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Cross-reactivity between major IgE core epitopes on Cry j 2 allergen of Japanese cedar pollen and relevant sequences on Cha o 2 allergen of Japanese cypress pollen

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ABSTRACT

Background: Cry j 2 and Cha o 2 are major allergens in Japanese cedar (*Cryptomeria japonica*; CJ) and Japanese cypress (*Chamaecyparis obtusa*; CO) pollen, respectively. Here, we assessed the epitopes related to the cross-reactivity between Cry j 2 and Cha o 2 using *in vitro* analyses.
Methods: Peptides were synthesized based on Cry j 2 sequential epitopes and relevant Cha o 2 amino acid sequences. Four representative monoclonal antibodies (mAbs) against Cry j 2 were used according to their epitope recognitions. Serum samples were collected from 31 patients with CJ pollinosis. To investigate cross-reactivity between Cry j 2 and Cha o 2, ELISA and inhibition ELISA were performed with mAbs and sera from patients with CJ pollinosis.
Results: Two of four mAbs had reactivity to both Cry j 2 and Cha o 2. Of these two mAbs, one mAb (T27) recognized the amino acid sequence ^{169}KVVNGRTV^{176} on Cha o 2. This is related to the core epitope ^{169}KWVNGREI^{176} on Cry j 2, which is an important IgE epitope. In addition, we found that these correlative sequences and purified allergens showed cross-reactivity between Cry j 2 and Cha o 2 in IgE of CJ patients.
Conclusions: We demonstrated the importance of ^{169}KVVNGRTV^{176} in Cha o 2 for cross-reactivity with the Cry j 2 epitope ^{169}KWVNGREI^{176}, which plays an important role in allergenicity in CJ pollinosis. Our results are useful for the development of safer and more efficient therapeutic strategies for the treatment of CJ and CO pollen allergies.

Introduction

Seasonal allergic diseases including allergic rhinitis and asthma occur worldwide, particularly in developed countries. Pollinosis is a commonly-noted seasonal allergic disease induced by pollen allergens. The pollinosis induced by Japanese cedar (*Cryptomeria japonica*; CJ) pollen is one of the most common allergic diseases in Japan.[1] According to a survey conducted in the central Hokuriku area of Japan in May and June of both 2006 and 2007, 36.7% of study participants (566 of the 1540 subjects) had allergic rhinitis to CJ pollen.[2] Japanese cypress (*Chamaecyparis obtusa*; CO) pollen is one of the most important aeroallergens relevant to allergic symptoms in Japan.[3] The social impact and economic loss related to pollinosis are estimated to be tremendous because of the impaired performance of the patients, often accompanied by a self-imposed ban on leaving home.

Allergen cross-reactivity has been reported at the immunochemical and clinical levels. The cloning and sequencing of allergen genes have provided a better understanding of their cross-reactivity. The first evidence for the existence of clinically relevant cross-reactive IgE antibodies was reported in pollen-food cross-reactive allergens.[4] Other cross-reactive allergenic systems have been induced by aeroallergens and food antigens.[5,6] Many efforts have been made to correlate the serological cross-reactivity with elicitation of symptoms in susceptible patients, because not all cross-reactive IgE antibodies give rise to clinical signs. Therefore, it

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has been accepted that serological cross-reactivity may be broader than clinical cross-allergenicity.[7]

Three major allergens, named Cry j 1, Cry j 2, and Cry j 3, have been isolated from CJ pollen. Cry j 1 was isolated as a 41–46 kDa allergen with pectate lyase enzyme activity,[8,9] whereas Cry j 2 is a 45 kDa allergen with polymethylgalacturonase enzyme activity.[10,11] Cry j 3 is a 27 kDa protein that has relatively high homology with thaumatin-like proteins in the pathogenesis-related-5 family proteins.[12] On the other hand, Cha o 1 and Cha o 2 were identified as major allergens of CO with a high degree of homology with Cry j 1 and Cry j 2, respectively.[3,13,14] Amino acid sequence homology between major allergens from CJ and CO results in cross-allergenicity.

Cross-allergenicity is observed between CJ and CO pollen allergens.[15] In our previous studies, animal models of Japanese monkeys and dogs sensitized to CJ pollen demonstrated IgE reactivity to CO pollen.[16,17] Cry j 2 was characterized as a major allergen in CJ pollinosis,[10] and more than 90% of patients (139/145 subjects) had the anti-Cry j 2 IgE.[18] It was demonstrated that the IgE antibody levels to Cry j 2 and Cha o 2 were strongly correlated,[14] suggesting that there are one or more epitopes with high similarity between Cry j 2 and Cha o 2.

Six sequential and one conformational epitopes on Cry j 2 were identified in our previous studies.[19,20] Conformational epitopes on allergens play an important role in initiating human IgE-mediated allergic reactions.[21–23] In fact, the conformational epitopes on Cry j 1 are considered dominant in IgE reactivity as compared to the sequential epitopes.[24] However, we suggested the importance of sequential epitopes on Cry j 2, especially ^{169}KWVNGREI^{176}, for allergenicity at variance with other well-known allergens.[20] To our knowledge, it has yet to be demonstrated whether monoclonal antibodies (mAbs) and patients' IgE to sequential epitopes on Cry j 2 can cross-react with the relevant epitopes on Cha o 2. This is the first report to indicate the cross-reactivity between sequential epitopes on Cry j 2 and relevant sequences on Cha o 2 by mAbs and patients' IgE. Here, we also analyze the cross-reactivity between the Cry j 2 core epitope, ^{169}KWVNGREI^{176}, reported in our previous studies[19] and the related epitope on Cha o 2 by inhibition ELISA. We used ^{169}KWVNGREI^{176} because it was the only core epitope reported in previous studies, and it is known to be a major epitope recognized by patients' IgE.

Table 1
Synthetic peptides and allergenic similarities between Cry j 2 linear epitopes (upper sequences) and relevant Cha o 2 amino acid sequences (lower sequences).

