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Epitope specificity of IgE antibodies to a major allergen (Cry j 1) of Japanese cedar pollen in sera of humans and monkeys with pollinosis

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SUMMARY

Japanese cedar (*Cryptomeria japonica*) pollinosis has been reported to occur naturally in Japanese monkeys (*Macaca fuscata*) as well as humans. Using monoclonal antibodies (mAb) specific to Cry j 1, a major allergen in Japanese cedar pollen, we identified five independent epitopes (EP-1 to EP-5) on the molecule. The epitopes recognized by IgE antibodies in the sera of humans and monkeys with the pollinosis were analysed by an IgE enzyme-linked immunosorbent assay inhibition method with these mAb. In human patients, the mAb to EP-1 strongly blocked the binding of IgE antibodies in all patients' sera to Cry j 1. The reaction patterns of IgE antibodies in monkeys, however, varied among the troops of monkeys. In some troops, the mAb to EP-1 showed a blocking pattern similar to that for human patients. In other troops, mAb to EP-4 and EP-5 blocked binding of IgE. These results indicate that some, but not all, monkeys have antibody responses to the major allergen similar to those of humans.

INTRODUCTION

Japanese cedar (*Cryptomeria japonica*, CJ) pollinosis is one of the most common allergic diseases in Japan.[1,2] The number of patients with this pollinosis has recently increased.[3] Our previous seroepidemiological study showed that about 30% of the general population between 20 and 39 years of age who reside in the Tokyo area have anti-CJ IgE antibody.[4] In another study of 892 university students, the percentage of carriers with CJ-specific IgE was found to be 27% and the frequency of CJ pollinosis sufferers was 12%.[5]

The natural occurrence of CJ pollinosis has also been reported in Japanese monkeys (*Macaca fuscata*).[6] The monkeys with the pollinosis show symptoms similar to those of human patients; tear production, eye redness, sneezing and rhinorrhoea. The skin test done by the injection of CJ extract produced a wheal in the monkeys. Monkey serum induced a skin allergic reaction to the extract on Prausniz–Küstner test. The presence of anti-CJ IgE in monkeys is assayable by the Pharmacia CAP system for human IgE.[7,8] The peripheral blood mononuclear cells (PBMC) from monkeys with the pollinosis showed CJ pollen allergens-specific histamine release.[9] Furthermore, the monkeys showed significant proliferation of PBMC in response to CJ pollen allergens.[9]

Two major allergens have been isolated from CJ pollen and characterized as Cry j 1 and Cry j 2.[10,11] It was reported that the five amino acid residues of the N-terminus of Cry j 1 were identical to those of the mountain cedar which grows in North America and belongs to the same order (Pines) as Japanese cedar.[12] Recently, c-DNA of Cry j 1 has been cloned and the Cry j 1 sequence shows some homology to the Amb a 1 and 2 family of genes for ragweed pollen allergens.[13,14] We found that humans[11,15] as well as monkeys[7,8] have specific IgE to Cry j 1 and Cry j 2.

Monoclonal antibodies (mAb) have been used to identify and characterize the allergenic epitopes of many allergens in pollen.[16–20] Recently, mAb specific to Cry j 1 have been established in some laboratories.[21–23] The B-cell-epitope of Cry j 1 was analysed with some mAb to Cry j 1.[22] An epitope on Cry j 1 recognized by a group of mAb was found to be an important determinant for allergic responses in human patients.[22]

In this study, we have examined the major epitopes on Cry j 1 that are recognized by IgE antibodies in the sera of humans and monkeys using an IgE enzyme-linked immunosorbent assay (ELISA) inhibition method with mAb.

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Abbreviations: BSA, bovine serum albumin; CJ, *Cryptomeria japonica*; ELISA, enzyme-linked immunosorbent assay; FU, fluorescence unit; mAb, monoclonal antibody; PBS, phosphate-buffered saline.

