicine (Seoul, Korea). HEK and HeLa cells were gifts from College of Life Sciences, Shenzhen University (China). BALB/c mice 6-8 weeks were obtained from Guangzhou Experiment Animal Center (Guangzhou, China).

Plasmid construction

pBC167 was used as a template for PCR with Taq polymerase with primers specific for Der p 2 (5'-CTG GAT CCG ATA TGG ATC AAG TCG ATG-3' and 5'-CGG GAA TTC TTA ATC GCG GAT TTT AGC-3'). This primer includes *Bam*H I and *Eco*R I sites for cloning. The amplified PCR products were then cloned into pcDNA3.1 eukaryotic expressing vector (Invitrogen, San Diego, CA, USA) and sequenced to verify the insertion of the right gene with appropriate open reading frame. Large-scale purification of both plasmids was conducted using a QBIOgene MAXIPREP GFII™ Endo-Free Kit (QBIOgene Inc, USA) according to the manufacturer's instructions.

Der p 2 proteins

Der p 2 were expressed as His-fusions from pET-24a (Navogen, CA) plasmids containing inserts that encode for Der p 2. The pET-24a-Der p 2 was obtained from Shenzhen University and protein expression was carried out. The Der p 2 were purified using the Ni-NTA His-Bind affinity chromatography. The His-rDer p 2 are hereafter referred to as rDer p 2.

Preparation of chitosan Der p 2 nanoparticles

Nanoparticles were made by complex coacervation of chitosan and DNA as reported (1). Briefly, chitosan (pH 5.5, 0.02% in a 25 mM sodium acetate-acetic acid) was heated for 10 min at 55°C and recombinant plasmid (10 μg) dissolved in 25 mM Na_2SO_4 was also heated for 10 min at 55°C. After heating, chitosan (100 μl) and DNA (100 μl) were mixed, vortexed vigorously for 30 s, and stored at room temperature until use.

DNase degradation test

To assess the efficiency of encapsulation and stability against nuclease digestion, 20 μl chitosan-DNA complex (containing 1 μg plasmid) was incubated with various concentrations of DNase I for 15 min at 37°C, pH 7.0. Adding EDTA stopped the reaction. Meantime, 20 μl chitosan-DNA complex (containing 1 μg plasmid) was incubated with 10 U DNase I for 15 min at 37°C, pH 3.0, pH 7.0 or pH 9.0. Then the undegraded (1 μg) and degraded plasmid, undegraded and degraded chitosan-DNA complex, were subjected to 1% (w/v) agarose gel electrophoresis.

Evaluation of cytotoxicity

The cytotoxicity of chitosan dissolved in acetic acid at various concentrations was evaluated using MTT colorimetry. HEK and HeLa cells were seeded at a density of 1×10^4 and 5×10^3 cells/well, respectively, in 200 μl of growth medium in 96-well microtitre plates and incubated for 24 h prior to the addition of filtered chitosan. Growth medium were replaced by fresh serum-free DMEM medium containing chitosan (0.05, 0.5, 5 and 25 μg/ml). Cells were incubated with chitosan for 48 h and replaced with 100 μl growth medium prior to the addition of the 20 μl/well 10 mg/ml MTT solution. After further incubation for 4 h, the absorbance was then read at 590 nm using a microplate reader. Chitosan-untreated cells in media were used as positive reference. The cell viability (%) was calculated according to the following equation: Cell viability (%) = ($OD_{570\ (sample)}$ / $OD_{570\ (control)}$) × 100%