Peptide (No†)	Position‡	mAb to a peptide§	Amino acid sequence
P1-1 (13)	166–186	T27	GQC**KWVNGREI**CNDRDRPTA
P1-2	166–186		----V----TV---------
P2-1 (25)	286–305	9E7	GRENSRAEVSYVHVNGAKFI
P2-2	286–305		--D-------H----R----
P3-1 (33)	366–385	J2A01	TYKNIRGTSATAAAIQLKCS
P3-2	366–385		-----H----------M--

The core determinant of P1-1 is shown in bold.
† Peptide numbers shown in brackets were linked to the study of Tamura *et al.*[19]
‡ Amino acid positions were described as complete sequences.
§ The mAb reactivity to each peptide was determined in our previous study.[20]

Methods

Antigens and mAbs

Cry j 2 in CJ pollen and Cha o 2 in CO pollen were purified as previously described.[10,14] Table 1 shows six peptides that were synthesized based on Cry j 2 sequential epitopes recognized by mAbs for Cry j 2 and relevant Cha o 2 amino acid sequences (Hokkaido System Science Co., Ltd., Sapporo, Japan). We also synthesized the core epitope on Cry j 2 (^{169}KWVNGREI^{176}) and the relevant Cha o 2 amino acid sequence (^{169}KVVNGRTV^{176}). Four representative mAbs for Cry j 2 (S1, T27, 9E7, and J2A01) were used according to their epitope recognitions.[20]

Subjects

Informed consent was obtained from all subjects. The study protocol was approved by the ethical committee at the Jikei University School of Medicine. Serum samples were collected from 31 patients with CJ pollinosis. The patients were selected based on clinical symptoms of seasonal allergic rhinitis and positive CAP (Phadia AB, Uppsala, Sweden) to CJ pollen. Their IgE were preliminarily confirmed to react with Cry j 2. To determine the cut-off value, serum samples obtained from 10 non-allergic subjects, who had been previously confirmed as negative for the crude pollen allergen were used as negative controls.

Colorimetric ELISA for Cry j 2, Cha o 2, and synthetic peptides with mAbs

As previously described, the reactions of the mAbs were measured using a colorimetric ELISA.[19] Briefly, Cry j 2, Cha o 2 (1 μg/ml), P1-1, P1-2, P3-1, and P3-2 (10 μg/ml) were immobilized in the wells of a microplate (F96 Maxisorp® NUNC-Immuno™ Plate, ThermoFisher Scientific, Waltham, MA, USA) overnight at 4°C. The microplate was then washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST) and incubated with biotin-labeled mAbs (1 μg/ml) for 1 h at room temperature. Next, streptavidin-peroxidase polymer (Sigma–Aldrich, MO, USA) was added to the wells. After a 1 h incubation period at room temperature, a substrate solution of o-phenylenediamine dihydrochloride was added. After the enzyme reaction was terminated with 2 M H_2SO_4, the optical density (OD) was measured using a multi-mode microplate reader (Powerscan MX, DS Pharma Biomedical, Osaka, Japan).

Colorimetric ELISA inhibition for Cry j 2 and Cha o 2 epitopes with mAbs

It was difficult to immobilize short peptides on the wells of the microplate. Thus, to evaluate the reactivity of the T27 mAb with ^{169}KWVNGREI^{176} (Cry j 2 core epitope) and ^{169}KVVNGRTV^{176} (relevant sequence within Cha o 2), an inhibition ELISA was conducted using these synthetic peptides as inhibitors.[25] Briefly, P1-1 and P1-2 (10 μg/ml) were immobilized in the wells of a microplate overnight at 4°C. The synthetic peptide inhibitors (final concentrations, 0–100 μg/ml) were incubated with equal volumes of T27 mAb (final concentration, 10 ng/ml) for 1 h at room temperature. Subsequent procedures were the same as described above. The inhibition ratio (%) was calculated as follows:

$$\left(1-\frac{\text{OD in presence of inhibitor}}{\text{OD in absence of inhibitor}}\right)\times 100$$

Fluorometric ELISA for Cry j 2 and Cha o 2 peptides

As previously described, specific IgE against synthetic peptides were measured in the sera of patients using fluorometric ELISA.[20] Briefly, synthetic peptides (20 μg/ml) were immobilized in the wells of a microplate (FLUOTRAC™ 600, Greiner Bio-One GmbH, Frickenhausen, Germany) overnight at 4°C. The microplate was then washed with PBST buffer and incubated with diluted (1:10) serum samples for 3 h at room temperature. The plates were washed, and anti-human IgE antibodies conjugated to β-D-galactosidase (diluted 1:10; Phadia AB) were added to each well. The enzyme reaction substrate, 0.2 mM 4-methylumbelliferyl-β-D-galactoside (Sigma–Aldrich) was added to the wells, and the plates were incubated at 37°C for 2 h. After quenching the reaction, fluorescence units (FU) were measured using a multi-mode microplate reader. Cut-off values were determined using sera from subjects without pollinosis as negative controls.

Fluorometric ELISA inhibition for Cry j 2 and Cha o 2 with human IgE

To evaluate cross-reactivity between Cry j 2 and Cha o 2, we used a fluorometric ELISA inhibition for Cry j 2 and Cha o 2 with human IgE.[20] Briefly, Cry j 2 or Cha o 2 (0.5 μg/ml) were immobilized in the wells of a microplate (FLUOTRAC™ 600) overnight at 4°C. Cry j 2 or Cha o 2 inhibitors (final concentrations, 0–10 μg/ml) were incubated with equal volumes of human sera (final dilution, 1:50) for 3 h at room temperature. The microplate was then washed with PBST buffer and incubated with diluted (1:10) serum samples for 3 h at room temperature. Subsequent procedures were the same as described above.

