

RESEARCH ARTICLE

A single administration of interleukin-4 antagonistic mutant DNA inhibits allergic airway inflammation in a mouse model of asthma

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Interleukin 4 (IL-4) is essential for the switching of B cells to IgE antibody production and for the maturation of T helper (Th) cells toward the Th2 phenotype. These mechanisms are thought to play a crucial role in the pathogenesis of the allergic airway inflammation observed in asthma. In the present study, we examined the anti-inflammatory effects of DNA administration of murine IL-4 mutant Q116D/Y119D (IL-4 double mutant, IL-4DM), which binds to the IL-4 receptor α and is an antagonist for IL-4. Immunization of BALB/c mice with alum-adsorbed ovalbumin (OVA) followed by aspiration with aerosolized OVA resulted in the development of allergic airway inflammation. A single administration of IL-4DM DNA before the aerosolized

OVA challenge protected the mice from the subsequent induction of allergic airway inflammation. Serum IgE level and extent of eosinophil infiltration in bronchoalveolar lavage (BAL) from IL-4DM DNA-administered mice were significantly lower than those in BAL from control plasmid-immunized mice. In our study, IL-4 or IL-4 mutants were not detected in sera from mice that had received a single administration of IL-4DM DNA. The results of this study provide evidence for the potential utility of IL-4 mutant antagonist DNA inoculation as an approach to gene therapy for asthma.

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Introduction

Asthma is characterized by airway hyper-responsiveness (AHR) to a variety of specific and nonspecific stimuli, chronic pulmonary inflammation with eosinophilia, excessive mucus production and high serum IgE levels. T helper (Th) 2 cells are thought to play a crucial role in the pathogenesis of asthma in association with production of interleukin (IL)-4, IL-5 and IL-13.^{1,2} Bronchoalveolar lavage (BAL) T cells from human asthmatics have been reported to express elevated levels of IL-4 and IL-5 mRNA.^{3,4} In addition, an inverse association between Th1 tuberculin responses and asthma has been observed.^{5,6} Current treatments for asthma are not satisfactory, and disease prevention is not possible. Given the high prevalence of this disease, improved and more effective therapeutic strategies are needed.

IL-4 has numerous biological activities relevant to the mediation of allergic inflammation.⁷ IL-4 stimulates proliferation of mast cells, B cells and T cells. IL-4 also induces class switching/secretion of IgE and IgG1 by B cells. IL-4 release is important in promoting Th2-mediated allergic immune responses.^{8,9} The action of

IL-4 is not limited to the initiation of Th2 responses but may also stimulate other cellular responses that contribute to the manifestations of allergic disease.⁸ Potential cellular sources of IL-4 include CD4⁺ T cells,⁹ mast cells,¹⁰ basophils,¹¹ NK1.1⁺ T cells,¹² $\gamma\delta$ T cells¹³ and eosinophils.¹⁴ These findings suggest that inhibition of the action of IL-4 may be particularly helpful in suppression of allergic reaction, in which IL-4 is responsible for the differentiation of allergen-specific Th2 cells.

Steroids are the most effective class of drugs for inhibiting the inflammatory reactions in asthma in humans.¹⁵ Various therapeutic trials with immunological tools have been carried out using experimental models of asthma. Administration of the Th1-associated cytokines IL-12 and interferon- γ (IFN- γ) has been shown to inhibit allergic inflammation in asthma.^{16–18} Inhibition of the action of IL-4 for treatment of allergic inflammation in a mouse model has been achieved in another trial on inhibition of Th2-type responses. In studies using a neutralizing antibody (Ab) to IL-4 during sensitization or mice deficient in IL-4, the development of airway eosinophilia, AHR and the increase in serum IgE seen following sensitization were reduced or abolished.^{19–21} Inhibition of *in vivo* binding of IL-4 to its receptor by using a soluble IL-4 receptor (sIL-4R) or antagonistic IL-4 mutant has also been reported to be effective for