Protein expression in mouse stomach and small intestine

BALB/c mice were divided randomly into PBS group (n = 5), chitosan nanoparticles group without DNA (n = 5), naked plasmid (Der p 2) (n = 5) and chitosan/Der p 2 nanoparticles group (n = 5). BALB/c mice were fed with either chitosan-DNA nanoparticles containing the Der p 2 gene (pDer p 2, 100 μg/mice) or naked plasmid DNA (pDer p 2), using animal feeding needles. Three days later, the mice were killed, with their stomachs and small intestines surgically removed. The tissues were fixed with 10% neutral-buffered formalin and embedded in paraffin. Sections (5 μm) of specimens were cut onto APES-coated slides. Tissue sections were deparaffinized and rehydrated with graded ethanol solutions, and endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide for 30 min at 25°C, nonspecific antigens were blocked by exposure to 10% nonimmune horse serum for 30 min at room temperature. After that, slides were then incubated with anti-Der p 2 mAb at dilutions of 1:100 at 4°C. The same process without the primary antibody was used as a control after incubation with secondary antibody labeled with biotin at 37°C for 1 hour. Then, the sections were incubated with biotinylated mouse anti-mouse antibody (1:100 dilution) and peroxidase-conjugated avidin (1:100 dilution). Lastly, the brown color reaction was developed with the addition of diaminobenzidine for 5 min. The pictures were transferred into a computer and adjusted for equal brightness and contrast using Adobe Photoshop.

Immunization

Thirty-two BALB/c mice were divided randomly into groups of PBS (n = 8), chitosan naonoparticles without pDer p 2 (n = 8), naked pDer p 2 (n = 8) and chitosan-pDer p 2 nanoparti-

cles (n = 8). BALB/c mice were immunized orally with various formulations using animal feeding needles attached to a 1 ml syringe. Immunized mice were fed with chitosan-pDer p 2 nanoparticles containing the Der p 2 gene (100 μg/mice). Control mice were treated with chitosan nanoparticles in the absence of DNA, with chitosan nanoparticles complexed without pDer p 2, or with naked DNA alone. They were boosted 1 weeks later. Two weeks after the final vaccine, mice were sensitized *i.p.* with 20 μg rDer p 2 adsorbed onto 4 mg alum and suspended in 0.5 ml of PBS. Blood was collected at weeks 4 (before sensitization) and 7 (after sensitization) through tail veins. Blood was collected from immunized and control mice to measure serum IgG2a and IgE.

Determination of Der p 2-specific IgE and IgG2a

Blood from mice of the four groups was collected on 4 and 7 weeks. Der p 2-specific IgE and IgG2a were determined by ELISA as follows. Ninty-six microtiter plate was coated overnight at 4°C with 100 μl of rDer p 2 (10 μg/ml in 0.1 M carbonate buffer, pH 9.6). The antigen-coated plates were washed five times with PBST (0.5% Tween-20 in PBS). Mouse sera were added to the antigen-coated wells, the plates were incubated with peroxidase-conjugated anti-mouse IgE and IgG2a antibody (SouthernBiotech, USA) overnight at 4°C, and then washed five times with PBST before adding citric acid-phosphate buffer (pH 5.0) containing 0.15 mg/ml of O-phenylenediamine (Sigma, USA). Color was developed at 37°C, and the reaction stopped with 2.5 M sulfuric acid, and measured at 450 nm.

Determination of IL-4 and IFN-γ

Serum from mice in the four groups was collected at week 7. Serum was stored at -70°C and the levels of IL-4 and IFN-γ were determined using ELISA kit (R&D systems, USA).

Statistical analysis

Specific IgG2a antibody and cytokine were expressed as means ± SD, and all group comparisons for specific IgG2a, IgE responses and cytokines were made using ANOVA, and the significant level was defined as a $p < 0.05$. The data were analyzed by SPSS statistical software.

Results

The construction and identification of pcDNA3.1-Der p 2 recombinant vector

Der p 2 coding region gene segment was added a Kozak consensus translation codon by PCR. The PCR segments digested with *Bam*H I and *Eco*R I were approximately 393 bp in length. They were cloned into vector pcDNA3.1 and recombinant plasmids, designated pcDNA3.1-Der p 2, were identified by digestion with restriction enzymes *Bam*H I and *Eco*R I. After digestion, two bands of 5.3 kb and 393 bp could be visualized on 1% agarose gels (Figure 1A). The inserted Der p 2 gene contain a Kozak translation initiation sequence and ATG start codon for proper initiation of translate (Figure 1B).