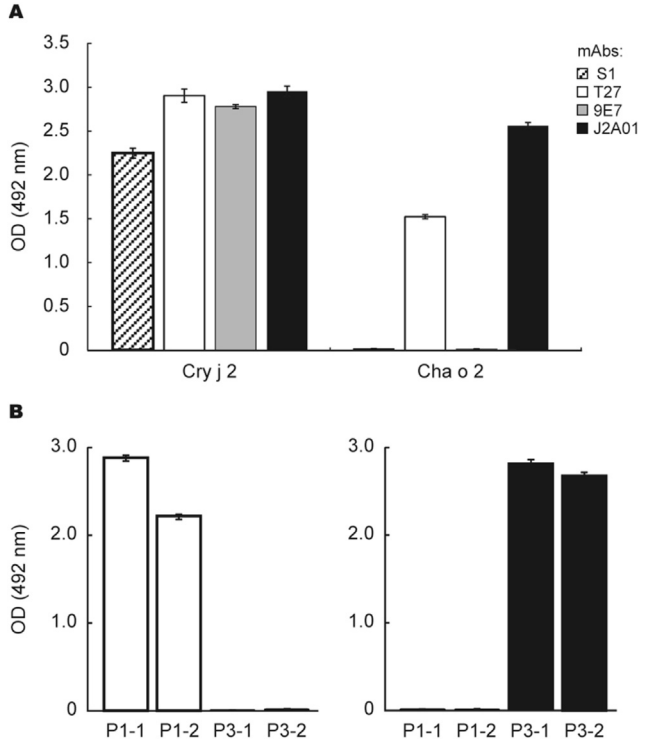

Fig. 1. Reactivity of Cry j 2 mAbs. Cry j 2 and Cha o 2 were immobilized on a microplate **(A)**. The peptides P1-1, P1-2, P3-1, and P3-2 were coated on a microplate **(B)**. Biotin-labeled S1, T27, 9E7, and J2A01 mAbs were reacted with each peptide. The binding activity of each mAb is expressed as optical density (OD). Experiments were performed in triplicate, and data are expressed as mean values ± SD from triplicate determinations. S1 and J2A01 mAbs share the same epitopes as bound by J2A07 and J2A03, respectively.[14]

Fluorometric ELISA inhibition for Cry j 2 and Cha o 2 peptides with human IgE

It was difficult to immobilize short peptides on the wells of the microplate. Therefore, to evaluate the reactivity of human IgE to ^{169}KWVNGREI^{176} (Cry j 2 core epitope) and ^{169}KVVNGRTV^{176} (relevant sequence within Cha o 2), an inhibition ELISA was conducted using these synthetic peptides as inhibitors. Briefly, P1-1 and P1-2 (10 μg/ml) were immobilized in the wells of a microplate (Nunc® Immobilizer™ Amino Plate, ThermoFisher Scientific) overnight at 4°C. The synthetic peptide inhibitors (final concentrations, 0–100 μg/ml) were incubated with equal volumes of human sera (final dilution, 1:100) for 3 h at room temperature. Subsequent procedures were performed as described above.

Results

Reactivity of Cry j 2 mAbs to Cry j 2 and Cha o 2 peptides

To examine the reactivity of four mAbs to Cry j 2 and Cha o 2 peptides, the binding of mAbs to each allergen was measured by ELISA. We found that T27 and J2A01 mAbs reacted with both Cry j 2 and Cha o 2 (Fig. 1A). These two mAbs have cross-reactivity to Cha o 2. However, the S1 mAb against a conformational epitope and 9E7 mAb against P2-1 reacted with Cry j 2, but not with Cha o 2 (Fig. 1A). These two mAbs have Cry j 2-specific binding. T27 mAb reacted with both Cry j 2-related peptide (P1-1) and Cha o 2-related

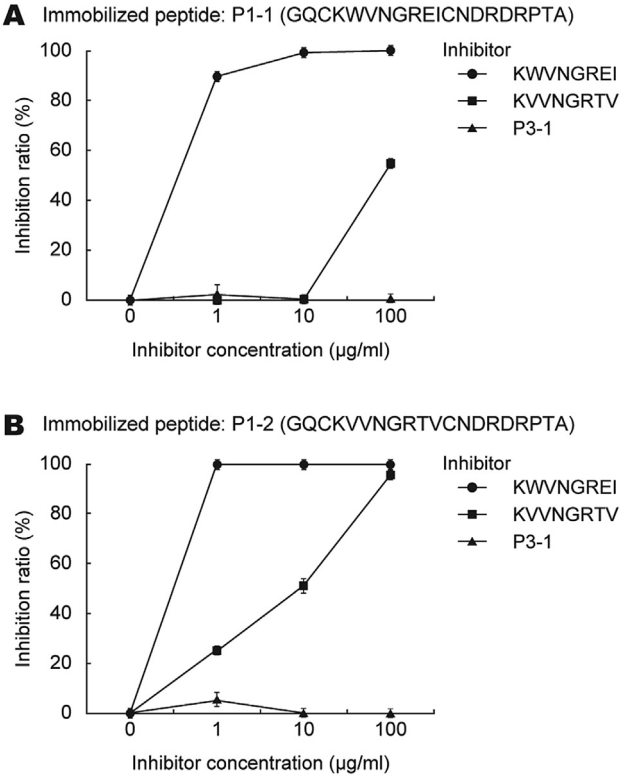

Fig. 2. Colorimetric ELISA inhibition using mAb against epitopes. Cry j 2 **(A)** or Cha o 2 **(B)** peptides (P1-1 or P1-2) were immobilized in the wells of a microplate. The Cry j 2 peptide ^{169}KWVNGREI^{176} (closed circles) and the Cha o 2 peptide ^{169}KVVNGRTV^{176} (closed squares) were incubated as inhibitors with equal volumes of T27 mAb. Unrelated peptide P3-1 was used as a negative control (closed triangles). Inhibition ratios were calculated as a percentage in the presence of homozygous or heterozygous inhibitors. Experiments were performed in triplicate, and data are expressed as mean values ± SD from triplicate determinations.

peptide (P1-2) (Fig. 1B). J2A01 mAb reacted with both Cry j 2-related peptide (P3-1) and Cha o 2-related peptide (P3-2) (Fig. 1B).