treatment of allergic inflammation.^{22–24} The IL-4 mutant Q116D/Y119D, which forms unproductive complexes with the IL-4R α -chain, acts as an antagonist by inhibiting the formation of heterodimers with other receptors.²⁵ These IL-4-binding inhibitors act not only by inhibiting IL-4 binding to its receptor but also by preventing IL-13 from eliciting its activity, since the IL-4R α -chain also forms a functional signaling component of the IL-13R heterodimer (IL-13R α 1-chain/IL-4R α -chain).^{26,27} However, results of experiments using animal models have shown that these IL-4 inhibitors have some disadvantages for clinical use. Although the pharmacokinetic half-lives of IL-4 mutant and sIL-4R protein are very short *in vivo*, a high concentration of these molecules in plasma must be maintained for a long period in order for them to have suppressive effects on allergic inflammation.^{22–24} Thus, these molecules must be administered in large quantity, many times and over a long period during the antigen (Ag) sensitization and challenging periods in order for them to have suppressive effects on allergic inflammation. In the present study, the applicability of plasmid encoding cDNA of antagonistic IL-4 mutant DNA to gene therapy of asthma was assessed. We demonstrated that inhibitory effects on development of allergic inflammation are readily obtained in a mouse model of asthma through the administration of IL-4 mutant DNA, even with only a single administration before or after Ag sensitization.

Results

Production of IL-4, IL-4 double mutant (IL-4DM) and IL-4 single mutant (IL-4SM) by transfected cells

The levels of IL-4, IL-4DM and IL-4SM in the supernatant of plasmid-transfected COS 7 cells were measured by an enzyme-linked immunosorbent assay (ELISA). All of the transfected COS7 cells secreted 9–11 ng/ml of each cytokine into the culture supernatant (Figure 1). There were no significant differences between the amount of IL-4 produced by transfected cells.

Marked inhibition of the development of allergic inflammation by administration of IL-4DM DNA

To test the efficacy of gene therapy using IL-4DM DNA for inhibition of the development of allergic inflammation, mice were intraperitoneally administered 100 μ g of IL-4DM DNA or control plasmid on day 0, 7, 14 or 21 (Figure 2). The levels of eosinophils and protein in BAL fluid from mice administered IL-4DM DNA were significantly lower than those in BAL fluid from mice vaccinated with control DNA (Figure 3a and b). However, these inhibitory effects in BAL fluid from mice injected with IL-4DM DNA on day 21 were weak compared with those in BAL fluid from the other three IL-4DM-injected groups of mice (Figure 3a and b). These results were confirmed by histopathological observation. Histopathological examinations of lungs of mice injected with IL-4DM DNA or control DNA and lungs of healthy naive mice were performed 25 days after the first inoculation of the plasmid. The lungs of mice that had been administered IL-4DM DNA on days 0, 7 and 14 did not show any pathological abnormalities compared with those of healthy naive mice, but the lungs of mice that had been administered IL-4DM DNA on day 21 showed

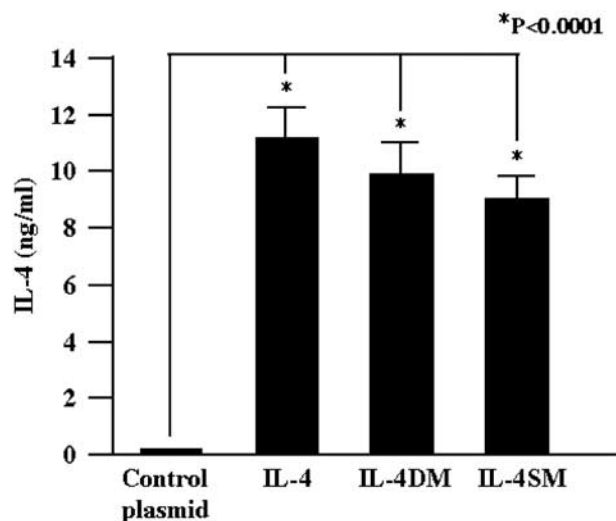


Figure 1 Production of IL-4, IL-4DM and IL-4SM in culture supernatant from COS7 cells transfected with plasmid DNA. COS7 cells were transfected with plasmid DNA encoding IL-4, IL-4DM, IL-4SM or a control by using lipofectamin. Culture supernatants of the cells were assessed 48 h after cultivation.