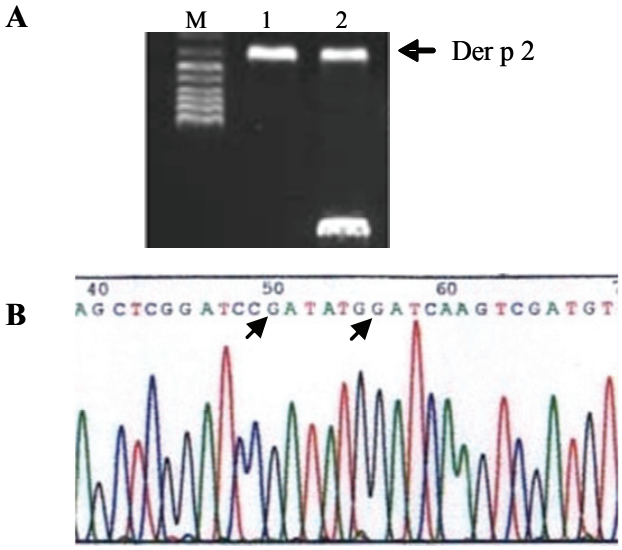

Figure 1. The construction and identification of pcDNA3.1-Der P2 recombinant vector. (A) Lane M, 100bp Marker; Lane 1, PCR amplification of Der p 2 cDNA; Lane 2, pcDNA3.1-Der p 2 cut by *Bam*H I/*Eco*R I. (B) The ATG initiation codon is shown.

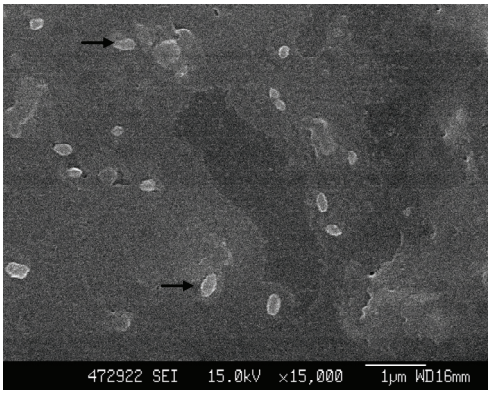

Figure 2. Appearance and size of DNA nanoparticles were characterized by scanning electron micrograph of chitosan-DNA nanoparticles. Scale bar represents 1 μm.

Particle characterization

The nanoparticles were synthesized by complexing high-molecular weight (about 390,000 Da) chitosan with plasmid DNA. Relatively uniform particles were obtained using 50 μg/ml DNA (DNA in 50 mM sodium sulfat) and 0.02% chitosan (pH 5.5). Scanning electron microscopy showed that chitosan-DNA nanoparticles were 100-400 nm in size and fairly spherical (Figure 2). The chitosan-DNA nanoparticles were analyzed by agarose gel electrophoresis along with non-encapsulated DNA as control. DNA was completed retained in the loading well after complexation with chitosan. This confirmed that the DNA was fully complexed into

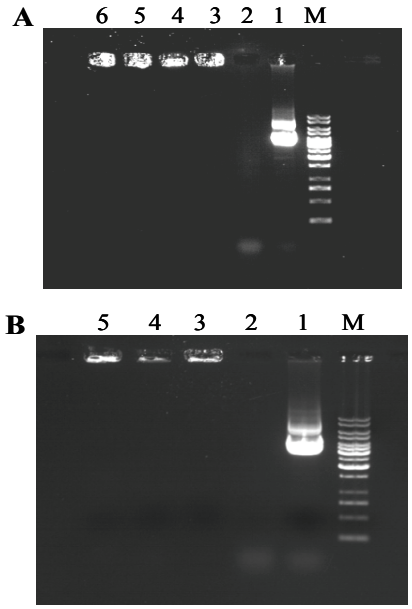

Figure 3. Electrophoretic mobility analysis of chitosan-DNA nanoparticles following DNase I digestion. (A) Naked DNA and nanoparticles were both incubated with different concentrations of DNase I for 15 min at 37°C. Lane M, molecular marker; Lane 1, control pasmid; Lane 2, naked plasmid DNA + DNase I (10 U); Lane 3, chitosan-DNA nanoparticles + DNase I (10 U); Lane 4, chitosan-DNA nanoparticles + DNase I (20 U); Lane 5, chitosan-DNA nanoparticles + DNase I (30 U); Lane 6, chitosan-DNA nanoparticles + DNase I (40 U). (B) Naked DNA and nanoparticles were both incubated with DNase I in different pH for 15 min at 37°C. Lane M, molecular marker; Lane 1, control pasmid; Lane 2, naked plasmid DNA + DNase I (10 U), pH 3; Lane 3, chitosan-DNA nanoparticles + DNase I (20 U), pH 3; Lane 4, chitosan-DNA nanoparticles + DNase I (20 U), pH 7; Lane 5, chitosan-DNA nanoparticles + DNase I (20 U), pH 9.