Cross-reactivity of T27 mAb between Cry j 2 and Cha o 2 peptides

To further evaluate the mAb reactivity with Cry j 2 and Cha o 2 peptides, we conducted ELISA inhibition using these peptides. The T27 mAb binding to Cry j 2 peptide (P1-1) was concentration-dependently inhibited by both ^{169}KWVNGREI^{176} (Cry j 2 core epitope peptide) and ^{169}KVVNGRTV^{176} (relevant Cha o 2 peptide) (Fig. 2A). In addition, the binding of this mAb to Cha o 2 peptide (P1-2) was inhibited by both Cry j 2 and Cha o 2 peptides (Fig. 2B). We found that this mAb exhibits cross-reactivity between the Cry j 2 core epitope in P1-1 and the relevant Cha o 2 peptide. In T27 mAb for Cry j 2, the inhibitory efficiency of the Cry j 2 peptide ^{169}KWVNGREI^{176} was higher than that of the Cha o 2 peptide ^{169}KVVNGRTV^{176}.

Reactivity of human IgE to Cry j 2 and Cha o 2 peptides in patients with Cry j 2-specific IgE

To evaluate IgE reactivity to Cry j 2 and Cha o 2 peptides, specific IgE to these peptides were measured in the sera of 31 human patients with Cry j 2-specific IgE. We summarize the FU titers of patients' IgE to each peptide in Table 2. The cut-off values used for each peptide were set as the mean value + 3SD of 10 negative controls. Of 31 patients, 10 patients had specific IgE to both P1-1 and P1-2, 13 patients had specific IgE to only P1-1, and 8 patients had no specific IgE to either peptide (Fig. 3A). Of 31 patients, 5 patients had specific IgE to both P2-1 and P2-2, 7 patients had specific IgE to only P2-1, 2 patients had specific IgE to only P2-2, and 17 patients had no specific IgE to either peptide (Fig. 3B). Of 31 patients, 10 patients had specific IgE to both P3-1 and P3-2, 10 patients had specific IgE to only P3-1, 1 patient had specific IgE to only P3-2, and 10 patients had no specific IgE to either peptide (Fig. 3C). Specific IgE to Cry j 2 showed the strongest reactivity to P1-1 and P1-2 among these peptides.

Cross-reactivity of human IgE between Cry j 2 and Cha o 2

In a representative patient with specific IgE to both P1-1 and P1-2 (Fig. 4A), allergenic cross-reactivity of Cry j 2 and Cha o 2 was investigated by ELISA inhibition. Incubation of the serum with the homologous Cry j 2 or Cha o 2 greatly inhibited binding (Fig. 4B). IgE binding to the solid-phase Cha o 2 was greatly inhibited by Cry j 2, but IgE binding to the solid-phase Cry j 2 was not greatly inhibited by Cha o 2 (Fig. 4B). We found cross-reactivity between Cry j 2 and Cha o 2, and Cry j 2 has a trend of greater inhibition than Cha o 2.

Cross-reactivity of human IgE between Cry j 2 and Cha o 2 peptides

To evaluate IgE cross-reactivity with the Cry j 2 peptide ^{169}KWVNGREI^{176} in P1-1 and the Cha o 2 peptide ^{169}KVVNGRTV^{176}

Table 2
Summarized results of total IgE fluorescence units of 31 patients.

Coated peptide	Minimum	Maximum	Median
P1-1	0	31,026	916
P1-2	11	10,242	79
P2-1	1	293	88
P2-2	0	399	62
P3-1	0	822	94
P3-2	0	1268	41

