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ORIGINAL RESEARCH

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

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Abstract
Purpose Japanese cedar (*Cryptomeria japonica*; CJ) pollinosis is a type I allergy induced by CJ pollen, and Cry j 2 is one of the major allergens in this pollen. In a previous study, we analyzed IgE epitopes on Cry j 2 in humans by using synthetic peptides. The main purpose of this study was to identify B-cell epitopes on Cry j 2 in patients with CJ pollinosis by using monoclonal antibodies (mAbs) for Cry j 2.
Methods We used ELISA with mAbs for the epitope analysis. Sera samples were collected from 80 patients with CJ pollinosis, and allergenic epitopes for mAbs and human IgE were identified using ELISA with synthetic peptides. The importance of the epitopes for human IgE was analyzed using an inhibition ELISA.
Results Four independent epitopes (epitope #1, #2, #3, and #4) were identified on Cry j 2 with the use of mAbs. Epitope #3 and #4, corresponding to peptides No. 25 and No. 33, respectively, were newly determined as epitopes for mAbs and human IgE. Inhibition ELISA showed that not only epitope #2 (sequential) but epitope #1 (conformational) may play an important role in the CJ pollinosis.
Conclusions Our results revealed 4 epitopes, including two new ones, on Cry j 2. We also found that inhibition ELISA with appropriate mAbs could be a viable method of evaluating the importance of the conformational and sequential epitopes for human IgE. These results are beneficial for the development of safer and more efficient therapeutic strategies for treating CJ pollinosis.

Keywords Japanese cedar pollen · pollinosis · Cry j 2 · IgE · epitope

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Introduction

Pollinosis is a typical type I allergy induced by pollens, and is defined as the appearance of respiratory symptoms such as rhinoconjunctivitis. Japanese cedar (*Cryptomeria japonica*; CJ) pollinosis is one of the most common allergic diseases in Japan [1]. In an epidemiological study conducted

in the central Hokuriku area of Japan in May and June of both 2006 and 2007 [2], 36.7 % of the study participants (566 of the 1,540 subjects) had allergic rhinitis to CJ pollen. Impaired performance and a voluntary ban on leaving home for patients with CJ pollinosis comprise the tremendous social impact and economic loss associated with this disease. Therefore, CJ pollinosis is one of the most serious public health problems in Japan.

Yasueda et al. [3] purified Cry j 1 as a major allergen from CJ pollen, and we isolated Cry j 2 as another major allergen [4]. Of 145 patients with CJ pollinosis tested for specific IgE levels, 134 (92 %) had specific IgE antibodies to both Cry j 1 and Cry j 2 [5]. Cry j 2 is completely different from Cry j 1 in terms of its chemical and immunophysical properties, N-terminal amino acid sequence, and its molecular mass of 37 kDa under non-reducing conditions [4]. Furthermore, we also recently isolated Cry j 3 as a causative allergen of CJ pollinosis [6].

The identification and characterization of allergenic epitopes are important for establishing safer and more efficient immunotherapy protocols [7, 8]. Previously, we used monoclonal antibodies (mAbs) to characterize the allergenic epitopes of Cry j 1 in CJ pollens, and identified five independent epitopes on Cry j 1 [9]. In our preliminary study [10], in which epitopes on Cry j 2 were analyzed using a synthetic peptide of Cry j 2, we found that ^{124}KWVNGREI^{131} is an important major linear epitope. However, we had not classified IgE epitopes on Cry j 2 with mAbs for the allergen, and the significance of a previously identified epitopes remains to be evaluated.

In the present study, we identified four independent epitopes on Cry j 2 by using mAbs for Cry j 2; two are new epitopes. Analysis of a large number of sera samples obtained from patients with CJ pollinosis has confirmed that they are also new epitopes for human IgE. We additionally predicted whether amino acids that made synthetic peptides recognizable by mAbs and human IgE could be exposed on Cry j 2 by means of bioinformatics tools. Finally, fluorometric and inhibition ELISA assays were used to evaluate the importance of conformational and sequential epitopes recognized by mAbs and human IgE.

Methods

The Cry j 2 Antigen and Monoclonal Antibodies

Cry j 2 was purified as previously described [4]. A library of peptides, consisting of 18, 20, 21, or 25 amino acids overlapping by 10 to 12, was commercially synthesized (Hokkaido System Science Co., Ltd., Sapporo, Japan) [10, 11]. The eight mAbs (S1, S2, S3, N26, T27, 24, 9E7, and J2A01) against Cry j 2 used in our laboratory were categorized according to cross-inhibition ELISA. These mAbs were kindly provided or generated by immunizing mice with Cry j 2 [10, 12–14].

