

Original article

The effect of vaccination with DNA encoding murine T-cell epitopes on the Der p 1 and 2 induced immunoglobulin E synthesis

Background: Immunization with naked plasmid DNA leads to strong and persistent cell-mediated and humoral immune response to plasmid encoded antigen. Vaccination of DNA encoded whole allergen has been tried, but little information is currently available on the efficacy of DNA encoding T-cell epitopes in allergic disease. The purpose of this study was to determine whether the vaccination of naked plasmid DNA encoding only T-cell epitopes suppresses the allergic reaction as effectively as naked DNA encoding whole segments of allergen.

Methods: We immunized mice with a mixed naked plasmid DNA encoding the five classes of murine T-cell epitopes on Der p 1 and Der p 2 three times at weekly intervals via an intramuscular injection of BALB/c mice. Control mice were injected with the pcDNA 3.1 blank vector. After 3 weeks, the mice were actively sensitized twice and allowed to inhale the Der p extracts intranasally six times at weekly intervals.

Results: The vaccinated mice showed a significant attenuated induction of Der p-specific immunoglobulin E synthesis compared to controls. In terms of the Der p-specific IgG2a antibody response, the vaccinated mice showed more prominent responses than the control mice group. In addition, analysis of the cytokine profile after Der p stimulation of the lymph-node cells revealed that the level of the mRNA expression of the interferon- γ gene was higher in the vaccinated mice than in the controls. Histologic studies showed a much reduced infiltration of inflammatory cells in lung tissue of the gene-vaccinated mice in comparison with the controls.

Conclusions: These results suggest that vaccination with DNA encoding T-cell epitopes effectively inhibits allergen-induced IgE synthesis and reduces cell infiltration in lung tissue. Thus, gene therapy using T-cell epitope-encoding DNA presents an ideal way of combating allergic disease in the future.

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The main characteristic of the allergic diathesis is high IgE response to allergens. It has been revealed that the relatively minor determinants of house-dust-mite (HDM) allergens show IgE binding with 40% or more of allergic sera (1–3). The most frequently implicated allergens are derived from the predominant species *Dermatophagoides pteronyssinus* (Der p) and *D. farinae* (Der f) (4). It has been reported that IgE-mediated allergy to mites is associated with diseases such as asthma, allergic rhinitis, and atopic dermatitis (5). Immunotherapy with subunit vaccination has been used to downregulate the allergic responses. However, these traditional vaccination methods have substantial limitations that would be overcome if the immunizing protein could be expressed in host cells.

Recently, it has been revealed that genetic vaccinations with naked DNA provide long-lasting cellular and

humoral immune responses (6–8). Long-term persistence of plasmid DNA and foreign gene expression in muscle suggests that muscle is an attractive target tissue for foreign gene vaccination (9). Many studies have subsequently revealed that gene immunization with plasmid DNA encoding allergens or protein antigens induces strong Th1 immune responses in mice and rats (10–13), but little information is currently available on the efficacy of DNA encoding T-cell epitopes in allergic disease.

Therefore, we examined the effect of vaccination with DNA encoding only murine T-cell epitopes on allergic responses. Our results suggest that genetic vaccination encoding only T-cell epitopes indeed induced the Th1 cytokine immune responses which reduced IgE antibody production and decreased the infiltration of inflammatory cells into the lung, and was finally

effective at suppressing allergic responses to Der p. Therefore, it would be useful to develop an alternative naked DNA vaccination method, which could be safer than injecting whole segments of the encoding region of Der p 1 or Der p 2.

Material and methods

Mice

Twenty BALB/c mice aged 6–8 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred at the University of Tennessee (Memphis, TN, USA). This study was performed in accordance with PHS policy on the humane care and use of laboratory animals, and the NIH Guide for the Care and Use of Laboratory Animal Welfare Act (7 U.S.C. *et seq.*); the protocol on animal use was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Tennessee.