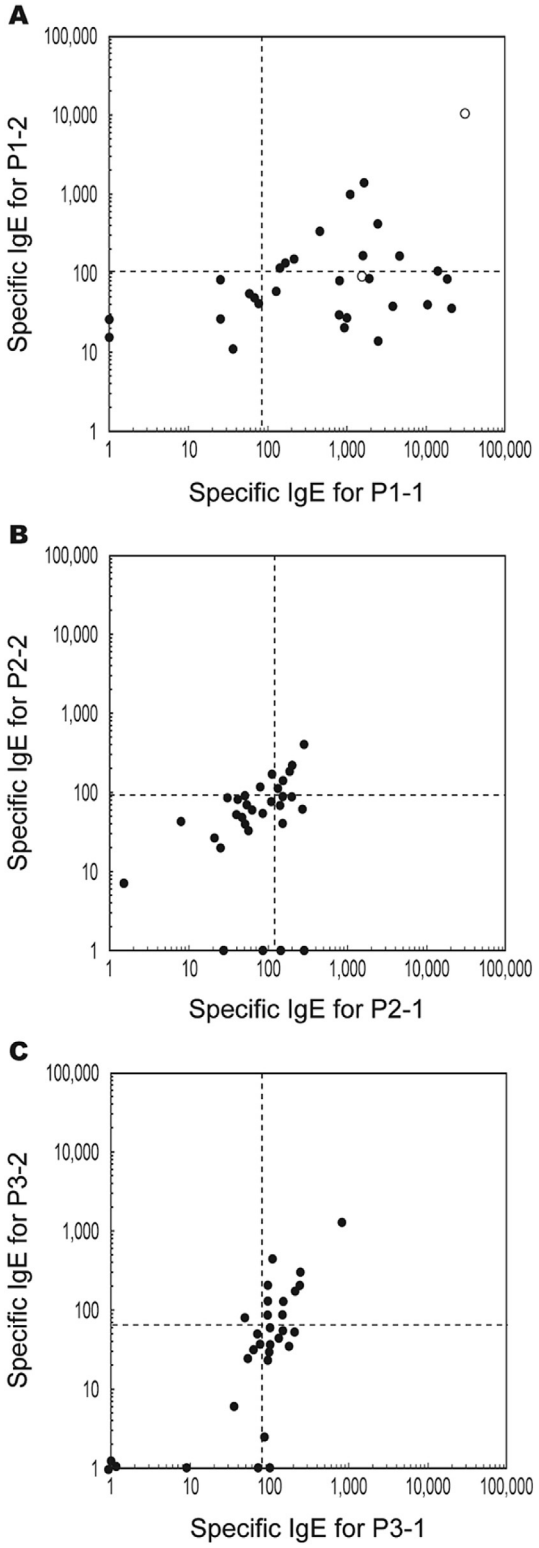

Fig. 3. IgE reactivity between Cry j 2 and Cha o 2 synthetic peptides. Binding activity is expressed in fluorescence units (FU). The x-axis is for Cry j 2-related peptides and the y-axis is for Cha o 2-related peptides. Serum samples from 31 patients were used to examine their IgE reactivity against P1-1 and P1-2 **(A)**, against P2-1 and P2-2 **(B)**, and against P3-1 and P3-2 **(C)**. The cut-off values (dashed lines) were determined as the mean + 3SD FU in negative controls. Open circles in **(A)** indicate representative patients in Fig. 4. Experiments were repeated at least three times.

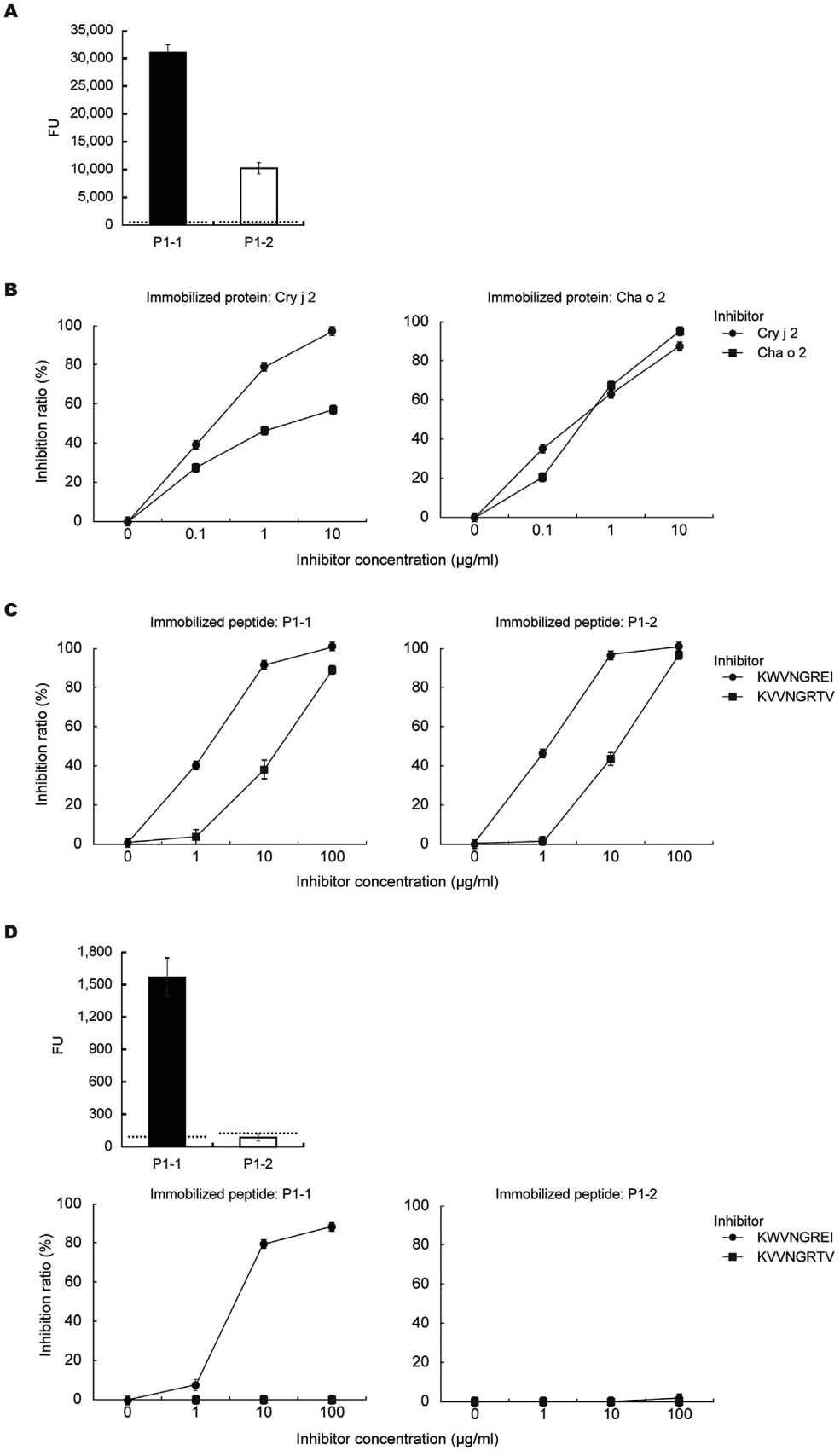

Fig. 4. Reactivity and inhibition ratios of IgE binding in representative patients. Binding activities of IgE from representative patients with double-positive reactivity to P1-1 and P1-2 **(A, patient no. 1)** and single positive reactivity to P1-1 **(D, patient no. 2)** are expressed in fluorescence units (FU). The dashed line represents the cut-off value which was determined as the mean + 3SD FU in negative controls. The cut-off values are 85.9 FU for the Cry j 2 peptide P1-1 and 110.0 FU for the Cha o 2 peptide P1-2. The inhibitory effects of the Cry j 2 (closed circles) and Cha o 2 (closed squares) on IgE binding to Cry j 2 and Cha o 2 were observed in patient no. 1 **(B)**. The inhibitory effects of the Cry j 2 peptide ^{169}KWVNGREI^{176} (closed circles) and Cha o 2 peptide ^{169}KVVNGRTV^{176} (closed squares) on IgE binding to P1-1 and P1-2 were observed in patient no. 1 **(C)**. In contrast, the inhibitory effect of Cha o 2 peptide ^{169}KVVNGRTV^{176} was not observed in patient no. 2 **(D)**. Inhibition ratios were calculated as a percentage under the presence of homozygous or heterozygous inhibitors. Experiments were performed in triplicate, and data are expressed as mean values ± SD from triplicate determinations.

