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ORIGINAL ARTICLE

Effect of Cry-consensus Peptide, a Novel Recombinant Peptide for Immunotherapy of Japanese Cedar Pollinosis, on an Experimental Allergic Rhinitis Model in B10.S Mice

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ABSTRACT

Background: We are developing an immunotherapeutic peptide, Cry-consensus peptide, for Japanese cedar pollinosis. Cry-consensus peptide is a recombinant polypeptide containing six major human T-cell epitopes derived from both Cry j 1 and Cry j 2, two major allergens of Japanese cedar pollen. We examined the effect of Cry-consensus peptide on an allergic rhinitis model in B10.S mice, which have one common T-cell epitope in the Cry-consensus peptide.

Methods: B10.S mice were sensitized with Cry j 1/alum, then the Cry-consensus peptide was administered subcutaneously once a week for 5 weeks from the last sensitization. Histamine was dropped in both nostrils (10 μL per nostril) of each mouse on the day before continuous intranasal instillation of Cry j 1. Soon after the final challenge with Cry j 1, the mice were observed for 5 minutes for the resulting number of sneezes. In addition, serum levels of Cry j 1-specific IgE and IgG2a antibody, eosinophil infiltration in nasal tissue, and Cry j 1-specific cytokine production from splenocytes were evaluated.

Results: Cry-consensus peptide markedly inhibited Cry j 1-induced sneezes, eosinophil infiltration, and eosinophil peroxidase (EPO) activity in nasal tissue. Cry-consensus peptide inhibited the production of anti-Cry j 1 IgE (Th2-mediated) and significantly enhanced anti-Cry j 1 IgG2a (Th1-mediated). In cytokine production from splenocytes, Cry-consensus peptide significantly decreased in IL-4/IFN-γ and IL-5/IFN-γ ratios.

Conclusions: It was concluded that Cry-consensus peptide effectively controlled allergic responses, which results from shifting from a Th2-dominated to a Th1-dominated immune response.

KEY WORDS

allergic rhinitis, Cry j 1, Cry j 2, Japanese cedar pollinosis, T-cell epitope

INTRODUCTION

Specific immunotherapy has been widely applied in the treatment of allergic diseases; it is based on the administration of increasing doses of disease-eliciting allergens to induce a state of unresponsiveness toward them.[1,2] The results of clinical studies have revealed the availability of specific immunotherapy,[3,4] although high doses of allergens caused various side effects, including anaphylactic reactions.[5]

Derivatives of several purified and recombinant allergens, including chemically modified allergens[6,7] and mutants of wild-type recombinant allergens, have recently been employed. These derivatives may be safer as immunotherapeutic agents for treating allergic diseases than the native allergens, since the binding capacity of the derivatives to allergen-specific IgE is reduced.

Furthermore, allergen-specific immunotherapy with synthetic peptides of T-cell epitopes in major al-

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lergens has been reported.[8,9] This has involved the use of short peptides, including T-cell epitopes, which, by virtue of their size, are incapable of cross-linking with allergen-specific IgE bound to the surface of mast cells and basophils. Therefore treatment with T-cell epitope peptides cannot pose the potential risk of cross-linking with IgE antibodies on mast cells.

Japanese cedar (*Cryptomeria japonica*, or Cj) pollinosis is one of the most serious type I allergic diseases in Japan. It is prevalent in 10% to 20% of the Japanese population and poses a significant social problem.[10] Therapies for Cj pollinosis include allergen avoidance, pharmacotherapy using antihistamines or corticosteroid nasal spray, and Cj-specific immunotherapy. Immunotherapy is an effective treatment for patients with allergic rhinitis/conjunctivitis, allergic asthma and allergic reactions from stinging insects. With this in mind, we produced a curative immunotherapeutic drug using Cry-consensus peptide, the cedar pollen epitope.

For Cry-consensus peptide, we selected multiple T-cell epitopes from patients with Japanese cedar pollinosis. This selection was based on differences among the types of restriction molecules capable of presenting these peptides. Cry-consensus peptide is a recombinant polypeptide containing six major T-cell epitopes derived from both Cry j 1 and Cry j 2, two major allergens of Japanese cedar pollen.