Plasmid construction

RNA was isolated from HDM, according to the instructions of the Perkin Elmer Gene Amp RNA PCR kit (Perkin Elmer, Branchburg, NJ, USA), as used as a template for reverse transcriptase. This cDNA was then used for PCR with Taq polymerase with primers specific for the Der p 1 three-epitope regions, 21–49 (5'-CCGGAATTCGCCGCCACCATGACTGTCACTCCCATTCGTATGC-3' and 5'-TGCTCTAGATTAAAGCCAAATAAGCTGATTCAGTTGC-3'), 78–100 (5'-CCGGAATTCGCCGCCACCATGCGTGGTATTGATACATCCAACAT-3' and 5'-TGCTCTAGATTATTCTCGTGC-AACGTATCGATAGTA-3'), 110–131 (5'-CCGGAATTCGCCGCCACCATGCGTTTCGGTATCTCAAATATTGC-3' and 5'-TGCTCTAGATTACAAAGCTTCACGAATTTTGTTC-3'), and Der p 2 two-epitope regions, 11–35 (5'-CCGGAATTCGCCGCCACCATGCATGAAATCAAAAAAGTTTGGTA-3' and 5'-TGC-TCTAGATTAGAATGGTTTACCACGATGAATGAT-3'), 87–129 (5'-CCGGAATTCGCCGCCACCATGGATATTAATATACATGGAATGTTCCGA-3' and 5'-TGCTCTAGATTAAATCGCGGATTTTAGCATGAGTAGC-3'). These primers cover the murine T-cell epitopes in both Der p 1 and Der p 2 (14), and include EcoRI and XbaI sites for cloning. The amplified PCR products were then cloned into pcDNA3.1 eukaryotic expression vector (Invitrogen, San Diego, CA, USA) and sequenced to verify the insertion of the right gene with the appropriate open reading frame.

DNA preparation and vaccination

Each plasmid construct was prepared with Maxi Prep (Qiagen, Chatsworth, CA, USA). The mice were vaccinated by injection with 300 µg of the pcDNA3.1 blank vector in 100 µl of PBS (control group) or the same amount of the mixed naked DNA encoding the murine T-cell epitopes on Der p 1 and 2 (vaccination group) three times at weekly intervals intramuscularly.

Immunization and inhalation of allergen by mice

Der p-induced sensitivity in a mouse model was performed as previously described (15). Der p was emulsified with an equal volume of complete Freund's adjuvant (CFA) for immunization. Three weeks after the final vaccination, the mice were sensitized subcutaneously at the base of the tail with 100 µg of Der p extract in CFA. The mice were also given an intraperitoneal dose of 300 ng of purified pertussis toxin 24 and 72 h after the first immunization. Seven days later, the mice were boosted again with the same amount of antigen in incomplete Freund's adjuvant. The mice were allowed to inhale intranasal 10 µg of Der p extract six times at weekly intervals from immunization (Fig. 1).

Determination of Der p-specific IgG1, IgG2a, and IgE

Blood from the six mice in the two groups was collected four times on weeks 0 (first immunization), 2, 4, and 6. Der p-specific IgG, IgG1, and IgG2a were determined by ELISA as follows. Fifty microliters of Der p (5 µg/ml in 0.1 M carbonate buffer, pH 9.6) was dispensed into each well of a polystyrene microtiter plate (Cost, Cambridge, MA, USA) and incubated overnight at 4°C. The antigen-coated plates were washed three times with 0.05% PBS-Tween 20 buffer (washing buffer) and incubated with mice sera overnight at 4°C. Plates were washed five times with washing buffer and incubated with peroxidase-conjugated antimouse IgG, IgG1, and IgG2a antibody (Sigma, St Louis, MO, USA) overnight at 4°C, and then washed a further five times before adding citric acid-phosphate buffer (pH 5.0) containing 0.15 mg/ml of *O*-phenylenediamine (Sigma, St Louis, MO, USA). Color was developed at room temperature, and the reaction stopped with 2.5 M sulfuric acid, and measured at 492 nm (Bio-Rad, Richmond, CA, USA). To measure Der p-specific IgE antibodies, we coated the plates with 25 µg/ml of HDM allergen in 0.1 M carbonate buffer (pH 9.6), and the serum samples were diluted fivefold in 10% FCS. The other procedures were the same as the measurement of Der p-specific IgG, which was used except for HRP-conjugated antimouse IgE detection mAb (clone R35-118; Pharmingen, San Diego, CA, USA). The level of Der p-specific IgE was compared to the standard serum pooled from six mice that were immunized with 100 µg of HDM twice, and that had nasally inhaled 10 µg of antigen six times. The standard serum is expressed as 100 ELISA units/ml.