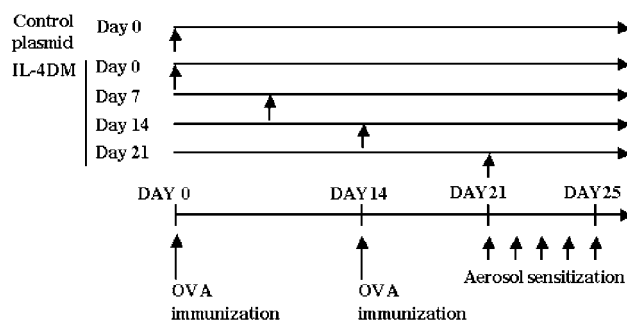


Figure 2 Experimental design used to investigate the effects of IL-4DM on OVA-induced AHR. Mice were subjected to an OVA sensitization scheme (see Materials and methods), and plasmid DNA of IL-4DM was intraperitoneally injected once on day 0, 7, 14 or 21. A control plasmid was administered on day 0.

mild inflammation due to infiltration of eosinophils (Figure 4). Mice administered control plasmid on days 7, 14 and 21 did not show any inhibitory effects on the development of allergic inflammations (data not shown). These results indicated that IL-4DM DNA administration was effective in inhibiting the development of allergic inflammations, especially in the early phase of Ag sensitization.

Inhibition of IgE synthesis by IL-4DM DNA administration

We also analyzed the degrees of antiovalbumin (OVA) IgE Ab responses in sera collected from experimental mice. The level of OVA-specific IgE in sera from mice in which AHR had developed was very high. Administration of IL-4DM DNA resulted in a significant reduction in the level of OVA-specific IgE. The inhibitory effect on IgE production was weak on day 21, in agreement with