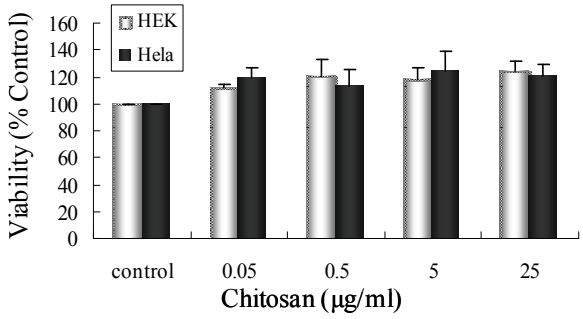

Figure 4. Cytotoxicity of chitosan against HEK cells and HeLa cells. At various concentrations (0.05-25 μg/ml), there were no differences in the cell viability of HEK and HeLa cells ($p > 0.05$), and cell viability over 100% was obtained.

chitosan-DNA nanoparticles, suggesting a 100% encapsulation efficiency.

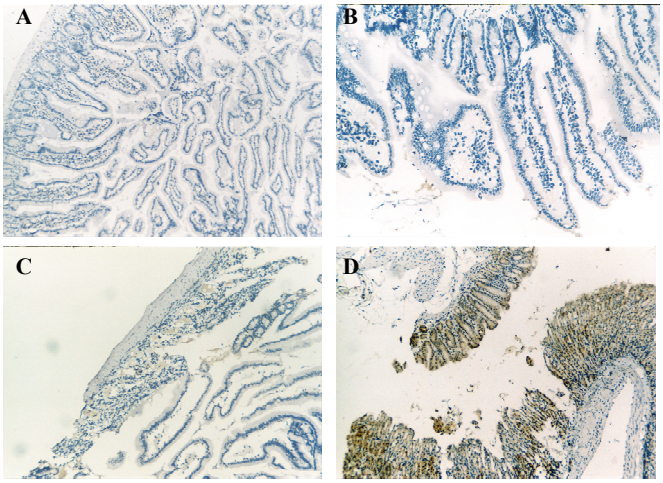

Figure 5. Immunohistochemical examination of Der p 2 expression in mouse stomach and small intestine 3 days after oral delivery of DNA nanoparticles. (A) Intestine from oral PBS (×100). (B) Intestine from oral naked DNA (pcDNA3.1) (×100). (C) Intestine from oral naked DNA (pDer p 2) (×100). (D) Intestine from oral chitosan-pDer p 2 nanospheres (×100).

DNase degradation test

The effect of protection to plasmid DNA from DNase degradation was examined using DNase I as a model enzyme. When incubated with 10 U DNase I at 37°C, naked plasmid DNA showed significant degradation in 15 min (Lane 2 of Figure 3A), whereas the plasmid DNA recovered from nanoparticles after the same treatment remained intact (Lane 3 of Figure 3A). At a range concerntration of DNase I from 10-40 U, plasmid DNA with chitosan-DNA complex remained intact during the same period of incubation (Lanes 3-6 of Figure 3A). At different pH condition, plasmid DNA with chitosan-DNA complex remained intact during the same period of incubation (Lanes 3-5 of Figure 3B), whereas naked plasmid DNA in pH 3 was completely degraded by DNase I. This suggested that chitosan-DNA complex render a significant protection of the plasmid.

Cytotoxicity of chitosan

We tested the cytotoxicity of chitosan at different concentrations used for the preparation of chitosan-DNA nanoparticles in two different cell lines. Chitosan displayed profiles being non-toxic at various concentrations (0.05-25 μg/ml). No effect on cell viability was observed in both cell types and average cell viability over 100% was obtained (Figure 4).