Histologic examination of lung tissue

Mice were anesthetized with a mixture of ketalar (35 mg/ml), rompun (0.6%/ml), and atropine (0.1 mg/ml), of which 0.2 ml was injected intramuscularly. The vascular bed of the lungs was then perfused with 0.01 M phosphate-buffered saline (PBS) and 4% paraformaldehyde 0.1 M PBS buffers. Whole lungs were removed and stored in 4% paraformaldehyde for 24 h at 4°C. After fixation, tissues were dehydrated and embedded in paraffin. Frozen sections of 5 µm were stained with hematoxylin and eosin and coded. The sections were evaluated by two observers using light microscopy. The number of mononuclear cells per section was scored by the method of Hessel et al. (16). This scoring method discriminates between the presence of mononuclear cells around blood vessels (score 0–3) and around bronchioli (score 0–3), and the number of patchy cellular infiltrates (score 0–3). Histologic scores were analyzed by the nonparametric Mann-Whitney *U* test. At least five mice from each of the two groups were examined.

Immunohistochemical staining for CD4+ and CD8+ T cells in lung

The lung tissues from the experimental and control groups were removed after the final intranasal inhalation. Tissues were fixed with periodate-lysine-paraformaldehyde solution for 24 h at 4°C. Frozen sections of 4–6 µm were rehydrated and rinsed in cold PBS. Endogenous pseudoperoxidase was blocked with absolute methanol containing 0.5% of hydrogen peroxide for 20 min at room temperature. Sections were then treated with 10% normal goat serum in PBS to reduce nonspecific binding. Biotin-conjugated rat antimouse CD8 or CD4 (Pharmingen, San Diego, CA, USA), diluted to 1:200 in PBS and containing 0.5% of bovine serum albumin, was applied to the sections and incubated overnight at 4°C. After rinsing, the sections were incubated with avidin-biotin peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature and rinsed with PBS. The reaction was developed with 0.02% 3,3'-diaminobenzidine in 0.05 M of Tris buffer (pH 7.6) with 0.005% hydrogen peroxide for 7 min. Sections were dehydrated, cleared in xylene, and mounted.

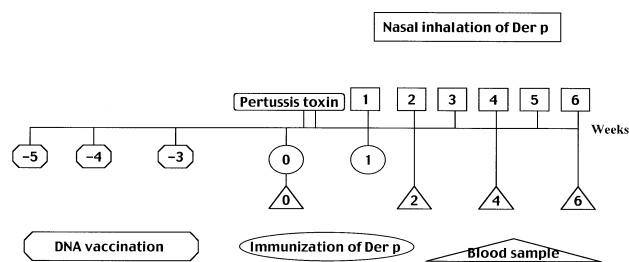


Figure 1. Vaccination and sensitization protocol.

Measuring cytokine mRNA expression

Four mice from each of the two groups were killed 10 days after immunization with Der p extract. The lymph nodes were removed and stimulated with Der p extract (100 µg/ml) *in vitro* for 18 h. Cells were washed with PBS buffer and mRNAs prepared (Biotex, Houston, TX, USA). With murine leukemia virus reverse transcriptase and random hexanucleotide primer, according to the instructions of the Perkin Elmer Gene Amp RNA PCR kit (Perkin Elmer, Branchberg, NJ, USA), first-strand cDNA was generated from 1 µg of total RNA and subjected to RT-PCR analysis. For determination of the relative abundance of each cytokine's mRNA expression, the amount of each cDNA for PCR was optimized by the intensity of the amplified DNA products of β-actin from each RNA. In the PCR reaction mixture, either β-actin (5'-GTGGGCCGC-TCTAGGCACCAA-3' and 5'-CTCTTTGATGTCACGCACGAT-TTC-3') as control primer, IL-2 (5'-TTCAAGCTCCACTTCA-AGCTCTACAGCGGAAG-3' and 5'-GACAGAAGGCTATCCA-TCTCCTCAGAAAGTCC-3'), IFN-γ (5'-TGCATCTTGGCTT-TGCAGCTCTTCCTCATGGC-3' and 5'-TGGACCTGTGGGT-TTGACCTCAAACCTGGC-3') (Clontech, Palo Alto, CA, USA), IL-4 (5'-CAGCTAGTTGTCATCCTGCTCTTC-3' and 5'-GTGATGTGGACTTGGACTCATTGATGG-3'), or IL-5 (5'-TG-TCTGGGCCACTGCCATGGAGATTC-3' and 5'-CCATTGC-CCACTCTGTACTCATCACAC-3') at final concentrations of 0.2 µM was added. The PCR conditions were as follows: 200 µM

