

Inhibition of specific IgE response *in vivo* by allergen-gene transfer

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Abstract

DNA immunization has been an attractive approach in altering the host immune response to antigen. To examine the utility of DNA immunization in allergic response, we examined the *in vivo* efficacy of an 'allergen-gene immunization' approach in the modulation of allergen-specific IgE responses in mice. Our results showed first that i.m. injection of a gene construct (pCMVD) containing an important house dust mite allergen gene (*Dermatophagoides pteronyssinus* group 5 allergen; Der p 5) results in the induction of Der p 5-specific IgG antibodies, but not IgE antibody. We next examined the effect of transduced allergen gene on the expression of specific IgE response in mice after i.p. challenge with recombinant Der p 5 (rDer p 5). Both vector (mock) control- and pCMVD-treated mice were i.p. sensitized with rDer p 5 at 3 weeks after injection of gene construct. Results showed that there is a 90% reduction in the level of specific IgE in pCMVD-treated mice when compared with mock-treated mice. Furthermore, the suppression of specific IgE response can be adoptively transferred with CD8⁺ T cells from pCMVD-treated mice and such inhibition is in an antigen-specific manner, since the level of specific IgE to an irrelevant allergen, Der p 1, remained unchanged in comparison to that of the mock-treated group. In addition, Der p 5-specific CD8⁺ T cells could produce high levels of IFN- γ which probably inhibit allergen-specific IgE responses. Taken together, our results suggest that allergen-gene transfer is effective in the modulation of allergen-specific IgE responses and may provide a novel therapeutic approach.

Introduction

Allergic diseases affect 20% of the population, and are important causes of morbidity and mortality. The main characteristic of the allergic diathesis is the propensity to develop a sustained IgE response to common environmental antigens (1). IgE production is highly dependent on IL-4 and strongly inhibited by IFN- γ . Other cytokines, such as IL-5, IL-6, IL-8, IL-12 and IL-13, as well as cell-surface molecules such as CD40 and CD23, may be involved (2,3). Recent studies using bronchoalveolar lavage fluids have shown that the production of IgE can be regulated by suppressor T cells (4). Furthermore, a successful outcome of immunotherapy has been associated

with the development of suppressor T cells which can down-regulate the allergic responses (5,6). There is also evidence for a defect in suppressor T cell function in atopic subjects, particularly children with allergic asthma (7). Recent data from animal experiments have also revealed that functionally distinct subsets of CD8⁺ T cells may play an important regulatory role in IgE production (8–10). It is therefore possible to generate antigen-specific regulatory T cells to modulate the IgE antibody response in atopic patients.

The intramuscular injection of DNA has been shown to result in the expression of the DNA-encoded protein by

muscle cells (11,12). Plasmids were shown to be maintained episomally and did not replicate (13). This could be a method of introducing allergen into the endogenous antigen-processing pathway that will result in the generation of CD8⁺ T cells which could regulate allergen-specific IgE synthesis. We therefore studied the ability of an injected plasmid vector encoding the important *Dermatophagoides pteronyssinus* allergen, Der p 5 (14), to generate Der p 5-specific CD8⁺ T cells and evaluated the regulatory role of these CD8⁺ T cells in specific IgE synthesis.

Methods

Animal

Female BALB/c, aged between 6 and 8 weeks, were obtained from the animal breeding center of the College of Medicine, National Taiwan University (originated from The Jackson Laboratory, Bar Harbor, ME). Mice were age and sex matched for each experiment.