ELISA Cross-Inhibition for Epitopes of Cry j 2 Recognized by mAbs

Epitopes recognized by the mAbs were grouped using the ELISA cross-inhibition test. This test is based on the ability of a mAb to inhibit the binding of a coexisting biotin-labeled mAb to solid-phase Cry j 2. Briefly, Cry j 2 (0.5 μg/mL) was adsorbed onto a microplate (F96 Maxisorp® NUNC-Immuno™ Plate, ThermoFisher Scientific, Waltham, MA, USA). Biotin-labeled mAbs (0.5 μg/mL) were then reacted with the solid-phase antigen in the presence or absence of unlabeled mAbs (5 μg/mL) for 1 h at room temperature. Next, a streptavidin-peroxidase polymer (streptavidin-HRP, Sigma-Aldrich, St. Louis, 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 quenched 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).

Patient Sera Samples

Informed consent was obtained from all subjects. The study protocol was approved by the ethics committee at the Jikei University School of Medicine. Sera samples from 80 patients with CJ pollinosis. The patents were selected on the bases of clinical symptoms of seasonal allergic rhinitis and positive CAP (Phadia AB, Uppsala, Sweden) to CJ pollen. For the negative control, sera samples were obtained from 25 non-allergic subjects who had previously been confirmed to be negative for the CJ pollen allergen.

Colorimetric ELISA for Overlapping Synthetic Peptides with mAbs

As previously described, the reaction of mAbs to Cry j 2 peptides was measured using a colorimetric ELISA [10]. Briefly, Cry j 2 protein (1 μg/mL) or synthetic peptides (10 μg/mL) were immobilized in the wells of a microplate (F96 MaxiSorp® NUNC-Immuno™ Plate) overnight at 4 °C. The microplate was then washed with phosphate-buffered saline containing 0.05 % Tween 20 (PBST buffer) and incubated with biotin-labeled mAbs (0.1 μg/mL) for 1 h at room temperature. The subsequent procedures were the same as those for the cross-inhibition ELISA described above.

Fluorometric ELISA for Overlapping Synthetic Peptides with Human IgE in Patient Sera

As previously described, specific IgE in the sera of patients with pollinosis for Cry j 2 peptides was measured using fluorometric ELISA [5]. Briefly, Cry j 2 protein (1 μg/mL)

or synthetic peptides (10 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 finally incubated with diluted (1:10) sera 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. As 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 on a multi-mode microplate reader. Cutoff values were determined using sera from pollinosis-negative patients as controls.

Inhibition ELISA for the Conformational and Sequential Epitopes with the Use of mAbs

To evaluate the importance of the conformational and sequential epitopes, inhibition ELISA was conducted using mAbs as inhibitors [9]. Briefly, Cry j 2 protein (1 μg/mL) was immobilized in the wells of a microplate overnight at 4 °C. Non-labeled mAbs S1 or N26 (20 μg/mL) were reacted with the immobilized protein for 1 h at room temperature. Plates were then washed with PBST buffer and subsequently incubated with diluted (1:10) sera samples for 3 h at room temperature. Sera samples from 50 patients were used for conducting the assay because of their sufficient volume (>100 μL). Subsequent procedures were the same as those of the fluorometric ELISA described above. The inhibition ratio (%) was calculated as follows:

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

Bioinformatics Tool

The surface exposure of the Cry j 2 amino acids was predicted using NetSurfP (http://www.cbs.dtu.dk/services/NetSurfP/) [15, 16].

Results

Epitope Specificity of Anti-Cry j 2 mAbs and Their Binding to Synthetic Peptides of Cry j 2

Four independent epitopes were identified on the Cry j 2 molecule by using the ELISA cross-inhibition test with mAbs. An inhibition reaction was not observed with a non-labeled mAb that recognized different epitopes. In the present study, the epitope on the Cry j 2 molecule related to mAbs S1, S2, and S3 was defined as epitope #1, mAbs N26, T27, and 24 as epitope #2, mAb 9E7 as epitope #3, and mAb J2A01 as epitope #4 (Table I).