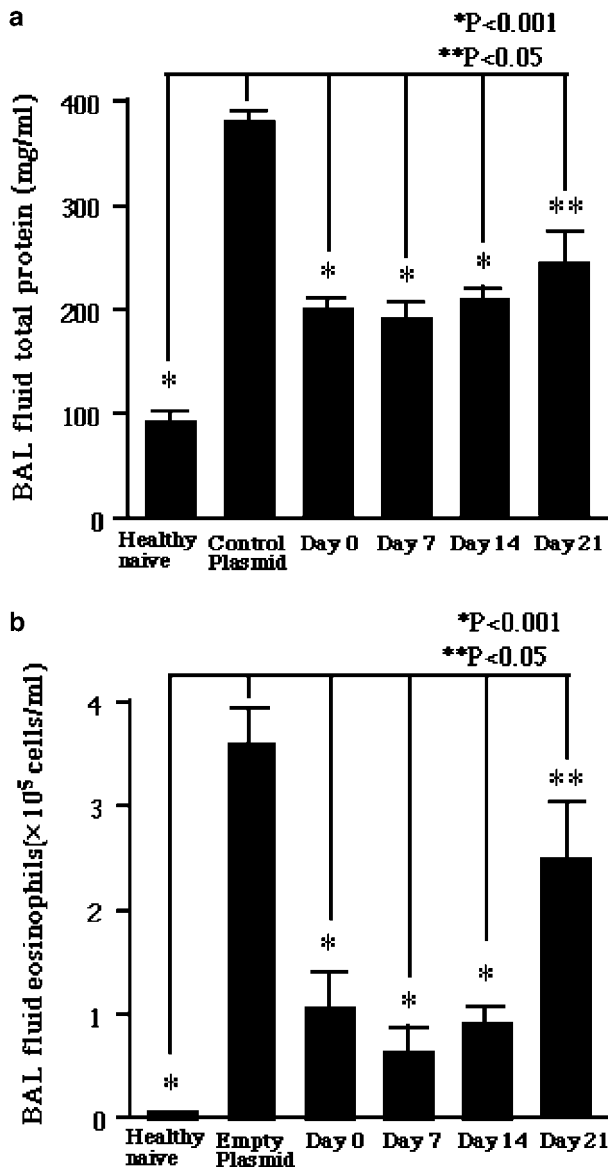


Figure 3 Inhibition of the development of AHR by administration of IL-4DM DNA. Total protein (a) and number of eosinophils (b) in BAL from experimental animals were investigated. Values shown are means and s.d. of five mice per group.

the results of analysis of BAL fluid and histopathological examinations (Figure 5).

Effects of IL-4DM DNA administration on production of cytokines in response to OVA

Responses of OVA-specific cytokines in lymphocytes obtained from experimental mice on day 25 were examined by two different methods. Production of IL-4 in BAL fluid was assessed by ELISA 25 days after the first OVA immunization. The amounts of IL-4 in BAL fluid from all IL-4DM-administered mice were much higher than those in BAL fluid from healthy naïve mice, but no significant difference was found between the amounts of IL-4 in BAL fluid from control DNA- and IL-4DM DNA-administered mice (Figure 6a). These results were confirmed by mRNA levels of Th1-type

cytokines (IL-2 and 12) and Th2-type cytokines (IL-4 and 13) in lymph node cells after *in vitro* stimulation with OVA. The mRNA expression levels of cytokines in lymph node cells obtained from IL-4DM DNA-administered mice in which allergic inflammations had developed and the levels in lymph node cells obtained from IL-4DM DNA-administered mice in which allergic inflammations did not develop were almost the same after *in vitro* stimulation with OVA (Figure 6b). Lymphocytes from both groups of mice showed strong IL-4 expression and weak IL-2, -12 and -13 expression of mRNA compared with the levels in anti-CD3 monoclonal Ab (mAb)-stimulated lymphocytes obtained from healthy naïve mice. IL-4 or IL-4 mutants were not detected in sera obtained from experimental mice that had received a single administration of IL-4DM DNA during the experimental period (data not shown). These results suggest that the existence of a small amount of IL-4DM for a long time might have played a role in the inhibitory effects on the development of allergic inflammations observed in this study.

Discussion

Bronchial asthma is a syndrome characterized by inflammation of the airways that causes AHR. In the case of allergic asthma, the airway wall is infiltrated by Th2 cells, eosinophils and mast cells,^{28–31} and AHR is considered to be the result of a combination of submucosal edema, infiltration of the airway epithelium by eosinophils and lymphocytes, damage to epithelial cells, and direct effect of mediators derived from mast cells and eosinophils. The occurrence of these phenomena has been confirmed in murine models in which Th2-type immune responses were induced. In the present study, we demonstrated the effectiveness of a single administration of antagonistic IL-4 mutant DNA as a new type of immunogene therapy.

Several strategies for disrupting the cytokine network in allergic airways inflammation have been reported. There are two possible strategies for inhibiting the development of allergic inflammation in asthma. One strategy uses Th1 cytokines for enhancing immune responses to Th1 in order to inhibit the induction of Th2 responses in asthma.^{32,33} IL-12 is the cardinal signal for the differentiation of Th1 lymphocytes, and considerable interest has been shown in possible utilization of its Th1-polarizing properties for treatment of Th2-driven pathologies, such as allergic asthma. In experimental animal models of asthma, administration of recombinant IL-12 during active sensitization transiently decreased the levels of IgG1 and IgE Abs and reduced IL-5 production,³⁴ and administration of IL-12 to actively sensitized mice, in which Th2-associated responses were established, suppressed recruitment of eosinophils to the airway.^{34,35} Although these findings suggest that IL-12 may be a useful therapeutic tool for the treatment of allergic asthma, it is necessary to consider the Th1-associated inflammatory responses for practical use. Indeed, administration of recombinant human IL-12 has been shown to be associated with several toxic side effects in human subjects.³⁶ IFN- γ , released from Th1 cells, is also a critical factor regulating the balance of Th1/Th2 development, exerting an inhibitory effect on