Molecular cloning of pCMVD recombinant plasmid

The Der p 5 cDNA was obtained by PCR amplification from λ gt10 clone WM as described previously (14). The sequences of the 5' and 3' primers are 5'-AAAAAGATCTATCAT-GAAATTCATC-3' (*Bgl* site underlined) and 5'-ATTA-AGCTTAACTTCAATCTTTTA-3' (*Hind*III site underlined) respectively, encompassing the entire Der p 5 sequence. The PCR product was digested with *Bgl*I and *Hind*III, and cloned into the eukaryotic expression vector pCMV2 originally derived from pcDNA3 (Invitrogen) and designated pCMVD. Plasmid propagation was first performed in SURE strain. Purification of the plasmid was then performed by the Wizard DNA purification system (Promega, Madison WI) according to manufacturer's instructions. The quality and quantity of the DNA were analyzed by absorption at 260 and 280 nm, and by agarose gel electrophoresis.

Immunohistochemical staining for the Der p 5

Immunostainings were performed according to the instructions of the Histomouse-SP kit (Zymed, San Francisco, CA). Briefly, frozen sections of muscles (5 μ m), after drying at room temperature, were fixed in pure cold acetone (10 min at 4°C) and first treated with Peroxo-Block (Zymed) for 45 s to quench the endogenous peroxidase activity. Sections were then blocked with 10% non-immune serum for 1 h. After blocking, mAb to Der p 5 (a gift from Dr R. H. Lin, National Taiwan University) at 1 μ g/ml was incubated with appropriate sections for 1 h. Der p 1 mAb (4C1, a gift from M. Chapman, UVA, VA) was used as control antibody. Rats that were similarly injected with a blank vector were used as negative controls. All incubations were performed at 25°C in a moist chamber. Incubation was followed by the addition of a biotinylated secondary antibody and the streptavidin-peroxidase conjugate. Signals were developed by the addition of 3-amino-9-ethylcarbazole. Finally, the slides were counterstained with hematoxylin solution.

Determination of Der p 5-specific IgG1, IgG2a and IgE

The amounts of Der p 5 and Der p 1-specific IgG1, IgG2a and IgE were determined by ELISA. Protein high binding

plates were coated with 100 μ l of purified Der p 5 or Der p 1 diluted in coating buffer (0.1 M NaHCO₃, pH 8.2) to a concentration of 5 μ g/ml. After overnight incubation at 4°C, plates were washed three times and blocked with 3% (w/v) BSA/PBS buffer for 2 h at 25°C. Sera were used at 1:100 dilution for IgG measurement and 1:10 dilution for IgE measurement in duplicate. After overnight incubation at 4°C, either biotin-conjugated monoclonal rat anti-mouse IgE mAb (PharMingen, San Diego, CA) or rat anti-mouse IgG mAb (PharMingen) diluted in 0.05% gelatin buffer was added for an additional 1 h. Avidin-alkaline phosphatase (Sigma, St Louis, MO) (1:1000) was then added and incubated for 1 h at 25°C, followed by six washes. The color reaction was developed with the addition of phosphatase substrate *p*-nitrophenylphosphate disodium salt (Sigma). Plates were read in a microplate autoreader (Metertech, Taiwan) at 405 nm. Readings were referenced to commercial isotype standards which were mouse anti-TNP mAb, G155-178 (IgG2a) and IgE-3 (IgE) (PharMingen).

T cell proliferation assay

Cells after purification were resuspended in complete tissue culture medium (RPMI 1640) supplemented with streptomycin (100 μ g/ml), penicillin (100 U/ml), glutamine (5 mmol/l), and 10% heat-inactivated FCS. Cells (1×10^5 /well) were cultured with Der p 5 (15 μ g) in 96-well, flat-bottom, tissue culture plates for 72 h. Cells were pulsed with 1 μ Ci [³H]thymidine for an additional 18 h and then harvested with a cell harvester. Thymidine incorporation was measured in a liquid scintillation counter (Beckman, Fullerton, CA).