We examined the binding of these mAbs to synthetic peptides of Cry j 2. Our observations were similar to those of a previous report [10]; we reconfirmed that mouse Cry j 2-specific mAb S1 did not react with any of the peptides, and mAbs N26 and 24 reacted with peptide No. 13 (^{121}GQCKWVNGREICNDRDRPTA^{140}). We found that the mAbs 9E7 and J2A01 reacted with peptides No. 25 (^{241}GRENSRAEVSYVHVNGAKFI^{260}) and No. 33 (^{321}TYKNIRGTSATAAAIQLKCS^{340}), respectively. It was determined that epitope # 2 is relevant to peptide No. 13, epitope #3 to peptide No. 25, and epitope #4 to peptide No. 33. We found that epitope #3 and epitope #4 were new epitopes recognized by mAbs.

Binding of IgE in Sera of Human Patients to Synthetic Peptides of Cry j 2

Figure 1 shows the reactivity of human IgE obtained from 80 patients with anti-Cry j 2 IgE to synthetic peptides. The cutoff values for Cry j 2 and peptides were obtained in the maximal FU values of 25 negative controls, because the values were higher than mean value+3SD. We reconfirmed that IgE from 61 patients (76 %) reacted with peptide No. 13 (relevant to epitope #2). We also found that IgE from 11 (14 %) patients reacted with peptide No. 25 (relevant to epitope #3), and IgE from 19 (24 %) patients reacted with peptide No. 33 (relevant to epitope #4). In our previous study, none of the samples obtained from 20 patients reacted with these peptides [10].

Exposed Amino Acids of Sequential Epitopes on Cry j 2

The solvent-accessible amino acids of epitopes epitope #2, epitope #3, and epitope #4 were predicted using NetSurfP (Table II). Core amino acids for epitope #2 (^{124}KWVNGREI^{131}), which were identified using small peptides of peptide No. 13 in the previous study [10], all seemed to be exposed on the Cry j 2 surface. The exposed regions of epitope #3 were predicted to be ^{242}R^{243}E^{244}N^{245}S^{246}R^{247}A^{248}E^{250}S^{256}G^{258}K^{260}I, and those of epitope #4 to be ^{323}Y^{324}N^{326}R^{328}T^{330}A^{331}T^{332}A. All computed results are shown in the Supplementary Table.

Evaluating the Significance of the Epitopes, Epitope #1 and #2, for Human IgE

We determined the inhibition rates of mAbs S1 (for epitope #1) and N26 (for epitope #2) against human IgE for comparison of their importance. Table III indicated FU value of human IgE to

Table I Classification of anti-Cry j 2 mAbs by inhibition ELISA

Unlabeled mAb[a]	Biotin-labeled mAb to							
	epitope #1			epitope #2			epitope #3	epitope #4
	S1	S2	S3	N26	T27	24	9E7	J2A01
S1	+	+	+	−	−	−	−	−
S2	+	+	+	−	−	−	−	−
S3	+	+	+	−	−	−	−	−
N26	−	−	−	+	+	+	−	−
T27	−	−	−	+	+	+	−	−
24	−	−	−	+	+	+	−	−
9E7	−	−	−	−	−	−	+	−
J2A01	−	−	−	−	−	−	−	+

[a]Inhibitor. +, >30 % inhibition. Experiments were repeated at least three times.

peptide No. 13 (relevant to epitope #2), and inhibition ratio of mAbs to epitope # 1 and #2 against human IgE for each subject. We found that their patterns were substantially different. Of the 10 sera samples that showed >50 % inhibition rates for mAb to epitope #1, eight sera samples had negative FU value for IgE to epitope #2 relative peptide.

Discussion

The bioinformatics tool NetSurfP can predict solvent-accessible regions on proteins. Using this tool, we predicted exposed amino acids in Cry j 2 epitopes (epitope #2, #3, and #4). A review article relevant to food allergies discussed that the majority of IgE epitopes are eight amino acids or longer [17]. However, it has been suggested that five to six amino acids play an important role in the binding between antibody and epitope [18], and pentapeptides have been reported as IgE epitopes in wheat gliadin [19]. All core amino acids of epitope #2 determined in our previous study [10] were predicted to be exposed, indicating that ^{124}KWVNGREI^{131} was a completely sequential epitope. Epitope #3 was predicted to contain seven consecutive amino acids, ^{242}RENSRAE^{248}, exposed on Cry j 2, suggesting that these amino acids may contribute as the epitope. Meanwhile, epitope #4 was not expected to contain three or more consecutive amino acids. Our result raises the possibility that the core determinants may result from discontinuous amino acids within epitope #4. Further study is needed to identify the core determinants of those epitopes.