In order to investigate new therapeutic approaches, we established a new experimental model of allergic rhinitis in B10.S mice where the symptoms are sneezing and eosinophil infiltration into the nasal mucosa, and induction of antigen specific IgE production after a continuous intranasal antigen challenge with histamine pretreatment. These symptoms mimic the major features of patients with allergic rhinitis.[11,12] In Cry j 1-sensitized B10.S mice, a major T-cell epitope has been identified,[13,14] and the sequence of this epitope coincides with that of the most prevalent epitope in patients. Cry-consensus peptide has one peptide that has been described as an important T-cell epitope in B10.S mice immunized with Cry j 1. We present direct evidence obtained by *in vitro* and *in vivo* analyses that the on-going allergic immune response can be modulated by the application of Cry-consensus peptide in an experimental model of allergic rhinitis.

METHODS

ANIMALS

Female B10.S mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The animals were housed under controlled conditions of temperature (21 ± 2℃) and relative humidity (55 ± 15%), fed a standard diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan), and given water *ad libitum*. All experiments were performed according to the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

ANTIGEN

Cry j 1 was purified from Japanese cedar pollen as described previously.[15] The absence of Cry j 2 in the purified Cry j 1 preparation was confirmed by Western blot analysis with two monoclonal antibodies (anti-Cry j 1 and anti-Cry j 2; Hayashibara Biochemical Laboratories Inc., Hayashibara Biochemical Laboratories Inc., Okayama, Japan). The purity of Cry j 1 determined by densitometric scanning after sodium dodecyl sulfate polyacrylamide gel electrophoresis and Coomassie brilliant blue staining was 88%.

CRY-CONSENSUS PEPTIDE AND DRUG

Cry-consensus peptide (Fig. 1) was expressed in E. coli and purified in our company.

Histamine dihydrochloride was purchased from Nacalai Tesque Inc. (Kyoto, Japan).

SENSITIZATION, NASAL ANTIGEN CHALLENGE AND ADMINISTRATION OF CRY-CONSENSUS PEPTIDE

Mice were sensitized with 10 μg of Cry j 1 mixed with 4 mg of aluminum hydroxide gel (Imject Alum, Pearce, Rockford, IL, USA) by subcutaneous injection once a week for 3 consecutive weeks. Before continuous intranasal Cry j 1 challenges—5 weeks after the final sensitization of Cry j 1 and prior to the intranasal Cry j 1 challenge—1 μg/mL of histamine dihydrochloride in phosphate buffered saline (PBS) was dropped into both nostrils (10 μL per nostril) the day before the first intranasal antigen challenge. On the day after the histamine pretreatment, 10 μL of Cry j 1 (200 μg/mL in PBS) was dropped into each nostril, using a micropipette, for 5 consecutive days. Soon after the last intranasal antigen challenge, the number of sneezes was counted for 5 minutes.

Twenty-four hours after the last intranasal antigen challenge, the mice were killed and histologically examined; eosinophil peroxidase (EPO) activity in nasal tissues was also evaluated, and the serum levels of Cry j 1-specific IgE, IgG1 and IgG2a were measured.

Cry-consensus peptide was administered subcutaneously once a week for 5 consecutive weeks.

HISTOLOGICAL EXAMINATION

For histological examination of the nasal mucosa, the mouse head was fixed in 10% buffered formalin solution 24 hours after the last intranasal antigen challenge. Then the head was severed between the upper and lower jaws and the facial skin stripped. The tip of the nose area was decalcified in Plank-Rychlo solution and embedded in paraffin. Frontal sections of the nasal tissues were stained with Biebrich scarlet-iron hematoxylin (Luna stain) to detect infiltrated eosinophils.

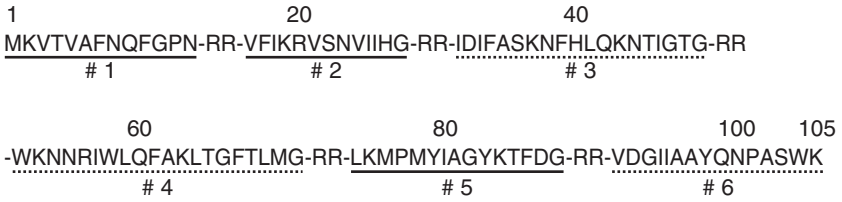

Fig. 1 Amino acid sequence of Cry-consensus peptide. The underlines show the amino acid sequences of human T cell epitopes; solid underlines; Cry j 1, and dotted underlines; Cry j 2. The #1 peptide (MKVTVAFNQFGPN) is a major T cell epitope in B10.S mice.