Lymphocyte preparation and cell transfer protocol

Purified CD4⁺ and CD8⁺ splenocytes were obtained by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, splenocytes obtained from pCMVD-immunized and control mice 3 weeks after immunization were incubated with superparamagnetic microbeads coated with monoclonal anti-CD4 or anti-CD8 antibody for 30 min at 4°C (Miltenyi Biotec) for additional 30 min. Labeled cells were separated from unlabeled cells using a MACS steelwool column placed into a magnetic field of 0.6 Tesla. The desired concentrations of splenocytes (10^6 per recipient) were resuspended in PBS in a final volume of 0.1 ml. The cell suspensions were injected into tail veins of age- and sex-matched syngeneic recipients. The recipients were then sensitized by i.p. injection of 10 μ g Der p 5 with 20 mg aluminum hydroxide (Wyeth Pharmaceuticals, Punchbowl, Australia). Venous blood was obtained weekly from the tail vein under anesthesia.

Cytokine production by splenocytes from pCMVD-immunized mice

IL-4 and IFN- γ were both measured by ELISA according to the protocol supplied by the manufacturer (PharMingen). Briefly, microtiter plates were coated with purified rabbit anti-mouse IL-4 and IFN- γ (2 μ g/ml). After incubation with samples or purified rIL-4 plates were developed with the biotinylated rabbit anti-mouse IL-4 or rabbit anti-mouse IFN- γ antibody followed by avidin-alkaline phosphatase conjugate which was revealed by *p*-nitrophenylphosphate disodium salt. IL-10 (R&D) and