of dNTP, 10 µCi [32P] dCTP, 50 µM Tris HCl (pH 9.0), 50 µM NaCl, 2 µM MgCl₂, 0.5 mM DTT, and two units of Taq polymerase (Perkin Elmer, Branchberg, NJ, USA) at a final volume of 20 µl. A negative control reaction was performed in parallel with each sample to verify that no PCR bands appeared in the absence of template. The optimal amplification conditions were as follows: 30 cycles of 45 s at 94°C for denaturation, 45 s at 67°C for annealing, and 1 min at 72°C for elongation. The amplified DNAs of β-actin, IFN-γ, IL-2, IL-4, and IL-5 were 540, 365, 413, 354, and 349 base pairs, respectively. Gels were dried on Whatman 3M paper and exposed to Kodak XAR film. In each electrophoresis run, intra- and inter-gel staining homogeneity was confirmed by the staining intensity of molecular weight markers at both ends of the gels. In general, amplification kinetics was monitored for each PCR run by examining aliquots of the products on the gel. Amounts of the PCR products were compared during the cycle where the amplification was attained before reaching saturation.

Statistical analysis

The results of immunoglobulin response were analyzed by Student's paired *t*-test for comparisons between the control and experimental groups. Histologic grades were analyzed by a nonparametric Mann-Whitney *U* test. Results are expressed as mean ± SD. A *P* value of <0.05 was considered significant.

Results

Der p-specific IgE, IgG1, IgG2a, and IgG antibody responses by gene vaccination

To examine immune response, we examined IgG, IgG1, G2a, and IgE antibody production by ELISA (Figs. 2 and 3). Gene vaccination with the murine T-cell epitope on Der p 1 and 2 showed about 70% inhibition of Der p-specific IgE antibody production compared with the control group at week 6 (Fig. 2). Thus, genetic vaccination can inhibit an *in vivo* allergen-specific IgE synthesis efficiently. The production of Der p-specific IgG2a antibodies in the vaccination group was increased more than that in the control group after 6 weeks (Fig. 3A), but in terms of the Der p-specific IgG1, and IgG antibody responses, the two groups were similar (Fig. 3B and C).

Cytokine gene expression by antigen stimulation *in vivo*

To determine whether the Th1- or Th2-dominant cytokines are involved in the genetic vaccination, we performed a RT-PCR analysis on the total RNA samples extracted from the lymph-node cells (1×10^7 cells per well) that were cultured in the presence of Der p (100 µg/ml) *in vitro* for 18 h. A higher mRNA expression of IFN-γ was detected in the vaccination group than in the controls. But the levels of the mRNA expressions of IL-2, -4, and -5 in the vaccination group were found to be no different from the controls (Fig. 4). These results indicate that vaccinations with Der p epitope DNA induced a dominant Th1 cytokine (IFN-γ) gene expression in the lymph node.

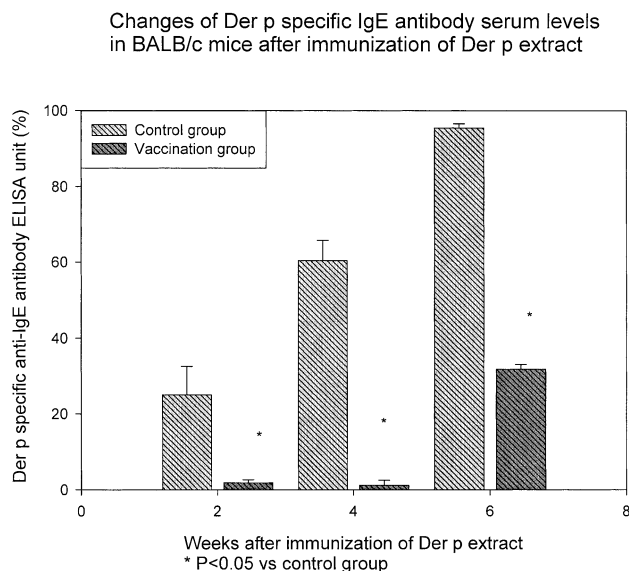
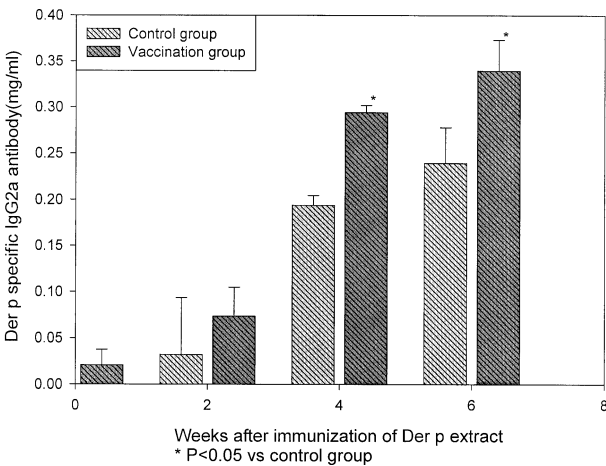
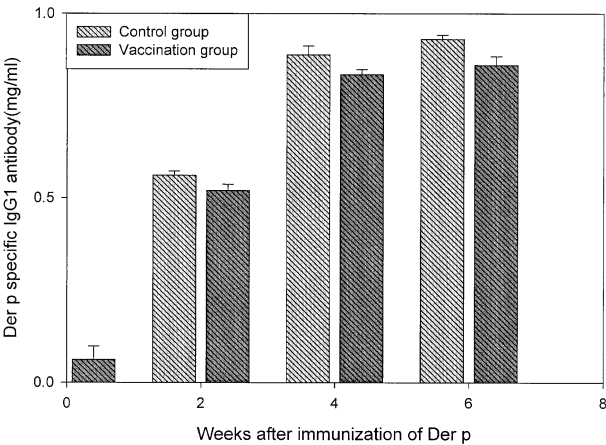