MEASUREMENT OF EOSINOPHIL PEROXIDASE (EPO) ACTIVITY

Twenty-four hours after the last intranasal antigen challenge, the tip of the nose area was removed and homogenized with 5 mL of 0.5% hexadecyltrimethylammonium bromide (HTAB) in PBS. The homogenate was freeze-thawed three times and the supernatant was assayed for EPO after centrifugation. The supernatant (50 μL) was mixed with the substrate solution (16 mmol/L *o*-phenylenediamine and 0.01% H_2O_2 in 50 mmol/L Tris-HCL, pH 8.0) (100 μL) and the mixture was incubated for 15 minutes at room temperature. The reaction was stopped by adding 4 mol/L H_2SO_4 (50 μL), and the optical density of the solution was read at 490 nm. Data were expressed in units of EPO activity, 1 unit being arbitrarily defined as the activity of 1×10^3 eosinophils isolated from guinea pigs.

EVALUATION OF SERUM LEVEL OF CRY J 1-SPECIFIC IgE

On the day following the final intranasal antigen challenge, mice sera were obtained and the Cry j 1-specific IgE titer in the serum was evaluated by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates (F16 Black Maxisorp, Nalge Nunc International, Rochester, NY, USA) were coated with 100 μL of purified rat anti-mouse IgE monoclonal antibody (50 μg/mL, BD Bioscience, San Jose, CA, USA) in bicarbonate buffer (0.1 mol/L, pH 8.2) 50 μg/mL overnight at 4℃. After removal of the coating solution, the plates were washed and blocked with PBS containing 1% bovine serum albumin for 30 minutes at room temperature (RT). After blocking, the serum samples and standard serum serially diluted were added to the plate in duplicate. The standard serum was obtained by intraperitoneal immunization of a mouse with Cry j 1 and arbitrary assigned a value of 10,000 relative units (RU)/mL of Cry j 1-specific IgE. Plates were then washed and incubated with 100 μL of 1/2000 diluted Cedar Pollen Allergen Cry j 1-Biotin (Hayashibara Biochemical Laboratories Inc.) for 1 hour at RT. The plate was then washed and subsequently incubated with streptoavidine-β-galactosidase conjugate (Molecular Probes, Eugene, OR, USA) for 30 minutes at RT. After a final wash, 100 μL of substrate solution, consisting of Tris-HCl with 0.034 mg/mL of 4-methylumberifenyl-β-D-garactopyranocide, was added to the well for 2 hours at 37℃. The reaction was stopped with 100 μL of glycine-NaOH, 0.2 mol/L, pH 10.3, and the fluorescence intensity measured by a microplate fluorescence reader (Fluoroskan Ascent, Thermo Electron Corp., Waltham, MA, USA) at wavelengths of 355 nm (excitation) and 460 nm (emission).

EVALUATION OF SERUM LEVEL OF CRY J 1-SPECIFIC IgG1 AND IgG2a

Ninety-six-well plates were coated with 50 μL of Cry j 1 (10 μg/mL) overnight at RT, and the plates were blocked with PBS containing 1% BSA. Then the plates were washed and incubated with the serum samples in serial dilution for 2 hours at RT. After the incubation of the samples, peroxidase-labeled anti-mouse IgG1 or IgG2a was added to the plate for 1 hour at RT. Tetramethylbenzidine substrate solution (TMB Substrate Kit, Pearce, Rockford, IL, USA) was added to the plate after it had been washed. Finally, 4 mol/L of phosphoric acid was added to stop the reaction and the absorbance at 450 nm was measured with a microplate reader.

CYTOKINE PRODUCTION INDUCED WITH CRY J 1

The spleen cells (5×10^5 cells/mL) from control mice and mice treated with Cry-consensus peptide were suspended in a 10% FBS in RPMI 1640 culture medium and then cultured in the presence or absence of Cry j 1 (1 μg/mL) for 48 hours at 37℃ in the 5% CO_2 incubator. The levels of IL-4, IL-5 and IFN-γ in the culture of the supernatant were measured with ELISA using Opt EIA sets (Pharmingen, San Diego, CA, USA).