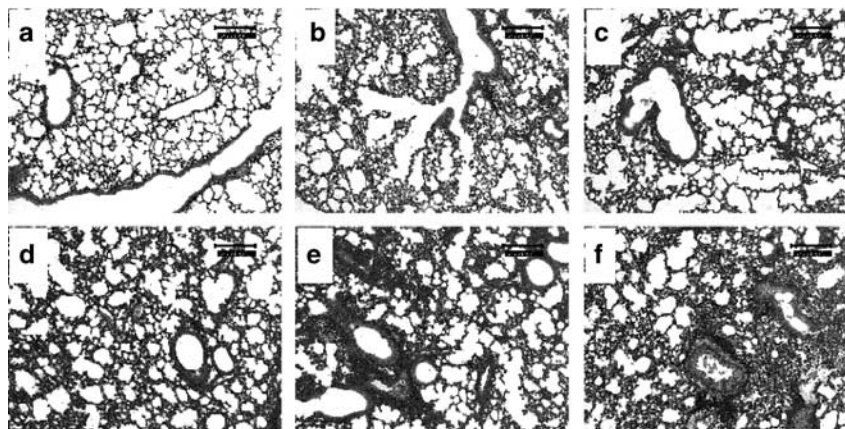


Figure 4 Results of histopathological examination of lungs of mice that had been administered IL-4DM DNA and lungs of mice that had not been administered IL-4DM DNA. All tissues were obtained 26 days after first OVA immunization. These tissues were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE). Mice that had been sensitized to OVA were each injected with 100 μ g of IL-4DM DNA once on day 0 (b), day 7 (c), day 14 (d) or day 21 (e). Results for healthy naïve mice (a) and control plasmid DNA-immunized mice (f) are also shown. Bars represent 200 μ m.

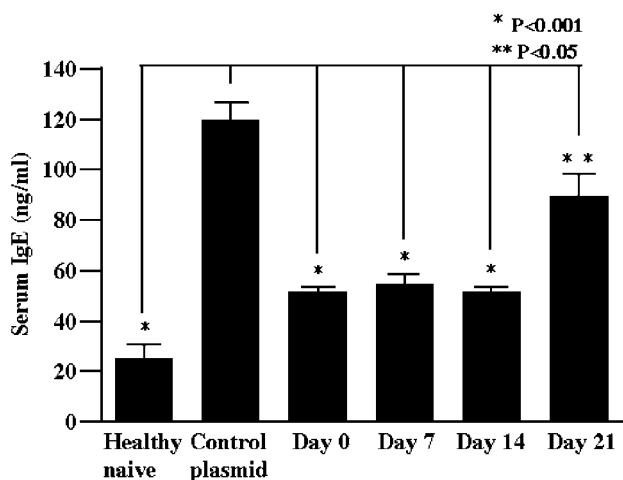


Figure 5 Inhibition of the production of IgE in OVA-sensitized mice by administration of IL-4DM DNA. Sera were collected 25 days after the first OVA immunization. Values shown are means and s.d. of five mice per group.

Th2 cells.³⁷ Administration of IFN- γ reduced lung Th2 cytokine levels and the degree of allergen-induced AHR with a concomitant reduction in BAL eosinophilia, while administration of an IFN- γ -blocking Ab led to an increase in airway CD4⁺ T cells and AHR in animal models.³⁸ Since IFN- γ had no effects on lung function, inhalation of IFN- γ has been evaluated as a possible means for preventing toxicity and enhancing the therapeutic effects of IFN- γ administration.³⁹ Trials on indirect induction of Th1-type immune responses have also been carried out using animal models. The administration of various bacteria that can induce Th1-type immune responses has also been reported to inhibit the development of allergic-induced AHR.^{40,41} These mechanisms have indirect inhibitory effects on the development of allergic AHR, since sensitization of hosts to a few species of bacteria, such as mycobacteria species, which can induce immune responses results in the elicitation of bacteria-specific Th1-type immune responses.