Figure 2. Effect of vaccination on allergen-induced immunoglobulin E production. Gene vaccination with murine T-cell epitopes on Der p 1 and 2 showed about 70% inhibition of Der p-specific IgE compared with controls at week 6. Results shown are mean ± SD (*n*=6 per group). **P*<0.05 compared with control mice.

A Changes of Der p specific IgG2a antibody serum levels in BALB/c mice after immunization of Der p extract



B Changes of Der p specific IgG1 antibody serum levels in BALB/c mice after immunization of Der p extract



C Changes of Der p specific anti-IgG antibody serum levels in BALB/c mice after immunization of Der p extract

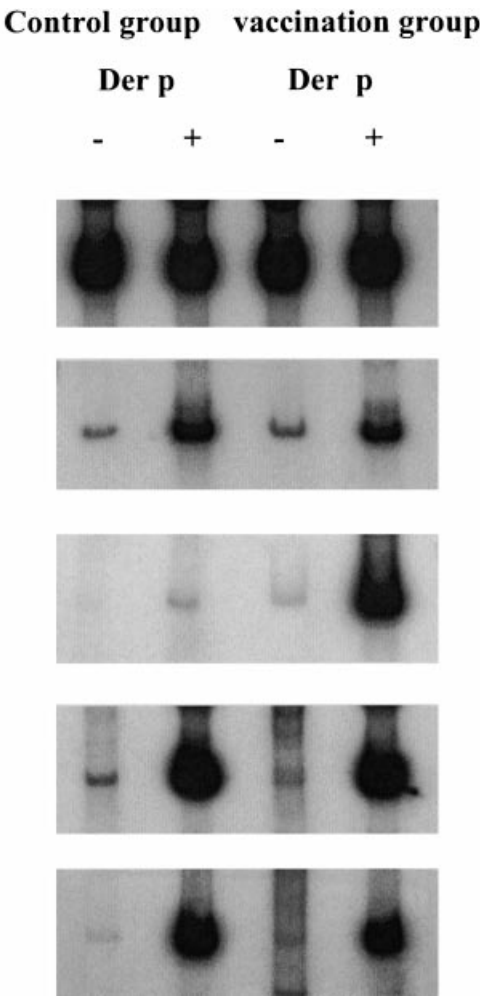
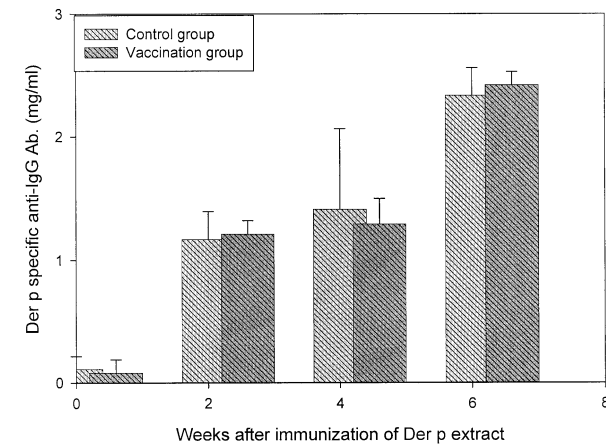


Figure 4. Cytokine gene expression. Total RNA was extracted with TRIzol reagent, and RT-PCR reactions were performed with cDNA with different primers specific for β-actin, IL-2, -4, -5, and IFN-γ. 1) Lymph-node cells from control mice were cultured without Der p and 2) in presence of Der p (100 μg/ml). 3) Lymph-node cells from vaccinated mice were cultured without Der p and 4) in presence of Der p (100 μg/ml). Higher mRNA expression of IFN-γ was detected in vaccination group than in control group.