DATA AND STATISTICAL ANALYSIS

The results are presented as means ± SEM For comparison among multiple groups, the data were analyzed for homogeneity of variance using Bartlett's test. When the variances were homogeneous, para-

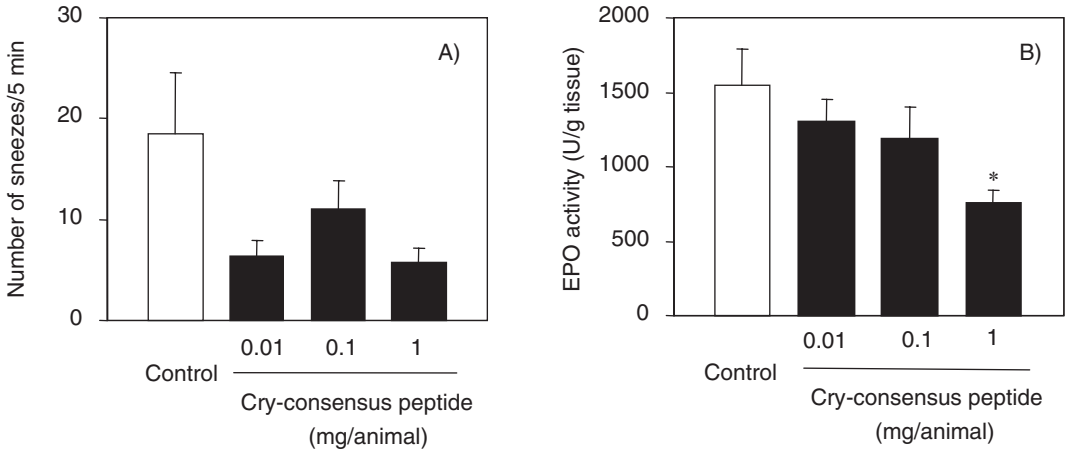

Fig. 2 Effects of the administration of Cry-consensus peptide on sneezing and EPO activity induced by continuous antigen challenge. **A**): number of sneezes. **B**): EPO activities in nasal tissues. Each value represents the mean ± S.E. of 15 mice. * : $p < 0.05$ (Dunnett's multiple comparison test), significant difference from the control group.

metric Dunnett's multiple comparison test was performed. When the variances were not homogeneous by Bartlett's test, nonparametric Dunnett's multiple comparison test was performed. Student's *t*-test or the Aspin-Welch test was used to evaluate differences between two groups. A probability value of $p < 0.05$ was considered to indicate significant differences.

RESULTS

EFFECTS OF CRY-CONSENSUS PEPTIDE ON CRY J 1-INDUCED SNEEZING AND EPO ACTIVITY

Treatment of the mice with Cry-consensus peptide suppressed sneezing at doses higher than 0.01 mg/mouse and decreased the number of sneezes to approximately 50% of that in the control group, although there were no significant differences between the control group and Cry-consensus peptide treatment group (18.5 ± 6.1, Fig. 2A).

To evaluate the number of infiltrating eosinophils, EPO activity was determined in the tissue homogenate. There was a significant difference between the control and the treated group—the mean value ± SEM was 1544 ± 247 in the control group, and 753 ± 86 U/g tissue in the group treated with 1 mg of Cry-consensus peptide ($p < 0.05$).

HISTOLOGICAL ANALYSIS OF NASAL TISSUES

A similar inhibiting effect of nasal infiltration of eosinophil was observed in the histological examination with Luna staining. On histological examination, eosinophils were infiltrated into the nasal septum resorptive epithelium, ethmoturbinal concha, back lateral wall and ventral aspect wall, and we classified the eosinophil infiltration into five degrees (0–4). The mice unsensitized with Cry j 1 but challenged intranasally with PBS showed no eosinophil infiltration (data not shown). In the control group, the number of eosinophils infiltrating into the lamina propria the day following the last continuous intranasal antigen challenge was increased (Fig. 3A). The administration of Cry-consensus peptide reduced eosinophil infiltration in a dose-dependent manner, especially the mice that received the dose of 1 mg/animal showed very little eosinophil infiltration (Fig. 3B). However, the number of mast cells was not affected by the Cry-consensus peptide treatment (data not shown) . EPO activity and histological examination in nasal tissue cannot be evaluated in the same mouse because the nasal tissue of one mouse has to be used for each examination. Therefore, we evaluated the EPO activity and histological examination in half of each group, respectively, and we evaluated the relation between EPO activity and the findings of histological examination.