in P1-2, we examined cross-reactivity by ELISA inhibition with human IgE. The binding of IgE from a representative patient was strongly inhibited by ^{169}KWVNGREI^{176} peptide and moderately inhibited by ^{169}KVVNGRTV^{176} peptide (Fig. 4C). We found that the IgE from a representative patient displayed cross-reactivity between the Cry j 2 core epitope in P1-1 and the relevant Cha o 2 peptide. In contrast, an inhibitory effect of ^{169}KWVNGREI^{176} was observed in a patient with single positive reactivity to P1-1, but this was not the case with ^{169}KVVNGRTV^{176} (Fig. 4D).

Discussion

Allergic symptoms such as rhinoconjunctivitis are induced by CJ and CO pollen allergens. The major causal allergen of pollinosis in Japan is CJ pollen because of the abundant pollination and widespread distribution of CJ. CO is the second most common pollinosis-inducing conifer in Japan. Cry j 2 is one of the major allergens in CJ pollen, and its homologue is isolated from CO as Cha o 2.[14] IgE levels between Cry j 2 and Cha o 2 were highly correlated in the sera of patients with pollinosis.[14] Moreover, bioinformatics tools such as the BLASTP (Protein Basic Local Alignment Search Tool; http://www.ncbi.nih.gov/blast) and SDAP (Structural Database of Allergenic Proteins; http://fermi.utmb.edu/SDAP/)[26,27] also suggest the existence of cross-reactive epitopes between Cry j 2 and Cha o 2. Until now, however, the contributing epitopes had not been identified on Cry j 2 and Cha o 2.

The T27 mAb reacted to P1-1 of Cry j 2 and P1-2 of Cha o 2 (Fig. 1B), and both the Cry j 2 core epitope peptide ^{169}KWVNGREI^{176} and the Cha o 2 relevant peptide ^{169}KVVNGRTV^{176} efficiently inhibited the binding of T27 mAb to P1-1 and P1-2 (Fig. 2). ^{169}KWVNGREI^{176} and ^{169}KVVNGRTV^{176} also blocked the binding of IgE from the patient with double-positive IgE to P1-1 and P1-2 (Fig. 4C). These results indicate that the ^{169}KVVNGRTV^{176} sequence in Cha o 2 is cross-reactive with the Cry j 2 epitope ^{169}KWVNGREI^{176} for mAb and patient IgE. This is another example indicating that antibodies can not only bind to the original epitope but also to sequences that may have a few different amino acids. This raises the intriguing possibility of cross-reactivity to non-homologous amino acids, which may have a large contribution to allergic diseases. On the other hand, in a patient with single positive reactivity to P1-1, the IgE binding to P1-1 was inhibited by ^{169}KWVNGREI^{176}, but not ^{169}KVVNGRTV^{176}. This result suggests the presence of another core epitope which partially overlaps with ^{169}KWVNGREI^{176}.

As shown in Fig. 1B, the J2A01 mAb reacted to both the Cry j 2 peptide (P3-1) and the Cha o 2 peptide (P3-2). Therefore, we tried to identify the core determinants for this antibody by using synthetic peptides with 10–20 residues but failed to do so (data not shown). This J2A01 mAb shares the same epitope as bound by J2A03 mAb used in our previous study.[14] In our previous study, it was suggested that the core determinants result from discontinuous amino acids within the synthetic peptides.[20] The present results may support the previous suggestion. The 9E7 mAb against P2-1 did not cross-react with the Cha o 2 peptide (Fig. 1A). However, of the 31 patients, IgE from the sera of 5 patients reacted with both P2-1 and P2-2 peptides, and there seemed to be a good correlation between these peptides (Fig. 3B). Our results suggest that the core determinant for the 9E7 mAb in P2-1 had fewer or no overlaps with P2-2. We did not determine the core epitope of P2-1, because we did not have a sufficient volume of sera from patients whose IgE reacted with this peptide. Further studies are needed to identify the core sequences for J2A01 and 9E7 mAbs by *in vitro* analysis.

We demonstrated IgE responses to Cry j 2 peptides in our previous studies,[19,20] and the cross-reactivity between the sequence epitopes on Cry j 2 and Cha o 2 in the present study. In addition to Cry j 2 and Cha o 2, other group 2 conifer allergens are some of the main pollen allergens: Jun a 2 for *Juniperus ashei* (Mountain cedar),[28] Jun o 2 (also known as Jun o 4) for *Juniperus oxycedrus* (Prickly juniper),[29] Tax d 2 for *Taxodium distichum* (Bald cypress), and Cup a 2 for *Cupressus arizonica* (Arizona cypress). They belong to the family *Cupressaceae* and cause pollinosis in areas such as North America[30,31] and the Mediterranean region.[32,33] In addition to *in vitro* analyses, we suggest the possibility that cross-reactive epitopes exist among some group 2 conifer allergens by *in silico* analyses (data not shown). The *in silico* analysis also estimated that ^{170}KTINGRTV^{177} for Jun a 2 [property distance index (PD) = 6.55], ^{139}KWINGREI^{146} for Tax d 2 (0.68), and ^{117}KTINGRTV^{124} for Cup a 2 (5.87) were epitopes relevant to the Cry j 2 epitope ^{169}KWVNGREI^{176}. The results suggest that cross-reactivity between the Cry j 2 core epitope and the related Tax d 2 and Cup a 2, but not Jun a 2, sequences, will be observed according to the threshold proposed by Ivanciuc *et al.*,[34] which may lie between 5 and 6.5. Conversely, sera from patients with CJ pollinosis demonstrated binding to Jun a 2,[28] suggesting that epitopes rather than P1-1 are important for cross-reactivity between Cry j 2 and Jun a 2. Our findings provide useful information for the development of therapeutic strategies for pollinosis.