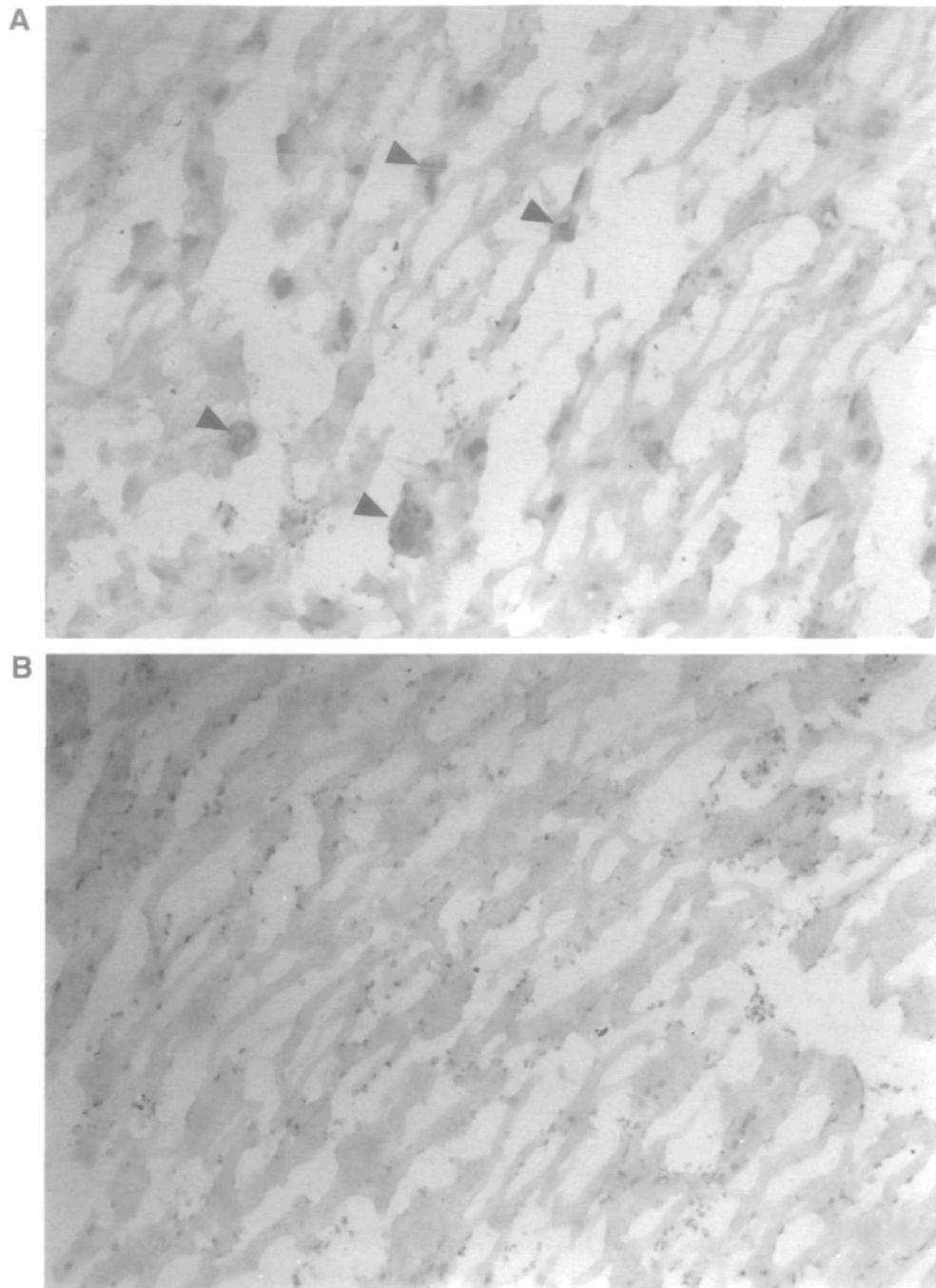


Fig. 1. *In situ* cytochemical staining of muscles for Der p 5 expression. Gross cryostat sections of a muscle taken 10 days after injecting 100 μ g of pCMVD DNA was stained with mAb to Der p 5 (A) or Der p 1 (B) at $\times 100$ optical magnification. The sections were counterstained with hematoxylin. Similar results were obtained in six individual injection sites. Arrows indicate positively stained cells. Control muscles injected with the blank vector showed no stained muscle cells.

transforming growth factor (TGF)- β (Promega) were measured with ELISA kits according to the manufacturer's protocols.

Statistical analysis

To assess the changes of *in vitro* proliferation response and cytokine production of splenocyte subsets from pCMVD-immunized mice, and IgE level after Der p 5 challenge,

repeated measurements for ANOVA were performed to compare the differences among different groups. Following analysis of variance, the Duncan multiple range test was used to differentiate differences between experimental and control groups. A $P < 0.01$ was used to indicate statistically significant difference. This work was calculated by the PHARM/PCS (Springer-Verlag, New York, NY) software.