EFFECT OF CRY-CONSENSUS PEPTIDE ON SERUM IMMUNOGLOBULIN LEVEL

There was marked increase in the concentration of specific IgE antibody in the sera of the control group (5958 ± 1603 R.U./mL). IgE production in the 1 mg Cry-consensus peptide-treated mice was inhibited more than 50% compared with that in the control group, although there were no significant differences from the control group (Fig. 4A). Regarding the antigen-specific IgG1 antibody titer in the sera, there was no difference between the mice treated with 1 mg/animal of Cry consensus peptide, (47.9 ± 10.7 R.U./mL) and the control group (48.8 ± 8.8 R.U./mL) (Fig. 4B). On the other hand, IgG2a titer (57.0 ± 20.2 R.U./mL) was higher than that in the control group (11.0 ± 5.2 R.U./mL) (Fig. 4C).

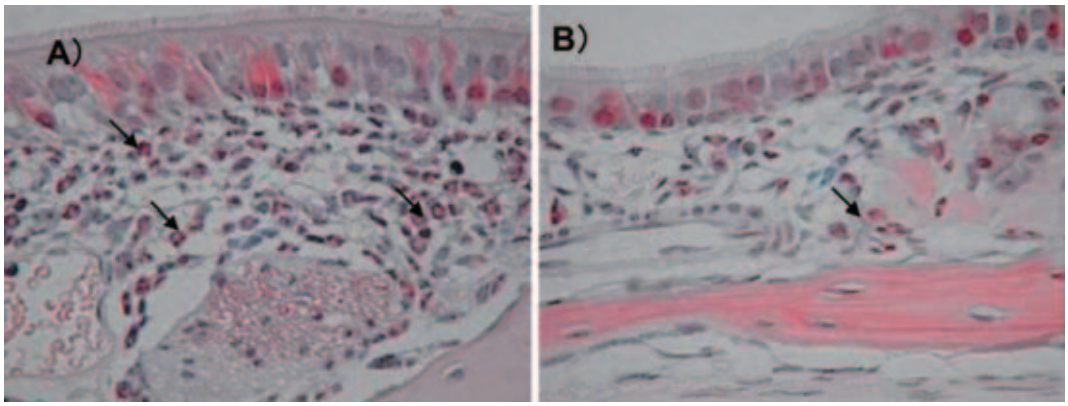

Fig. 3 Histopathological features of Cry j 1-induced allergic rhinitis in B10.S mice at 24 hours after the last nasal antigen challenge. **A**): Control group, **B**): Cry-consensus peptide treatment group (1 mg). Eosinophils were stained with Luna and are marked by the arrows. Original magnification×200.