Another type of immunotherapy for allergic inflammation of asthma utilizes direct blocking of Th2 responses to inhibit the induction of Th2 responses in asthma such as neutralizing Ab treatment to Th2 cytokines.^{42–44} The administration of sIL-4R or antagonistic IL-4 mutant proteins is a novel approach to inhibit allergic inflammation. These proteins directly inhibit IL-4 binding and do not induce host immune responses to these proteins. In our previous preliminary experiment, none of the experimental animals that had been administered IL-4DM showed Ab responses to IL-4 mutant protein during the observation period (at least 50 weeks, data not shown). However, results obtained by using experimental animals have shown that application of these trials to humans is difficult. Since pharmacokinetic half-lives of IL-4 mutant and sIL-4R protein are very short *in vivo* (IL-4 mutant: $t_{1/2}$ =0.83 h, sIL-4R: $t_{1/2}$ =4.6 h),^{23,24} a high concentration of these molecules in plasma must be kept for a long period in order for inhibitory effects on the development of allergic inflammation to be obtained. In fact, these molecules were administered in large quantity, many times and over a long period during Ag sensitization and challenging periods in order for them to have inhibitory effects on allergic inflammation in experimental animal models in previous studies.^{22–24} This problem must be resolved for application to suppression of allergic inflammation in humans. In our experiments, IL-4DM protein was not detected at any time point during the experimental period. In most studies on gene therapy and DNA vaccines combined with a cytokine gene for tumors or pathogens, cytokines in plasma were not observed, but effective immune responses to Ag were obtained. Recently, we also reported that administration of plasmid DNA having cDNA of IL-4 completely inhibited the development of insulinitis, which is a Th1-type autoimmune disease, although no IL-4 was detected in plasma.⁴⁵ These results suggest that IL-4DM inhibits the allergic inflammation by persistent secretion over a long period.

Nonviral approaches are advantageous for immunogene therapy. In human applications, both the efficacy and safety of any delivery system used for gene transfer