The mAb S1 did not react with any of the synthetic peptides. This result coincides with that from our previous

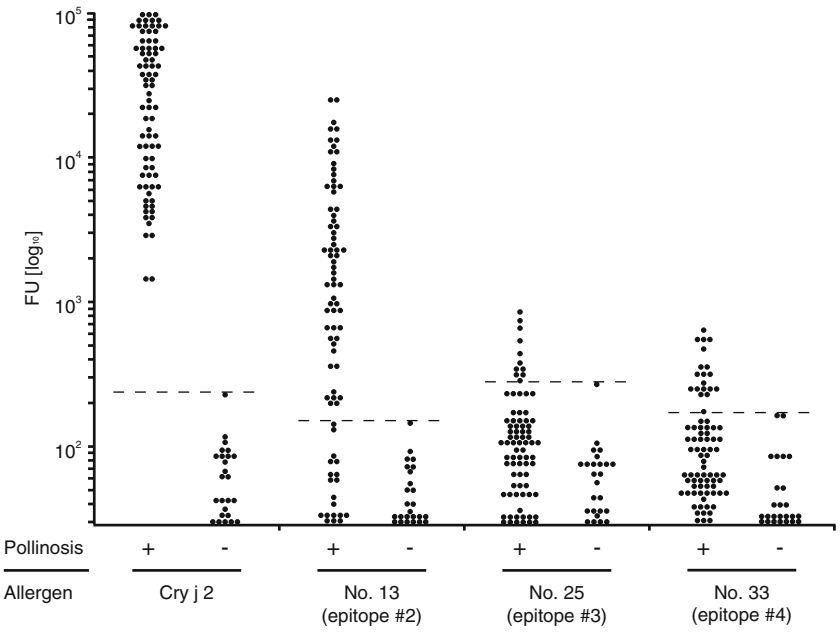

Fig. 1 Binding of human IgE to Cry j 2 and synthetic peptides. Related epitope numbers are indicated within parentheses below to the synthetic peptide numbers. Binding activity is expressed in fluorescence units (FU). The dashed line represents cutoff values, which were determined as highest FU in negative control. The cutoff values are 233.5, 156.0, and 185.5 from the left column. Sera samples from 50 patients were used to provide adequate amounts, and experiments were repeated at least three times

Table II Prediction of solvent-accessible regions of Cry j 2 amino acids using NetSurfP

Epitope	Position	Amino acids
epitope #2[a]	124–131	KWVNGREI ********
epitope #3	241–260	GRENSRAEVSYVHVNGAKFI -*******-*-----*-*-*
epitope #4	321–340	TYKNIRGTSATAAAIQLKCS --**-*-*--**--------

[a] Core sequence for epitope #2 was identified by Tamura et al. [10]

Surface exposure of Cry j 2 amino acids was predicted by NetSurfP, and each amino acid was assigned to "Exposed (*)" or "Buried (-)". See also supplementary table

study, in which the mAb S1 also did not bind to any synthetic peptides [10]. Along with decreasing reaction of S1 to heat treated Cry j 2 [20], these findings indicate that epitope #1 could be a conformational epitope. Although it has been shown that a number of patient IgE reacted to peptide No. 13 (relevant to epitope #2), the importance of a conformational epitope on Cry j 2 for human IgE had not been previously reported. Therefore, the responsiveness of human IgE to epitope #1 (a conformational epitope) and epitope #2 (a sequential epitope) was compared by inhibition ELISA. The reaction of mAb with the epitope #1 inhibited the binding of human IgE to Cry j 2 in a different manner in the reaction of mAb with epitope #2. Eighty percent of the tested sera samples showing >50 % inhibition rates for mAb to epitope #1 were negative for epitope #2. Conformational epitopes on allergens are considered to play an important role in initiating human IgE-mediated allergic reactions [21–23]. Our results suggested that not only conformational but also sequential epitope may play an important role in the CJ pollinosis. Variations in responsiveness to Cry j 2 epitopes could have been influenced by unknown factors such as an individual's genetic background and/or clinical history. It may be worthwhile to analyze IgE-binding epitopes to understand the etiology of this allergy and develop safer and more efficient therapeutic strategies for treating CJ pollinosis.