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Table 1. Classification of anti-Cry j 1 mAb by sandwich ELISA

Immobilized mAb	Biotinylated mAb as detector									
	EP-1			EP-2		EP-3		EP-4		EP-5
	B01	013	S14	S32	052	S36	S131	S46	S84	S95
B01	−*	−	−	+++	+++	+++	+++	+++	++	+++
013	−	−	−	+++	++	+++	+++	++	++	++
S14	−	−	−	+++	+++	+++	+++	++	++	+++
S32	+++	+++	+++	−	−	+++	+++	++	++	+++
052	+++	+++	+++	−	−	+++	+++	+++	++	+++
S36	+++	+++	+++	++	+++	−	−	+++	++	+++
S131	+++	+++	+++	+++	+++	−	−	+++	++	+++
S46	+++	+++	+++	+++	+++	+++	+++	−	−	+++
S84	++	+	++	++	++	+	+++	−	−	+
S95	+++	+++	+	+	+	+	+++	+	+	−

*Results are presented by absorbance at 492 nm: −, $<0{\cdot}5$; +, $0{\cdot}5 \leqslant OD_{492} < 1{\cdot}0$; ++, $1{\cdot}0 \leqslant OD_{492} < 2{\cdot}0$; +++, $\geqslant 2{\cdot}0$.

Table 2. Classification of anti-Cry j 1 mAb by the ELISA inhibition

Unlabelled* mAb	Biotinylated mAb									
	EP-1			EP-2		EP-3		EP-4		EP-5
	B01	013	S14	S32	052	S36	S131	S46	S84	S95
B01	+†	+	+	−	−	−	−	−	−	−
013	+	+	+	−	−	−	−	−	−	−
S14	+	+	+	−	−	−	−	−	−	−
S32	−	−	−	+	+	−	−	−	−	−
052	−	−	−	+	+	−	−	−	−	−
S36	−	−	−	−	−	+	+	−	−	−
S113	−	−	−	−	−	+	+	−	−	−
S46	−	−	−	−	−	−	−	+	+	−
S84	−	−	−	−	−	−	−	+	+	−
S95	−	−	−	−	−	−	−	−	−	+

*Inhibitor.
†+, $\geqslant 30\%$ inhibition.

MATERIALS AND METHODS

Antigen

Cry j 1 was purified as described previously.[9]

Monoclonal and polyclonal antibodies

Ten mAb against Cry j 1 tested were obtained from three laboratories: B01 from Dr Hiroshi Yasueda, National Sagamihara Hospital, Sagamihara;[23] 013, 052 from Dr Yoshifumi Taniguchi, Hayashibara Biochemical Laboratories, Okayama;[22] and kw-S14, S32, S36, S40, S84, S95 and S131 from Dr Takao Nagoya, Kowa Research Institute, Tsukuba.[21] Polyclonal anti-Cry j 1 IgG from rabbit immunized Cry j 1 was used.[10]

ELISA for the epitope specificity of mAb

The epitopes recognized by the mAb were grouped by two methods. One was the colorimetric sandwich ELISA. Briefly, anti-Cry j 1 mAb (2 μg/ml) were immobilized on a microplate, after which Cry j 1 (100 ng/ml) was added to the wells. After 1 hr of incubation at room temperature, biotinylated mAb (1 μg/ml) were added to the wells. Then, horseradish peroxidase labelled streptavidin (Sigma, St Louis, MO) was added to the wells. After 1 hr of incubation at room temperature, *o*-phenylenediamine dihydrochloride and hydrogen peroxide were added. After the enzyme reaction was stopped, absorbance was measured with a colorimetric microplate reader. The other method was the cross-inhibition ELISA test which is based on the ability of mAb to inhibit the binding of coexisting biotin-labelled mAb to solid-phase Cry j 1. Briefly, Cry j 1 (1 μg/ml) was adsorbed to a microplate. Biotin-labelled mAb (0·5 μg/ml) were reacted with the solid-phase antigen in the presence or absence of unlabelled mAb (20 μg/ml). The following procedures were the same as those in the colorimetric sandwich ELISA method mentioned above.