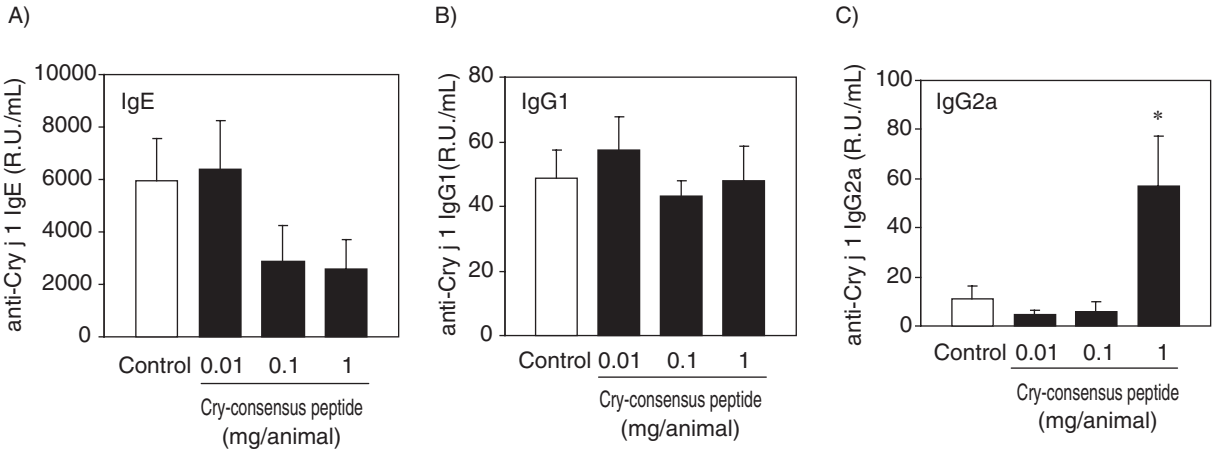

Fig. 4 Effects of the administration of Cry-consensus peptide on Cry j 1-specific IgE (**A**), IgG1 (**B**) and IgG2a (**C**) antibody levels in mice sera. Each value represents the mean ± S.E. of 15 mice. * : $p < 0.05$ (Dunnett's multiple comparison test), significant difference from the control group.

EFFECT OF CRY-CONSENSUS PEPTIDE ON CYTOKINE PRODUCTION

Cytokine production from spleen cells after continuous intranasal challenge with Cry j 1 was examined. Cry j 1-stimulated spleen cells from the control group produced detectable amounts of IL-4 (34.8 ± 4.0 pg/mL), IL-5 (2662.8 ± 312.1 pg/mL) and IFN-γ (1328.1 ± 187.2 pg/mL). Application of 1 mg of Cry-consensus peptide decreased IL-4 (23.9 ± 4.8 pg/mL) and IL-5 (1894.1 ± 362.9 pg/mL) secretions from spleen cells, although there were no significant differences from the control group (Figs. 5A, 5B). On the other hand, IFN-γ production was slightly increased by the Cry-consensus peptide treatment (1455.9 ± 262.0 pg/mL, Fig. 5C). The IL-4/IFN-γ and IL-5/IFN-γ ratios were significantly lower in the mice treated with 1 mg/animal of Cry-consensus peptide than in the control group (Fig. 6).

DISCUSSION

Immunotherapy using allergen extracts was initially reported by Noon in 1911.[1]

It takes several years to acquire tolerance, requires frequent injections, and immunotherapy has a serious side effect, namely anaphylaxis.[2] Recently, allergen-specific immunotherapy using short lengths of synthetic peptides has been reported.[6,7] The major cat allergen, Fel d 1, the house dust mite allergen, Der p 1 (*Dermatophagoides pteronyssinus*), and the birch pollen allergen, Bet v 1, have been applied in the specific immunotherapy of these allergies.[8,9,16] Peptide immunotherapy is safe and clinically effective, and several clinical and experimental studies have been reported on the use of T-cell peptides in immunotherapy.[17]

In Japan, the number of patients presenting with pollinosis has continued to increase from the 1970s,