Gene expression in vivo

To assess the expression *in vivo* after oral chitosan-DNA nanoparticles delivery, we fed BALB/c mice either chitosan-DNA naonoparticles containing the Der p 2 gene (pDer p 2) or naked plasmid (pDer p 2), PBS and chitosan naonoparticles without pDer p 2. We determined the tissue expression of Der p 2 in stomach and small intestine 3 days

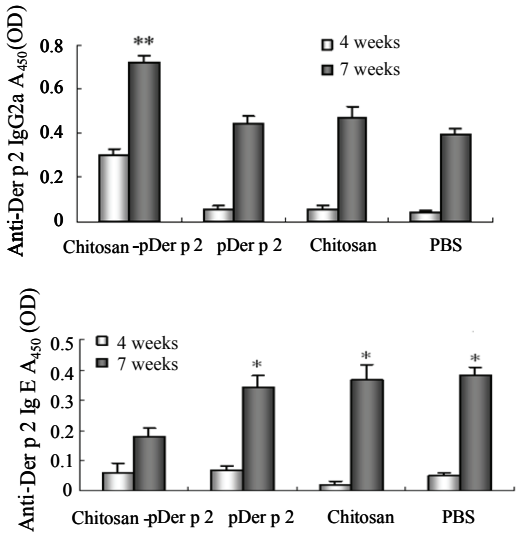

Figure 6. The levels of anti-Der p 2 IgG2a and anti-Der p 2 IgE by ELISA in serum. Serum 1:200 dilution for IgG2a. $^{**}p < 0.01$, compared with pDer p 2, chitosan and PBS groups. Serum 1:20 dilution for IgE. $^{*}p < 0.01$, compared with pDer p 2, chitosan and PBS groups.

after the oral administration. Mice fed with the chitosan-DNA naonoparticles showed a higher level of gene expression in the epithelial cells of both stomach and small intestine, whereas mice fed the naked plasmid DNA (pDer p 2), chitosan naonoparticles without pDer p 2 or PBS showed some background staining (Figure 5). The result showed oral gene delivery with chitosan-DNA nanoparticles can generate a higher-level expression of gene *in vivo*.

Antibody response against Der p 2

To assess the potential utility of oral nanoparticle-mediated gene immunization in modulating the immune response, we measured the change of serum IgG2a (1:200) and IgE (1:20) antibody. Four weeks after the first immunization, there was a substantial induction of serum anti-Der p 2 IgG2a in chitosan-DNA nanoparticles treated mice. Mice receiving naked pDer p 2, PBS or chitosan nanoparticles without pDer p 2 showed no detectable levels of anti-Der p 2 IgG2a. After sensitization, those receiving chitosan-DNA nanoparticles had moderate increases of IgG2a antibody responses, but conversely suppressed IgE responses. Whereas the controls receiving naked pDer p 2, PBS or chitosan nanoparticles without pDer p 2 had rapidly increased IgG2a, and their specific IgE antibody responses were increased (Figure 6).

The levels of IL-4 and IFN-γ in serum

We assessed cytokine production in serum on 7 week. The levels of IL-4 and IFN-γ in serum were analyzed, The levels of IL-4 in chitosan-DNA nanoparticles treated mice were lower than chitosan naonoparticles without pDer p 2 and naked pDer p 2, as compared with chitosan naonoparticles without pDer p 2 and naked pDer p 2 ($p < 0.01$, Figure 7).

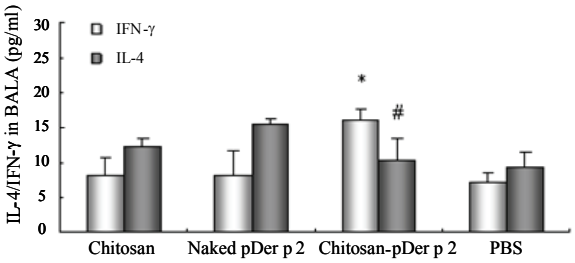

Figure 7. IL-4 and IFN-γ were determined by ELISA in t serum. $^{*}p < 0.01$, compared with the groups of PBS group, chitosan naonoparticles without pDer p 2 and naked pDer p 2. $^{\#}p < 0.01$, compared with the group of PBS, chitosan naonoparticles without pDer p 2 and naked pDer p 2.