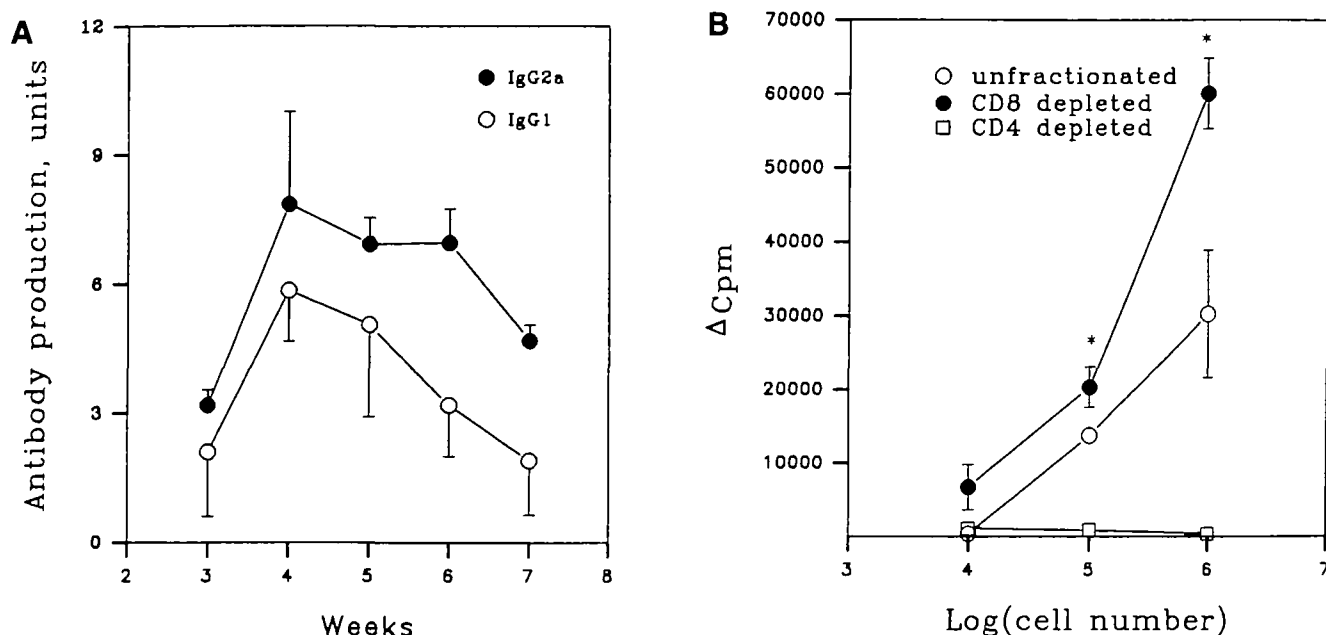


Fig. 2. (A) Immune responses after i.m. injection of pCMVD. Sera of BALB/c mice that had received a pCMVD injection were collected weekly and anti-Der p 5 IgG1, IgG2a and IgE titers were measured as described in Methods. There was no detectable Der p 5-specific IgE in this group. Mice injected with the blank vector had no Der p 5-specific immune responses. One unit of antibodies corresponds to 1 μ g IgG2a/ml and 100 ng IgE/ml. (B) *In vitro* proliferation response of splenocyte subsets from pCMVD-immunized mice. The depletion of subsets was as described in Methods. By FACSscan analysis the purified cell populations were shown to contain <0.5% contaminating cells. Then CD4⁺ or CD8⁺ cells from spleens of naive BALB/c mice were added to the cell preparations to replace the depleted cells. Cells were cultured with Der p 5 (15 μ g/ml) for 72 h. Proliferation was assayed by [³H]thymidine incorporation. Data shown are the mean $\pm$ SD of triplicate cultures. Cells did not proliferate in response to Der p 1 antigen stimulation. * $P < 0.01$ among the CD8⁺, CD4⁺ and unfractionated groups.

Results

To examine whether the uptake of pCMVD DNA by muscle cells could produce immune responses, we injected 100 μ g pCMVD in PBS into the quadriceps of female BALB/c mice. Control animals were injected with PBS or the appropriate blank vector lacking the inserted Der p 5 gene. The expression of the transferred gene in muscle cells was confirmed by *in situ* cytochemical immunostaining using Der p 5 mAb (Fig. 1). In addition, antigen-specific immune responses were indicated by production of Der p 5-specific IgG1 and IgG2a antibodies, which peaked at 4 weeks after immunization, and then decreased gradually. Der p 5-specific IgE antibodies were undetectable in immunized mice (Fig. 2A).

To examine the Der p 5-specific T cell responses after pCMVD injection, splenocytes from immunized mice were obtained at 2–6 weeks after immunization and then cultured with Der p 5. Proliferation was assayed by [³H]thymidine incorporation. Der p 5-specific T cell responses were demonstrated 3 weeks after immunization (Fig. 2B). Furthermore, the proliferative response to Der p 5 antigen shown by unfractionated splenocytes was suppressed by depletion of CD4⁺ cells, but enhanced by depletion of the CD8⁺ cells, which suggests that CD4⁺ cell proliferation is inhibited by the CD8⁺ population.