Histologic and immunohistochemical examination of lung tissue

To examine whether the genetic vaccination affected lung cellular response, we stained lungs at the end of the experiment for histologic and immunohistochemical examination. The lungs of the control group showed a greater infiltration of inflammatory cells around bronchioli, blood vessels, and interstitium (Table 1, Fig. 5). The immunohistochemical stain for CD4+ and CD8+ T cells showed that more CD8+ T cells infiltrated the submucosa and mucosa of the lungs of

Figure 3. IgG2a (A), IgG1(B), and IgG (C) antibody responses of BALB/c mice after immunizing with Der p extract. Production of Der p-specific IgG2a antibodies in vaccinated mice was higher than that of controls after 3 weeks (A). But, in terms of Der p-specific IgG1 and IgG response, no difference was found between two groups (B and C). Results shown are mean ± SD (n = 6 per group). *P < 0.05 compared with the control mice.

Table 1. Inflammatory cells in lung tissue after immunization with Der p

Group	Around bronchioli	Around blood vesels	Patch cellular infiltration
Control	1.5616 ± 0.7262	1.6438 ± 1.2733	1.6471 ± 0.4926
Vaccination	$0.6835 \pm 0.4947^*$	$0.3924 \pm 0.5168^*$	$0.3333 \pm 0.4815^*$

* $P < 0.05$ compared with control group.

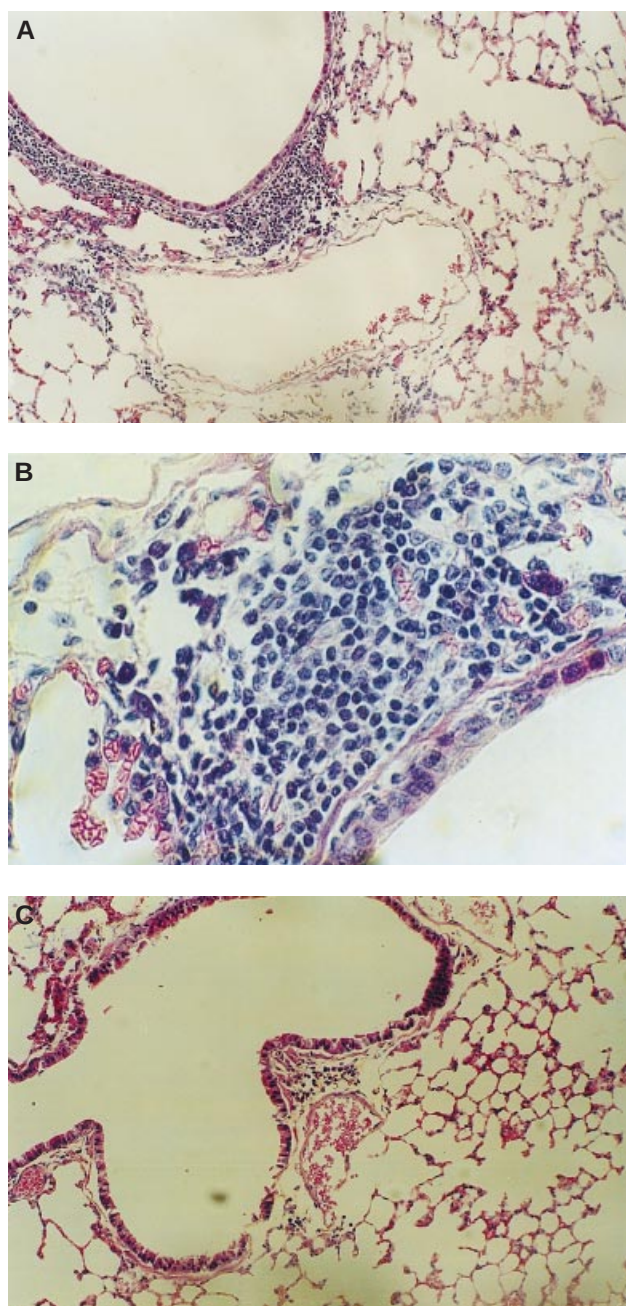


Figure 5. Histopathologic examination of lung. Lungs from control and experimental groups were removed on day 45 after immunization. A) Lung from control mouse ($\times 100$); B) lung from control mouse ($\times 400$); C) lung from vaccinated mouse ($\times 100$).