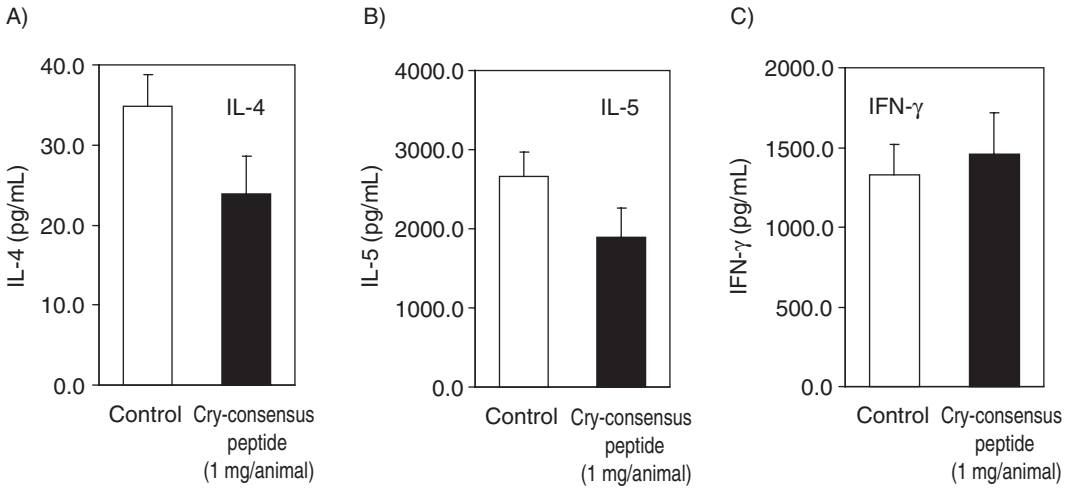

Fig. 5 Effects of the administration of Cry-consensus peptide on Cry j 1-induced IL-4 (**A**), IL-5 (**B**) and IFN-γ (**C**) production. Each value represents the mean ± S.E. of 15 mice.

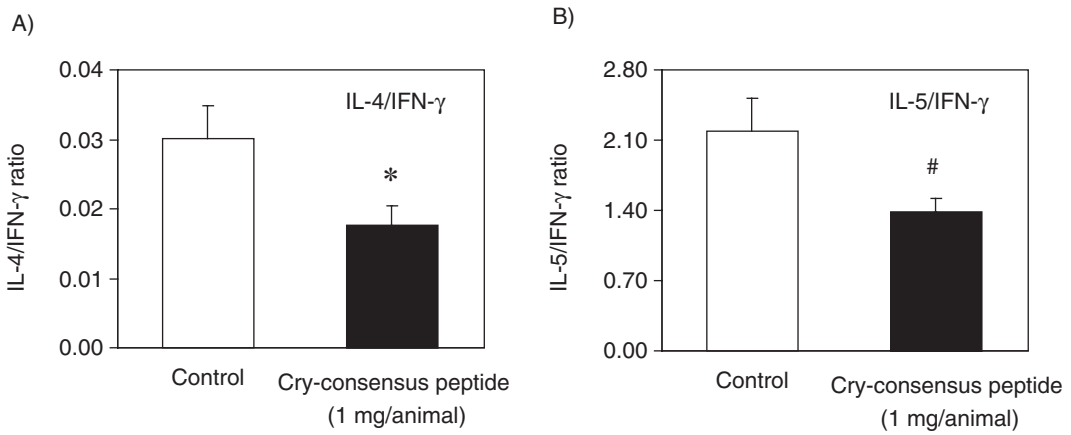

Fig. 6 Effects of Cry-consensus peptide on the periodic change in Th1/Th2 balance. **A:** Ratio of IL-4 to IFN-γ production, **B:** Ratio of IL-5 to IFN-γ production. Each ratio was calculated from the results of Figure 5
* : $p < 0.05$ (Student's-*t*), #: $p < 0.05$ (Aspin-Welch), significant difference from the control group.

and 35% of Japanese people are estimated to have antibodies to cedar pollen, and about 20% suffer from pollinosis. We have developed Cry-consensus peptide for the treatment of Cj pollinosis. Cry-consensus peptide is a recombinant polypeptide containing six types of T-cell epitope with an arginine dimer. In our preliminary study, no binding of IgE in sera from the allergic patients to Cry-consensus peptide was observed. So Cry-consensus peptide did not function as an allergen to induce allergic reactivity. In a previous study, we established an allergic rhinitis model in B10.S mice.[18] In this model, Cry j 1, one of two major allergens in Japanese cedar pollen, induced sneezing, nasal eosinophil infiltration, and increased serum levels of Cry j 1-specific IgE as a result of repeated Cry j 1 challenges, administered intranasally. In our rhinitis model, histamine pretreatment enhances the local sensitivity to allergen at the nasal mucosa and also the systemic reactions to allergen, such as allergen-specific IgE production. Pretreatment with histamine may cause the opening of the intraepithelial tight junctions, allowing the penetration of allergen into the epithelium and lamina propria, where allergen further interacts with sensitized mast cells.[19] In this study, we have demonstrated the efficacy of Cry-consensus peptide in the allergic rhinitis model in B10.S mice. Administration of Cry-consensus peptide at a dose that was higher than 0.01 mg/mouse suppressed sneezing, and reduced its frequency by approximately 50%, when compared with the control group.