We evaluated the *in vivo* efficacy of pCMVD DNA injection to determine whether a protective response to allergen challenge was functionally significant. Both mock-treated and pCMVD-treated mice were challenged i.p. with allergen Der p 5 at 3

weeks after immunization. The presence of anti-Der p 5 IgE in the serum 3 weeks after allergen challenge was assayed by an ELISA. Der p 5-specific IgE increased significantly in the mock-treated group; in contrast, pCMVD-treated mice showed >90% inhibition of Der p 5-specific IgE synthesis (Fig. 3A). The inhibition of IgE synthesis by plasmid pCMVD DNA injection was specific to Der p 5, because pCMVD-treated mice challenged with another mite allergen, Der p 1, could produce Der p 1-specific IgE. Thus, direct gene transfer could inhibit an *in vivo* allergen-specific IgE synthesis efficiently and in an allergen-specific manner.

Because antigen expressed endogenously is usually presented by MHC class I molecules and triggers CD8⁺ T cells (15), we therefore examined whether suppression of Der p 5-specific IgE *in vivo* was caused by CD8⁺ T cells. Adoptive-transfer experiments have been used to provide direct evidence for the involvement of CD8⁺ T cells in the suppression of IgE. Unfractionated, CD8⁺-depleted or CD4⁺-depleted splenocytes were adoptively transferred to naive recipients. The recipients were then challenged with Der p 5 and alum adjuvant, and Der p 5-specific IgE responses were determined. Both CD4⁺ cells and the unfractionated group showed significant inhibition of Der p 5-specific IgE production. In contrast, the CD8⁺ group demonstrated no inhibitory effects, indicating CD8⁺ T cells could probably down-regulate the ongoing production of IgE (Fig. 3B).

Because previous studies have indicated that CD8⁺ T cells could suppress antigen-specific antibody responses by killing