IgE antibodies to CJ have also been found in monkeys with pollinosis[19,24] and dogs with atopic dermatitis.[35] Monkeys with CJ pollinosis have symptoms similar to those of human patients. Dogs live in the same environment as humans and naturally develop a pruritic dermatitis that is extremely similar to human atopic dermatitis. Moreover, monkeys[16] and dogs[17] sensitized to CJ pollen were reported to have cross-reactive IgE to CO pollen. Although allergenic cross-reactivity between Cry j 1 and Cha o 1 was demonstrated in those studies, cross-reactivity between Cry j 2 and Cha o 2 remains to be elucidated. Because of the remarkable similarity with the human diseases, monkeys and dogs are considered prime animal models for allergic diseases. Further *in vivo* studies using such animal models will provide a better understanding of cross-reactivity of CJ and CO.

In summary, we showed that the amino acid sequences in Cha o 2 cross-react with Cry j 2 epitopes at the mAb and human IgE levels. We also provided evidence of the importance of ^{169}KVVNGRTV^{176} in Cha o 2 cross-reactivity with the Cry j 2 epitope ^{169}KWVNGREI^{176}, which plays an important role in allergenicity in CJ pollinosis. Induction of blocking IgG4 that can recognize IgE epitopes is responsible for clinically successful allergen-specific immunotherapy.[36] Therefore, targeting epitopes related to cross-reactivity may help in the development of immunotherapy against multiple allergic diseases. Our results are useful for the development of safer and more efficient therapeutic strategies for treating CJ and CO pollen allergies.

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Conflicts of interest

The authors have no conflict of interest to declare.

Authors' contributions

KM conceived and designed the experiments. KM analyzed the data and drafted the manuscript. NO helped KM to conduct the experiments. AS and HY helped to design the experiments and analyze the data. NO, YT, and HS assisted in data

analysis. SS and MS assisted in the study design, sample preparation, and editing of the manuscript. All authors have read and approved the final manuscript.