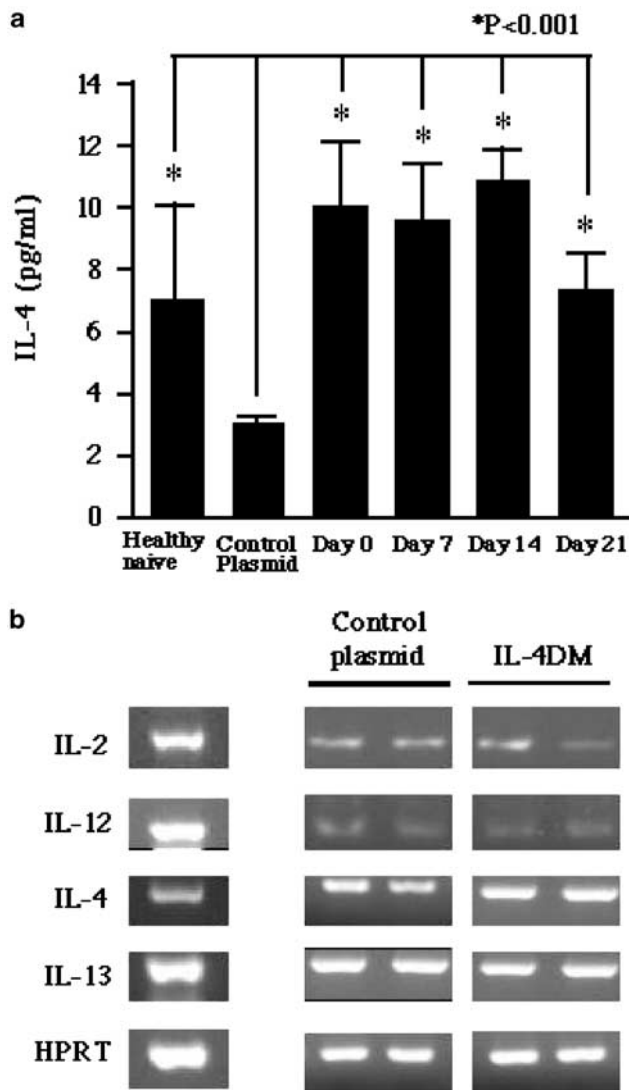


Figure 6 IL-4 production in BAL fluid and expression of mRNAs of cytokines from lymphocytes. (a) The amount of IL-4 in BAL fluid was measured by ELISA 25 days after the first OVA immunization. Values shown are means and s.d. of five mice per group. (b) Thoracic lymph node cells were stimulated in vitro with OVA for 1 day in culture. Lymphocytes stimulated by anti-CD3 mAb in naive mice were used as controls. Reaction products were analyzed on 2% agarose, Tris-buffered EDTA TBE gels. The profiles are representative of three independent experiments.

are major concerns. Tissue-specific gene transfer could be achieved naturally and effectively through the cell specificity of virus receptors.⁴⁶ However, there is a risk of vector toxicity through viral infection of the host cells. Also, the limited size of transgenes often presents a serious obstacle. Moreover, immune responses to viral vectors are also induced, and the effects of transgenes are eliminated by immune responses to the vectors. A defective recombinant adeno-associated virus (rAAV) carrying cDNA of IL-4DM for inhibiting development of asthma was recently reported.⁴⁷ An inhibitory effect on development of asthma was observed when a very high titer of rAAV-IL-4DM was infected (5×10^{11} CFU). However, it is difficult to use such a large amount of virus not only in animal experiments but also in trials using human subjects. Moreover, this vector cannot be used several times, since AAV usually elicits strong AAV-

specific immune responses. DNA vaccines are capable of inducing potent biological effects in a variety of experimental systems.⁴⁸ One of the characteristic features of DNA vaccines is their ability to induce lasting immunity. This mechanism might be the same as that by which inhibitory effects on the development of allergic inflammation were induced in experimental animals injected with plasmid DNA carrying cDNA of IL-4DM in our study. The animals that had been administered IL-4DM DNA did not develop allergic inflammation even in the case of only a single administration before or after Ag sensitization. In the present study, we showed usefulness of immunogene therapy using IL-4 mutant DNA, and the results of this study have provided evidence of the potential utility of IL-4 mutant DNA for the prevention and treatment of AHR.

Materials and methods

Mice

BALB/c female mice were housed in the Laboratory Animal Center of Mie University School of Medicine.

Preparation of mouse cDNA of IL-4 and IL-4 mutants

The IL-4 expression vector used in this study was described previously.⁴⁹ The mouse IL-4-374 plasmid was purchased from the Japan Health Science Foundation. The cDNA coding region of IL-4 was amplified by polymerase chain reaction (PCR) based on the cDNA sequence of IL-4. The mouse IL-4 fragment was inserted into *Bam*HI and *Eco*RI-filled in pcDNA3.1(+) (Invitrogen, CA, USA) under the TPA leader sequence. A QuikChangeTM Site-Directed Mutagenesis Kit (Stratagene, CA, USA) was used for mutagenesis of mouse IL-4. The oligonucleotide primers used to prepare a mouse IL-4DM (IL-4DM, Q116D/Y119D) were CTAAAGAGCAT CATGGATATGGATGACTCGTAGTCTAGAG and CTCTAGACTACGAGTCATCCATATCCATGATGCTCTTTAG, and the oligonucleotide primers used to prepare an IL-4 single mutant (IL-4SM, Q116D) were CTAAAGAGCAT CATGGAAATGGATTACTCGTAGTC and GACTACGAGTAATCCATTTCATGATGCTCTTTAG. Mouse IL-4 inserted into pcDNA3.1(+) was digested by *Bam*HI and *Sac*I. The IL-4 mutant fragments were ligated into pcDNA3.1(+). Mouse IL-4, IL-4DM and IL-4SM plasmid DNA were extracted with Wizard Midipreps (Promega, WI, USA).