Sera

Human sera from 21 patients who have CJ pollinosis and anti-CJ IgE were used as a source of IgE antibody. Sera from 19 monkeys with CJ pollinosis and anti-CJ IgE from four open enclosure troops in zoos and provisioned free-ranging troops were used as a source of IgE antibody.[8]

ELISA inhibition test

To analyse whether the epitopes on the Cry j 1 recognized by mAb are also recognized by human and monkey IgE, a fluorometric ELISA inhibition[7,15] was performed. Cry j 1 (1 μg/ml) was adsorbed to microplate wells. Anti-Cry j 1 mAb (20 μg/ml) was added to the Cry j 1–coated wells at room temperature for 2 hr. Without washing, sera of patients with CJ pollinosis (1 : 10 diluted) were added and incubated for 1 hr at room temperature. The plates were washed and anti-human IgE antibody conjugated with β-D-galactosidase (Pharmacia, Uppsala, Sweden) diluted 1 : 30 was added to each well. As the enzyme reaction substrate, 0·2 mM 4-methylumbelliferyl-β-D-galactoside (Sigma) was added to the wells and the plates were incubated at 37° for 2 hr. After 0·1 M glycine–NaOH (pH 10·3) was added to stop the reaction, fluorescence units (FU) were measured on a fluorometric microplate reader (Fluoroskan; Flow Laboratories, McLean, VA).

The percentage of inhibition was calculated as follows.

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

Thermal treatment

The effect of heating on the antigenicity of epitopes on the Cry j 1 was evaluated by two methods. Cry j 1 (100 μg/ml) in phosphate-buffered saline (PBS) was incubated at 4° (control), 37° or 57° for 1 hr, or at 100° for 5 min. Cry j 1 or heat-treated Cry j 1 (0·5 μg/ml) was adsorbed to the microplate wells. Method one: biotin-labelled anti-Cry j 1 mAb reacted with the solid-phase antigen. The subsequent procedures were the same as the colorimetric sandwich ELISA mentioned above. Method two: IgE antibody from the patients' sera reacted with the solid-phase antigen for 3 hr at room temperature. The plates were washed and anti-human IgE antibody conjugated with β-D-galactosidase diluted 1 : 30 was added to each well. The following procedures were the same as those in the fluorometric ELISA test.

RESULTS

Epitope specificity of anti-Cry j 1 mAb

We obtained 10 mAb to Cry j 1 derived from three laboratories. These mAb were grouped by the epitopes on Cry j 1 using sandwich ELISA (Table 1). Five independent epitopes (EP-1 to EP-5) were identified on the Cry j 1 molecule. The grouping of the mAb mentioned above was confirmed also by cross-inhibition ELISA tests (Table 2).

Major allergenic epitopes on Cry j 1 identified by ELISA inhibition with each epitope-specific mAb

The possibility that the epitopes recognized by the mAb are allergenic was assessed by the ability of the mAb to inhibit

Table 3. Inhibition of human IgE binding to Cry j 1 by anti-Cry j 1 mAb*

Patient no.	mAb to: EP-1 B01	EP-1 013	EP-1 S14	EP-2 S32	EP-2 052	EP-3 S36	EP-3 S131	EP-4 S46	EP-4 S84	EP-5 S95	pAb†
1	49	45	47‡	25	23	55	51	12	12	33	98
2	58	54	49	5	2	63	49	2	0	34	98
3	42	38	37	11	14	46	32	4	0	38	87
4	33	32	36	10	9	38	36	4	0	37	87
5	29	32	46	0	0	47	37	0	0	45	99
6	56	53	47	17	18	67	23	24	15	73	98
7	40	38	67	14	14	73	64	14	9	40	98
8	47	47	47	6	10	52	34	15	5	4	96
9	26	38	36	6	12	42	31	5	0	9	98
10	46	32	33	5	3	49	51	0	0	8	99
11	48	46	51	20	19	35	33	10	7	18	98
12	41	35	34	0	2	51	49	0	0	13	98
13	43	39	43	1	16	6	17	22	12	45	96
14	61	59	54	10	3	8	10	10	2	33	97
15	30	39	39	7	16	0	0	0	0	38	95
16	52	53	43	0	13	7	1	7	0	3	95
17	54	50	58	14	9	7	18	0	1	14	93
18	52	47	58	4	10	19	8	0	0	19	95
19	78	72	73	0	0	0	0	0	0	6	98
20	44	48	42	4	3	14	12	1	0	15	95
21	51	52	41	0	0	0	0	0	0	10	97

*The values are expressed as percentage inhibition of the binding of IgE to Cry j 1 (FU) in the presence of each mAb.