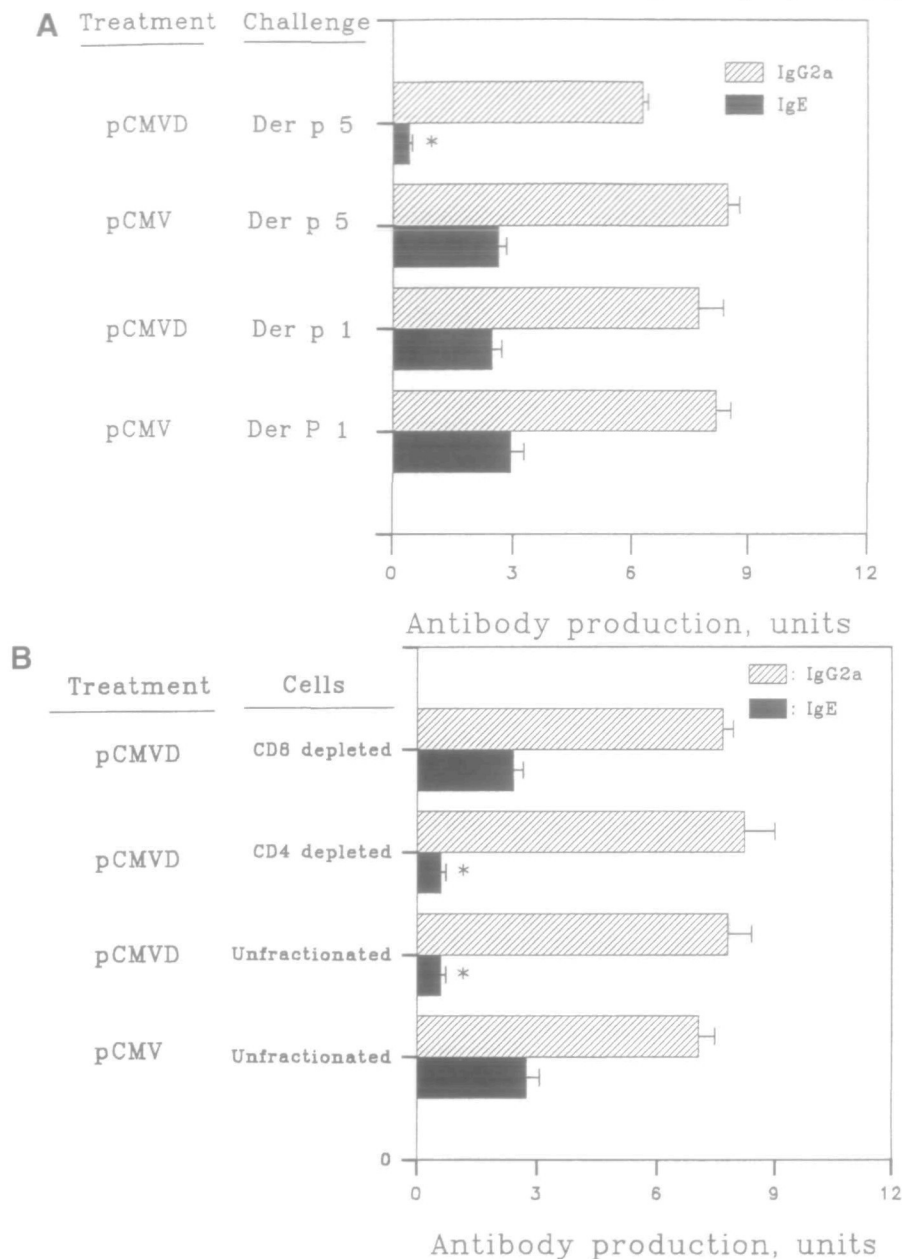


Fig. 3. (A) Inhibition of primary IgE responses by pCMVD injection (i.m.) in an antigen-specific manner. Mice ($n = 6$ per group) were immunized with blank vector or pCMVD as described. Three weeks after immunization, mice received Der p 5 (10 μ g with alum) or Der p 1 (10 μ g with alum), respectively. Data shown are the mean $\pm$ SD ($n = 6$ per group) at day 21 after challenge. Antigen-specific IgG2a and IgE titers were determined as described. * $P < 0.01$. (B) Suppression of Der p 5-specific IgE responses by adoptive transfer of splenic cells from pCMVD-immunized mice. The CD4⁺ and CD8⁺ populations used here contained $<0.5\%$ of contaminants as shown by FACScan analysis. Unfractionated splenocytes from vector-immunized mice were used as controls. Data shown are the mean $\pm$ SD ($n = 6$ per group) at day 21, and anti-Der p 5 IgG2a and IgE titers were determined as described. * $P < 0.01$. One unit of antibodies corresponds to 1 μ g of IgG2a/ml and 100 ng of IgE/ml.

antigen-reactive B cells (16) or by producing soluble factors, such as IFN- γ (17), IL-4 (18), IL-10 (19) and TGF- β (20), we investigated the *in vitro* activation and cytokine production by splenic T cells from pCMVD-treated mice. Spleens of mice were removed 3 weeks after immunization with pCMVD and cultured with Der p 5. Supernatants were collected after 48 h culture and analyzed by ELISA for the concentrations of IFN- γ , IL-4, IL-10 and TGF- β . Unfractionated splenocytes

from DNA-immunized mice secreted high levels of IFN- γ in response to specific antigen and this response was markedly reduced by depletion of CD8⁺, but not CD4⁺ cells. At the same time, a small amount of IL-4 was produced by the CD8⁺ population. In contrast, IL-4 and IL-10 production was more marked in the CD4⁺ population. There was no difference in TGF- β production between these two groups (Table 1)