Table III Inhibition rates of mAbs binding to epitope #1 and #2 for evaluating the importance of the conformational epitope (epitope #1) on Cry j 2 for human IgE

Inhibition rate (%)				Inhibition rate (%)			
Subject	FU value	epitope #1	epitope #2	Subject	FU value	epitope #1	epitope #2
1	0	27.1	0.0	26	1038	47.0	**50.6**
2	0	46.3	21.2	27	1214	32.4	**80.7**
3	0	**50.5**	40.5	28	1229	23.3	**71.0**
4	0	**57.2**	8.5	29	1317	29.0	**50.9**
5	1	34.5	16.5	30	1719	20.0	20.9
6	7	**50.1**	39.8	31	1885	42.3	**59.0**
7	8	**58.0**	41.6	32	2061	21.1	39.4
8	30	46.5	30.8	33	2071	31.2	**72.6**
9	47	36.6	28.4	34	2207	25.9	34.8
10	52	**63.4**	27.4	35	2257	35.7	**54.4**
11	56	34.6	16.9	36	2303	23.1	48.5
12	80	**61.0**	28.4	37	2322	**62.3**	44.8
13	93	41.7	25.6	38	2371	19.3	**54.4**
14	135	**50.6**	25.6	39	2670	3.2	44.2
15	219	26.4	19.7	40	2953	12.4	**72.7**
16	226	**50.1**	44.7	41	3306	34.5	**79.2**
17	230	44.0	26.9	42	3857	37.0	**67.4**
18	235	**54.7**	36.3	43	4214	19.6	**75.2**
19	464	31.5	**68.2**	44	6490	29.1	**54.5**
20	576	37.3	**50.3**	45	6686	22.7	**59.6**
21	670	17.9	35.8	46	7944	9.4	**57.1**
22	676	47.6	28.9	47	9159	24.6	**71.7**
23	815	43.2	41.5	48	11372	12.2	**65.6**
24	849	20.4	42.5	49	13088	6.4	35.7
25	983	45.0	44.6	50	26236	7.5	22.2

FU values of human IgE were calculated based on the activity to epitope #2 relative peptide No. 13, and the cutoff value is 233.5 (see also Fig. 1). The epitope #2 was chosen as a target for comparison, since the affinity of human IgE to peptide No. 13 was higher than to other peptides. Sera samples from 50 patients were used to have enough volume (>100 μL) to conduct inhibition ELISA. Inhibition rate over 50 % is shown in bold. The experiments were repeated at least three times.

A previous study reported that no patients' sera included reactive IgE to the synthetic peptides No. 25 and No. 33 [10]. Our present results show that these peptides were recognized by the mAbs and human IgE (Fig. 1), suggesting that the determinants are newly detected epitopes. The lack of detection in our previous analysis is probably because those sites are minor epitopes and were thus incidentally not recognized in the small sample size. In fact, we tested 80 patients with CJ pollinosis, and only 11 (14 %) patients for peptide No. 25 (relevant to epitope #3) and 19 (24 %) patients for peptide No. 33 (relevant to epitope #4) were detected as being epitope-specific IgE-positive at low FU compared to peptide No. 13 (relevant to epitope #2). Moreover, differences between the methodologies used in previous studies and our study, especially with regard to the detection procedures, may have contributed to the fact that those epitopes were not detected by the other investigators.

Many studies have analyzed IgE epitopes of group 1 conifer pollen allergens, such as Cry j 1 and Jun a 1 [9, 21, 24–26]. Using mAbs for Cry j 1, a major allergen of the CJ pollen, we identified five independent epitopes on Cry j 1 in human patients [9]. Major mountain cedar allergen, Jun a 1, contains conformational and sequential IgE epitopes [26]. Other group 2 conifer pollen allergens, such as Jun a 2 and Cha o 2 from cypress pollen, have also been identified [27–29]. However, few studies have analyzed IgE epitopes in group 2 conifer pollen allergens [10]. In our previous study, we identified important sequential IgE epitopes of Cry j 2 by using synthetic peptides on this allergen [10]. In the present study, we have identified two new IgE epitopes by using mAbs for Cry j 2. These studies concerning IgE epitopes on Cry j 2 will help in developing new allergen vaccines against cedar and cypress pollinosis [30].

Conclusion

Four epitopes, including two new epitopes, were identified on Cry j 2 by using mAbs, and IgE from patients with pollinosis also reacted to the epitopes. The bioinformatics tool NetSurfP could predict the candidate amino acids that accounted for the core determinants in one of the two new epitopes. Our study also demonstrated that inhibition ELISA using mAbs is a viable method to evaluate the importance of the conformational and sequential epitopes for human IgE. These results are useful for the development of safer and more efficient therapeutic strategies for treating CJ pollinosis.

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