References

1. Ishizaki T, Koizumi K, Ikemori R, Ishiyama Y, Kushibiki E. Studies of prevalence of Japanese cedar pollinosis among the residents in a densely cultivated area. *Ann Allergy* 1987;**58**:265–70.
2. Sakashita M, Hirota T, Harada M, Nakamichi R, Tsunoda T, Osawa Y, et al. Prevalence of allergic rhinitis and sensitization to common aeroallergens in a Japanese population. *Int Arch Allergy Immunol* 2010;**151**:255–61.
3. Mori T, Yokoyama M, Komiyama N, Okano M, Kino K. Purification, identification, and cDNA cloning of Cha o 2, the second major allergen of Japanese cypress pollen. *Biochem Biophys Res Commun* 1999;**263**:166–71.
4. Anderson Jr LB, Dreyfuss EM, Logan J, Johnstone DE, Glaser J. Melon and banana sensitivity coincident with ragweed pollinosis. *J Allergy* 1970;**45**:310–9.
5. Hirschwehr R, Valenta R, Ebner C, Ferreira F, Sperr WR, Valent P, et al. Identification of common allergenic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J Allergy Clin Immunol* 1992;**90**:927–36.
6. Valenta R, Duchene M, Ebner C, Valent P, Sillaber C, Deviller P, et al. Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 1992;**175**:377–85.
7. van Ree R. Clinical importance of cross-reactivity in food allergy. *Curr Opin Allergy Clin Immunol* 2004;**4**:235–40.
8. Yasueda H, Yui Y, Shimizu T, Shida T. Isolation and partial characterization of the major allergen from Japanese cedar (*Cryptomeria japonica*) pollen. *J Allergy Clin Immunol* 1983;**71**:77–86.
9. Taniguchi Y, Ono A, Sawatani M, Nanba M, Kohno K, Usui M, et al. Cry j I, a major allergen of Japanese cedar pollen, has pectate lyase enzyme activity. *Allergy* 1995;**50**:90–3.
10. Sakaguchi M, Inouye S, Taniai M, Ando S, Usui M, Matuhasi T. Identification of the second major allergen of Japanese cedar pollen. *Allergy* 1990;**45**:309–12.
11. Ohtsuki T, Taniguchi Y, Kohno K, Fukuda S, Usui M, Kurimoto M. Cry j 2, a major allergen of Japanese cedar pollen, shows polymethylgalacturonase activity. *Allergy* 1995;**50**:483–8.
12. Fujimura T, Futamura N, Togawa A, Goldblum RM, Yasueda H, Saito A, et al. Isolation and characterization of native Cry j 3 from Japanese cedar (*Cryptomeria japonica*) pollen. *Allergy* 2007;**62**:547–53.
13. Suzuki M, Komiyama N, Itoh M, Itoh H, Sone T, Kino K, et al. Purification, characterization and molecular cloning of Cha o 1, a major allergen of *Chamaecyparis obtusa* (Japanese cypress) pollen. *Mol Immunol* 1996;**33**:451–60.
14. Yasueda H, Saito A, Sakaguchi M, Ide T, Saito S, Taniguchi Y, et al. Identification and characterization of a group 2 conifer pollen allergen from *Chamaecyparis obtusa*, a homologue of Cry j 2 from *Cryptomeria japonica*. *Clin Exp Allergy* 2000;**30**:546–50.
15. Ito H, Ssuzuki M, Mamiya S, Takagi I, Baba S, Tomita K, et al. Analysis of the allergenic components of Hinoki cypress (*Chamaecyparis obtusa*) pollen by immunoblotting with the sera from patients with Japanese cedar pollinosis. *Allergol Int* 1996;**45**:181–6.
16. Kobayashi C, Nigi H, Saito S, Ide T, Taniguchi Y, Inouye S, et al. IgE reactivity and cross-reactivity of Japanese monkeys (*Macaca fuscata*) to Japanese cedar (*Cryptomeria japonica*) and cypress (*Chamaecyparis obtusa*) pollen allergens. *Clin Exp Allergy* 1999;**29**:856–61.
17. Sakaguchi M, Masuda K, Yasueda H, Saito S, DeBoer DJ, Tsujimoto H. IgE reactivity and cross-reactivity to Japanese cedar (*Cryptomeria japonica*) and cypress (*Chamaecyparis obtusa*) pollen allergens in dogs with atopic dermatitis. *Vet Immunol Immunopathol* 2001;**83**:69–77.
18. Hashimoto M, Nigi H, Sakaguchi M, Inouye S, Imaoka K, Miyazawa H, et al. Sensitivity to two major allergens (Cry j I and Cry j II) in patients with Japanese cedar (*Cryptomeria japonica*) pollinosis. *Clin Exp Allergy* 1995;**25**:848–52.
19. Tamura Y, Kawaguchi J, Serizawa N, Hirahara K, Shiraishi A, Nigi H, et al. Analysis of sequential immunoglobulin E-binding epitope of Japanese cedar pollen allergen (Cry j 2) in humans, monkeys and mice. *Clin Exp Allergy* 2003;**33**:211–7.
20. Miyaji K, Yurimoto T, Saito A, Yasueda H, Takase Y, Shimakura H, et al. Analysis of conformational and sequential IgE epitopes on the major allergen Cry j 2 of Japanese cedar (*Cryptomeria japonica*) pollen in humans by using monoclonal antibodies for Cry j 2. *J Clin Immunol* 2013;**33**:977–83.
21. Varshney S, Goldblum RM, Kearney C, Watanabe M, Midoro-Horiuti T. Major mountain cedar allergen, Jun a 1, contains conformational as well as linear IgE epitopes. *Mol Immunol* 2007;**44**:2781–5.
22. Padavattan S, Flicker S, Schirmer T, Madritsch C, Randow S, Reese G, et al. High-affinity IgE recognition of a conformational epitope of the major respiratory allergen Phl p 2 as revealed by X-ray crystallography. *J Immunol* 2009;**182**:2141–51.
23. Tiwari R, Negi S, Braun B. Validation of a phage display and computational algorithm by mapping a conformational epitope of Bla g 2. *Int Arch Allergy Immunol* 2011;**157**:323–30.
24. Sakaguchi M, Hashimoto M, Nigi H, Yasueda H, Takahashi Y, Watanabe M, et al. Epitope specificity of IgE antibodies to a major allergen (Cry j 1) of Japanese cedar pollen in sera of humans and monkeys with pollinosis. *Immunology* 1997;**91**:161–6.
25. Tamura Y, Sasaki R, Inouye S, Kawaguchi J, Serizawa N, Toda M, et al. Identification of a sequential B-cell epitope on major allergen (Cry j 1) of Japanese cedar (*Cryptomeria japonica*) pollen in mice. *Int Arch Allergy Immunol* 2000;**123**:228–35.
26. Ivanciuc O, Schein CH, Braun W. Data mining of sequences and 3D structures of allergenic proteins. *Bioinformatics* 2002;**18**:1358–64.
27. Ivanciuc O, Schein CH, Braun W. SDAP: database and computational tools for allergenic proteins. *Nucleic Acids Res* 2003;**31**:359–62.
28. Yokoyama M, Miyahara M, Shimizu K, Kino K, Tsunoo H. Purification, identification, and cDNA cloning of Jun a 2, the second major allergen of mountain cedar pollen. *Biochem Biophys Res Commun* 2000;**275**:195–202.
29. Tinghino R, Barletta B, Palumbo S, Afferni C, Iacovacci P, Mari A, et al. Molecular characterization of a cross-reactive *Juniperus oxycedrus* pollen allergen, Jun o 2: a novel calcium-binding allergen. *J Allergy Clin Immunol* 1998;**101**:772–7.
30. Pence HL, Mitchell DQ, Greely RL, Updegraff BR, Selfridge HA. Immunotherapy for mountain cedar pollinosis: a double-blind controlled study. *J Allergy Clin Immunol* 1976;**58**:39–50.
31. Ramirez DA. The natural history of mountain cedar pollinosis. *J Allergy Clin Immunol* 1984;**73**:88–93.
32. Tas J. Hayfever due to the pollen of *Cupressus sempervirens*, Italian or Mediterranean cypress. *Acta Allergol* 1965;**20**:405–7.
33. Panzani R, Centanni G, Brunel M. Increase of respiratory allergy to the pollens of cypresses in the South of France. *Ann Allergy* 1986;**56**:460–3.
34. Ivanciuc O, Midoro-Horiuti T, Schein CH, Xie L, Hillman GR, Goldblum RM, et al. The property distance index PD predicts peptides that cross-react with IgE antibodies. *Mol Immunol* 2009;**46**:873–83.
35. Masuda K, Tsujimoto H, Fujiwara S, Kurata K, Hasegawa A, Taniguchi Y, et al. IgE-reactivity to major Japanese cedar (*Cryptomeria japonica*) pollen allergens (Cry j 1 and Cry j 2) by ELISA in dogs with atopic dermatitis. *Vet Immunol Immunopathol* 2000;**74**:263–70.
36. Fujimura T, Kawamoto S. Spectrum of allergens for Japanese cedar pollinosis and impact of component-resolved diagnosis on allergen-specific immunotherapy. *Allergol Int* 2015;**64**:312–20.