Eosinophils release proinflammatory mediators successively, including cysteinyl leukotrienes, cationic proteins, and eosinophil peroxidase.[20] Eosinophils play an important role in nasal hypersensitivity in allergic rhinitis,[21] and they are the predominant

cells in the chronic inflammatory process that is characteristic of the late-phase allergic response. Continuous intranasal antigen challenges led to infiltration by eosinophils into the nasal tissue. EPO was used as a marker enzyme to measure tissue eosinophil content.[22] The results of the histopathological examination and EPO activity in this allergic rhinitis model showed that Cry-consensus peptide suppressed eosinophilic infiltration into sites of allergic inflammation in a dose-dependent manner. Another rhinitis model that is being established in Cry j 2-sensitized BALB/c mice has been reported which enables the evaluation of symptoms and reactions associated with allergic rhinitis.[23,24] In this model, increases in sneezing frequency, the vascular permeability of the nasal mucosa, and hyperreactivity to histamine occurred by challenging the mice with a Cry j 2 solution, intranasally. In our model, eosinophilic infiltration can be evaluated by measuring the level of EPO activity in the nasal tissue. These results suggest that treatment with Cry-consensus peptide may inhibit the development of rhinitis.

In our rhinitis model, repeated and continuous intranasal antigen challenge significantly increased anti-Cry j 1 IgE levels when compared with those levels measured before the intranasal antigen challenge. Treatment with Cry-consensus peptide reduced levels of anti-Cry j 1 IgE in a dose-dependent manner. This reduction in anti-Cry j 1 IgE levels may relate to the decrease in the frequency of sneezing. On the other hand, Cry-consensus peptide significantly raised the production of IgG2a. IgE is positively regulated by IL-4, which is secreted by Th2 cells.[25] Th1 cells promote immunoglobulin class switching to IgG2a due to the production of IFN-γ.[26] These results suggest that Cry-consensus peptide induces a qualitative alteration from Th2 to Th1.

To clarify the mechanisms involved in the inhibition of allergic rhinitis, we examined cytokine production from spleen cells that had been taken from mice with rhinitis and stimulated with Cry j 1 *in vitro*. When animals had been administered Cry-consensus peptide at a dose of 1 mg/animal, the production of Th2 cytokines, such as IL-4 and IL-5, was lower than that in the control group. In contrast, the production of the Th1 cytokine, IFN-γ, was a little higher in the group of animals administered Cry-consensus peptide at a dose of 1 mg/animal than in the control group.

The immunopathological features of allergic rhinitis are associated with the effects of Th2-derived cytokines.[27,28] Higher levels of Th2-derived cytokines, including IL-3, -4, -5, -13, and GM-CSF, have been described in allergic rhinitis. The Th2-type cytokines, IL-4 and IL-5, thought to play a crucial role in type I allergy, were down-regulated, while the Th1 cytokine, IFN-γ, was higher or unchanged after successful immunotherapy.[29,30] In this study we also observed the down-regulation of IL-4 and IL-5 production in splenocytes. The cytokine ratios IL-4/IFN-γ and IL-5/IFN-γ, produced from the splenocytes, were lower in the mice treated with Cry-consensus peptide at a dose of 1 mg/animal than in the animals in the control group. Determination of the IL-4/IFN-γ and IL-5/IFN-γ ratio could be a sensitive approach towards evaluating the Th1/Th2 balance. The immune responses of the mice could be altered qualitatively based on their cytokine secretion patterns from Th2 to Th1 through treatment with Cry-consensus peptide. It was clear that the ratio of Cry j 1-specific IgE/IgG2a directly correlated with the ratio of IL-4/IFN-γ and IL-5/IFN-γ *in vitro*. The mechanism of action of Cry-consensus peptide may involve switching from a Th2 to a Th1 cytokine profile.

In conclusion, our results demonstrate that treatment with Cry-consensus peptide can inhibit nasal tissue eosinophilia, antigen-specific IgE production, and the production of Th2 cytokines from splenocytes in an allergic rhinitis mice model. These results suggest that Cry-consensus peptide could be a new immunotherapeutic agent effective against Cj pollinosis by shifting from a Th2-dominated to a Th1-dominated immune response.

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