The levels of IFN-γ in chitosan-DNA nanoparticles treated mice were higher than PBS group, chitosan naonoparticles without pDer p 2 and naked pDer p 2, as compared with PBS groups, chitosan naonoparticles without pDer p 2 group and naked pDer p 2 group ($p < 0.01$, Figure 7).

Discussion

Oral delivery is attractive due to factors such as ease of administration, leading to improved patient convenience and compliance, thereby reducing overall healthcare costs (4). The oral delivery of peptide, protein, vaccine and nucleic acid-based biotechnology products is the greatest challenge facing the drug delivery industry. Chitosan is a natural biodegrable mucoadhesive polysaccharide derived from crustacean shells. This slowly degradable polymer has been shown to increase transcellular and paracellular transport of macromolecules across intestinal epithelial monolayers (8). Chitosan has more recently been used successfully to deliver a reporter gene (encoding chloramphenicol acetyl transferase) orally to enterocytes, Peyer's patches and mesenteric lymph nodes (9). Chew et al. demonstrated that mice fed with chitosan-pDer p 1 (encoding mite dust allergen Der p 1 gene) successfully primed Th1-skewed immune responses against Der p 1. Roy et al. have confirmed the effectiveness of orally delivered chitosan-DNA nanoparticles in inducing protective immunity in the peanut allergy mouse model (10). It had been reported that DNA vaccine can be easily delivered into fish by feeding with chitosan nanoparticles (11).

In the present study, we succeeded construction and identification of pcDNA3.1-Der p 2 recombinant vector. DNA-chitosan nanoparticles had also been prepared by a complex coacervation method. The fairly spherical particles sizes ranging from 100-400 nm were observed *via* scanning electron microscopy. Our findings confirmed that the DNA was fully complexed into chitosan-DNA nanoparticles, suggesting a 100% encapsulation efficiency. Several previous studies suggested that particles of up to 10 μm diameter could be phagocytosed by M cells. Chitosan-pDer p 2 nanoparticles, prepared by a complex coacervation method,

had an range from 100-400 nm, should be good candidates for vaccination. The greatest challenges faced by oral gene therapy that need to be overcome, are the acid pH, the nucleases, lipases and peptidases present in GI tract. As physiological concentrations of enzyme can merely be estimated, protection against DNase was checked by incubation of the chitosan/DNA complexes or naked DNA with DNase I as a model enzyme at different concentrations and pH, followed by gel electrophoresis. Our results demonstrated that chitosan was able to protect DNA against degradation more efficiently at different concentrations and pH. Meantime, chitosan displayed profiles being non-toxic. No effect on cell viability was observed in both cell types and average cell viability over 100% was obtained. The data indicated that chitosan is a good candidate for the development of conventional and novel gastrointestinal drug and gene delivery systems.

In mouse allergic disease model, it is confirmed that IgG2a and IgE antibody isotypes is a reflection of an altered T-lymphocyte response. IFN-γ is an important switch factor for IgG2a synthesis by murine B lymphocytes, and low concentrations of IFN-γ inhibit IL-4-induced increases in IgE synthesis. Thus, the concomitant increase in IgG2a levels in conjunction with the decline in IgE levels suggests that a shift in the cytokine profile of antigen-specific lymphocytes from a Th2- to Th1-type response (12). This study was designed to investigate the effect of chitosan-DNA nanoparticles on immune response in mice by oral delivery of chitosan-DNA nanoparticles as a potential treatment for allergen-induced airway eosinophil dominant inflammation. Our result demonstrated, 3 days after the oral chitosan-DNA nanoparticle administration, a higher level of gene expression was found in the epithelial cells of both stomach and small intestine. The present study has also shown BALB/c mice immunized by oral chitosan-pDer p 2 nanoparticles can preferentially induce IFN-γ and thus prevents subsequent sensitization of Th2 cell-regulated specific IgE responses. This finding is consistent with recent reports (13).

In summary, our results demonstrate that chitosan is a good candidate for the development of novel gene delivery systems. Oral chitosan-pDer p 2 nanoparticles can preferentially activate specific Th1 immune responses and thus prevents subsequent sensitization of Th2 cell-regulated specific IgE responses. It may also be a promising gene delivery system for Th1-skewed immunity.

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