Induction of allergic inflammation in mice

Mice were sensitized to OVA (Sigma, MO, USA) and challenged with aerosolized according to a modification of the method of Krinzman *et al.*⁵⁰ Briefly, mice were intraperitoneally immunized with 10 μ g of OVA complexed with alum on days 0 and 14. On days 21–25 after the first immunization, mice were challenged with an aerosol of 5% OVA in PBS in a chamber for 20 min (Figure 2).

Administration of DNA

Mice were intraperitoneally administered 100 μ g of plasmid DNA encoding IL-4DM once on day 0, 7, 14 or 21. An empty plasmid (pcDNA 3.1) vector was used as a control (Figure 2).

BAL fluid collection

BAL fluid was obtained by injecting and recovering two 0.5-ml aliquots of PBS via a tracheal cannula. Cells in the lavage fluid were counted using a hemacytometer, and the differentials were determined by utilizing light microscopy to count 300 cells on cytospin preparations. The concentration of inflammatory protein was measured by Protein Assay Reagent (Bio-Rad, CA, USA).

Measurement of IL-4

The production of IL-4 in BAL fluids and culture supernatants of COS7 cells transfected with IL-4, IL-4DM, IL-4SM or an empty plasmid was quantified using a standard ELISA kit (BioSource International, CA, USA).

Quantitation of IgE

IgE levels in sera were measured using ELISA kits according to the procedure recommended by the manufacturer (Shibeyagi Co. Ltd, Japan).

Detection of cytokine RNA from lymphocytes using RT-PCR

Total RNA was purified from OVA-stimulated lymphocytes derived from thoracic lymph nodes using ISOGEN (NIPPONGENE, Japan) following the manufacturer's instructions. Lymphocytes stimulated by an anti-CD3 mAb were used as controls. For RT reaction, a reverse transcription system (PROMEGA, WI, USA) was used. PCR was performed in a total volume of 50 μ l of 1 $\times$ PCR buffer (Takara Shuzo, Japan) containing 0.5–1.0 μ g of cDNA, 0.25 mM of each dNTP, 2 μ M of each primer and 2.5 U of *Taq* DNA polymerase (Takara Shuzo, Japan). The specific primer pairs used were as follows: IL-2, 5'-AAGATGAACCTTGGACCTCTGCGG-3' (sense) and 5'-CCTTATGTGTTGTAAGCAGGAGG-3' (antisense); IL-4, 5'-ATGGGTCTCAACCCAGCTAGT 3' (sense) and 5'-GCTCTTTAGGCTTTCCAGGAAGTC-3' (antisense); IL-12, 5'-TCCTGCACTGCTGAAGACATC-3' (sense) and 5'-TCTCGCCATTATAGATTCAGAGAC 3' (antisense); IL-13, 5'-GACCCAGAGGATATTGCATG -3' (sense) and 5'-CCAGCAAAGTCTGATGTGAG-3' (antisense); and mouse HPRT, 5'-GATACAGGCAGA CTTTGTGG-3' (sense) and 5'-GAGGGTAGGCTGGCC TATAGG-3' (antisense). The samples were amplified for 30–35 cycles using the following conditions: annealing for 30 s at 56°C, extension for 1 min at 73°C and denaturation for 30 s at 93°C. The reaction products were analyzed on 2% agarose, Tris-buffered EDTA TBE gel.

Statistical analysis

Statistical analysis was performed using Mann-Whitney's *U*-test and the Kruskal-Wallis test. Values are expressed as means $\pm$ s.d. A 95% confidence limit was taken as significant ($P < 0.05$).

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