the vaccination group ($94.5 \pm 6.75/\text{mm}$) than those of the control group ($49 \pm 4.966/\text{mm}$) (Fig. 6), but staining for CD4+ T cells showed no difference between the two groups (vaccination group $98.5 \pm 13.44/\text{mm}$ vs control group $114 \pm 11.31/\text{mm}$). These results suggest that the effect of the genetic vaccination on the development of CD+8 T and CD+8 T cells induced protection against a subsequent allergenic challenge through the inhibition of inflammatory cell infiltration in the lungs.

Discussion

Diseases such as allergic asthma and rhinitis, and atopic dermatitis are all characterized by elevated levels of serum IgE. Total and specific IgE positivity also has a close relationship with the clinical symptoms of atopic allergy (17). Various approaches to target the suppression of IgE have been proposed, such as synthetic peptides and T-cell vaccine. However, synthetic peptides proved to be poor immunogens and needed to be introduced at significantly higher levels than those derived intracellularly from processed antigens (18).

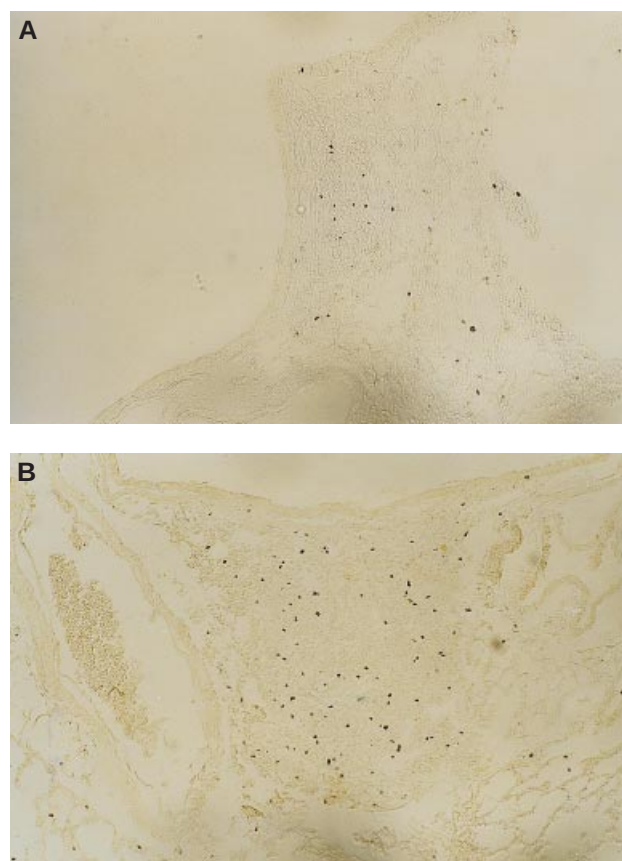


Figure 6. Immunohistochemical examination of lungs. Lungs from control and vaccination group were removed on day 45 after immunization and stained for CD8+ T cells. A) Lung from control mouse ($\times 100$); B) lung from vaccinated mouse ($\times 100$). More CD8+ T cells were observed in vaccinated mice than in control mice.

Recently, a new method to solve that problem has been reported by Hsu et al. (10). These workers showed that the gene immunization of rats with plasmid encoding Der p 5 prevented the induction of IgE synthesis (10). These results suggest that pDNA immunization with the gene containing the minor HDM allergen Der p 5 may induce Th1 immune responses to the encoded antigens. The Der p 5 allergen reacts with about 40% of allergic sera, and Der p 1 and 2 allergens react with about 80% of allergic sera (1, 3). We analyzed the effects of gene vaccination with plasmid DNA encoding only the murine T-cell epitopes in allergic responses to whole Der p extract. Our results showed about 70% inhibition of Der p-specific IgE, 6 weeks after immunization with Der p, using only T-cell epitopes (Fig. 2). Our results reinforce the clinical efficacy of the T-cell epitope for the effect of vaccination with DNA encoding epitopes, as well as whole structure, on the allergic responses.