Table 1. Cytokine production by splenocytes from pCMVD-treated mice

Cells	Cytokine secretion			
	IFN- γ (ng/ml)	IL-4 (pg/ml)	IL-10 (U/ml)	TGF- β (pg/ml)
Unfractionated	16.5 $\pm$ 2.3	870.4 $\pm$ 130.5	3.96 $\pm$ 0.4	362.0 $\pm$ 27.5
CD4 ⁺	11.4 $\pm$ 1.6	198.6 $\pm$ 46.3	1.08 $\pm$ 0.3	219.3 $\pm$ 21.4
CD8 ⁺	0.9 $\pm$ 0.5 ^a	719.1 $\pm$ 58.6	4.02 $\pm$ 0.5	365.2 $\pm$ 30.2
Media	0.7 $\pm$ 0.4	15.1 $\pm$ 8.0	0.39 $\pm$ 0.2	365.1 $\pm$ 20.4

Four groups of BALB/c mice ($n = 3$ per group) were immunized with plasmid pCMVD. Three weeks after immunization, splenocytes were cultured with recombinant Der p 5 (15 μ g/ml) as indicated. Culture supernatants were collected at 48 h for IFN- γ and IL-4, and at 72 h for TGF- β and IL-10 for cytokine concentrations determination by ELISA. Results represent pooled data from two independent experiments and are expressed as mean $\pm$ SD.

^a $P < 0.01$ between CD8⁺ group versus CD4⁺ or unfractionated group

Discussion

Most current studies in atopy are directed towards treatment of established allergic disease. In particular, treatments are focused on controlling the release and actions of various inflammatory mediators, including cytokines, from the effector cells at the end of the immunoinflammatory cascade (21). Our experiments suggest that i.m. injection of a DNA expression vector encoding an allergenic protein results in the generation of a significant 'split tolerance' against subsequent allergen challenge. The main characteristic immune response of pCMVD-treated mice is the production of allergen-specific CD8⁺ T cells which produced high levels of IFN- γ . IFN- γ has been shown to counteract IgE production in various *in vivo* and *in vitro* systems in both humans and mice (22,23). Besides, CD8⁺ T cells may regulate IgE production by suppressing IgE synthesis via the inhibitory effect of IFN- γ on B cells and/or by affecting the differentiation and function of T_H2-like CD4⁺ T cells, which support IgE production. An alternative explanation may be that CD8⁺ T cells interact physically with B cells or CD4⁺ T cells and may provide suppressive signals through cognate interaction (24). Further experiments are required to determine the precise regulatory function(s) of allergen-specific CD8⁺ T cells generated by direct gene transfer.

The inhibition of IgE synthesis by plasmid pCMVD DNA injection was in an allergen-specific manner. This very selective approach will offer considerable advantages over non-selective treatments in alleviating allergic diseases, because it is very important to avoid interfering with normal host defense mechanisms. Despite the benefits of this approach, numerous questions remain to be addressed with regard to the basic mechanisms that are involved in inducing an immune response upon inoculation of plasmid DNA, the pathway of processing and presentation of vector encoded antigen, and the reason for the apparent antigenic persistence in spite of an ongoing immune response. We have performed hematoxylin & eosin staining of muscle biopsies obtained from mice 10 days, 1 month and 6 months after the i.m. injection of naked DNA, and found no evidence of myositis (data not shown). The antigenic persistence might indicate that the majority of the antigen-expressing cells is being ignored by the immune effector mechanisms, which might be a reflection of the low levels of MHC class I determinant on muscle cells (25).

The main focus of this study is on the immunoprophylaxis of IgE-mediated disorders. However, it is pertinent to know the outcome of injecting DNA in Der p 5-sensitized mice. We have already performed experiments to evaluate the therapeutic potentials of direct allergen-gene transfer on the on-going allergic IgE responses in mice and the data showed that gene transfer could down-regulate the on-going IgE synthesis in sensitized mice (manuscript in preparation). This suggests that immunization with plasmid DNA encoding allergen may provide a novel strategy of immunotherapy (gene therapy) for allergic diseases.

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Abbreviations

Der p 5 *Dermatophagoides pteronyssinus* group 5 allergen
TGF transforming growth factor

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