Murine models have established that Th2 responses are mediated by T helper cells that secrete cytokines such as IL-4 and IL-5, which induce antibody production in B cells and, above all, the formation of IgE, which plays a central role in the allergic response (20, 21). IFN- γ is the Th1 cytokine that inhibits IL-4-mediated IgE responses and promotes the formation of IgG2a (22). Previous reports have shown that protein immunization induced a Th2 response, as shown by IgG1 and IgE antibody formation and IL-4- and IL-5-secreting T cells. In contrast, gene immunization with plasmid DNA induced a Th1 response with IgG2a antibody production and IFN- γ -secreting T cells (13). Our data show that the mRNA expression of IFN- γ in lymph nodes from plasmid DNA encoding the murine T-cell epitopes on Der p 1 or 2 increased more than that of the control group, and was associated with suppression of IgE synthesis. In terms of the Th2 response, no difference was found between the two groups. Our results suggest that genetic immunization might induce a Th1 immune response to the encoded antigen or allergen. After genetic immunization, the Th1 response dominates the Th2 response and suppresses pre-existing IgE antibody formation (23). IFN- γ promotes the formation of IgG2a and inhibits IgE production (13), although the mechanism through which IFN- γ exerts its stimulatory effects on IgG2a production has not been established. Our results also show that the production of IgG2a-anti-Der p antibody was higher in the vaccination group than in the control group. Therefore, DNA vaccination with encoded T-cell epitopes showed increased expression of IFN- γ -mRNA and increased production of IgG2a, suggesting the indirect effect of IFN- γ , although the level of the cytokines was not determined directly. Therefore, these results support the notion that genetic immunization suppresses IgE production by inducing Th1 response from T helper cells.

After inhalation challenge, the inflammatory cells

migrated from the peripheral blood to the site of inflammation in the bronchial mucosa, and the bronchoalveolar fluid showed dominant Th2-type cytokines (24, 25). Allergic asthma is characterized as a chronic inflammatory disease of the bronchi, and it is well established that various cells, including mast cells, eosinophils, and lymphocytes, play a role in this process (26). Our histologic study showed that gene vaccination induced the reduction of inflammatory cell infiltration in lung tissue (Fig. 5). It suggested that the change in the function of T cells might affect the reduction of inflammatory cells around bronchi as a major targeted organ, and finally induce the inhibition of the basic allergic responses. T cells have been considered to play a key role in orchestrating the interaction of participating cells since they are able to release an array of cytokines that can attract, prime, and activate other cell types (26). Thus, the result of the decreased level of inflammatory cells around bronchi might be affected by a change in the role of T cells according to the increased Th1 function response.

Genetic immunization might affect not only humoral immune responses but also cellular responses. A successful outcome of immunotherapy has been associated with the development of suppressor T cells, which can downregulate the allergic response (27, 28). A recent report has also revealed that functionally distinct subsets of CD8+ T cells may play an important regulatory role in IgE production (29–33). However, Manickan et al. showed that the mechanism of genetic immunization primarily involved CD4+ T cells, and not CD8+ T cells (34). Our immunohistochemical study showed that more CD8+ T cells were detected in the lung of the vaccination group than the controls (Fig. 6), but the number of CD4+ T cells was similar in the two groups. Peptides derived from cytosolic proteins (35) are generally presented to CD8+ T cells by major histocompatibility complex (MHC) class I molecules, which are expressed on virtually all somatic cells. Therefore, DNA vaccination with T-cell epitopes might induce the presentation of antigens in the cytosol from antigen-presenting cells. Hence, we suggest that DNA vaccination might induce such endogenous production of allergenic protein and might be a useful means to induce regulatory CD8+ T cells capable of conferring protection against a subsequent allergenic challenge. We have studied the effect of vaccination of pDNA for Der p 1, Der p 2, Der p 3, Der f 1, Der f 2 and Der f 3, as well as pDNA for human T-cell epitope peptides on HDM-allergic responses in mice. The results are similar to the results observed in the current study (36). Preliminary data showed that this mode of vaccination is also effective in ongoing allergic responses (37).

In conclusion, the vaccination with mixed naked DNA encoding only T-cell epitopes might induce abrogation of the allergic response in mice as effectively as DNA encoding whole segment allergen, and might

inhibit inflammatory cell infiltration of the lung. Thus, genetic vaccination using DNA encoding T-cell epitopes might be a means of very effectively combating allergic disease.

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