†Rabbit polyclonal anti-Cry j 1 antibody.

‡≥30% inhibition underlined.

Table 4. Inhibition of monkey IgE binding to Cry j 1 by anti-Cry j 1 mAb*

Monkey troop‡ & no.	mAb to: EP-1 B01	EP-1 013	EP-1 S14	EP-2 S32	EP-2 052	EP-3 S36	EP-3 S131	EP-4 S46	EP-4 S84	EP-5 S95	pAb†
A-1	50	50	55§	20	12	45	32	14	14	51	96
A-2	44	45	39	0	2	9	7	1	0	44	95
A-3	29	33	35	21	9	10	2	10	5	42	98
B-1	2	13	0	18	20	17	26	67	54	52	90
B-2	2	3	0	9	8	24	23	49	39	39	87
B-3	17	16	8	24	21	24	17	50	36	35	88
B-4	19	18	13	19	22	18	14	48	33	33	96
B-5	2	11	0	20	13	21	15	56	39	34	92
C-1	14	24	10	19	23	38	31	48	35	39	97
C-2	10	8	4	8	0	34	47	73	48	54	88
C-3	4	13	0	23	20	25	25	50	38	35	95
C-4	0	15	0	10	12	12	14	40	29	30	90
D-1	48	55	50	10	16	38	50	19	3	53	97
D-2	51	37	34	11	0	31	24	15	0	31	99
D-3	41	38	21	12	18	1	10	10	0	48	96
D-4	55	35	35	0	0	0	0	0	0	32	97
D-5	42	50	34	5	10	0	0	2	0	35	98
D-6	0	0	0	31	36	27	36	69	54	55	86
D-7	0	0	0	10	2	4	8	36	28	34	90

*The values are expressed as percentage inhibition of the binding of IgE to Cry j 1 (FU) in the presence of each mAb.

†Rabbit polyclonal anti-Cry j 1 antibody.

‡Location of the troops. A, Miyazaki; B, Gunma; C, Kyoto; D, Hyogo prefectures.

§≥30% inhibition underlined.

the binding of the IgE antibodies in the patients' sera to Cry j 1 on the solid phase. In the preliminary experiments, a concentration of 20 μg/ml for each mAb was shown to be optimal for maximal inhibition of the binding of the IgE to Cry j 1 without affecting the binding of other epitope-specific mAb (data not shown). Table 3 shows that each mAb was capable of inhibiting the binding between Cry j 1 and IgE from the sera of 21 human patients. The mAb to EP-1 strongly blocked the binding of IgE antibodies in almost all patients' sera to Cry j 1. The mAb to EP-3 and EP-5 inhibited binding in some patients. The mAb to EP-2 and EP-4 did not strongly inhibit the binding. Rabbit polyclonal anti-Cry j 1 IgG strongly inhibited the binding of IgE to Cry j 1. These data suggest that the EP-1 on Cry j 1 is the predominant allergenic epitope.

In the monkeys, the inhibition patterns by the epitope-specific mAb varied among the four troops of monkeys (Table 4). In troop A, the mAb to EP-1, EP-3 and EP-5 inhibited the binding of IgE to Cry j 1. These patterns were similar to those for human patients. In troops B and C, the mAb to EP-1 did not inhibit the binding of IgE, but mAb to EP-4 and EP-5 blocked the binding, which was a different inhibition pattern from that of humans. In troop D, however, the inhibition pattern was heterogeneous among monkeys; it was similar to the human pattern in monkeys D-1 to D-5, but different in monkeys D-6 and D-7. In all troop, the mAb to EP-5 blocked the binding of monkey IgE to Cry j 1.

Effect of heat treatment of Cry j 1 on its binding activities to mAb and IgE antibodies

To characterize the structure of the epitopes on Cry j 1, we investigated the effect of heat treatment of Cry j 1 on binding of the epitope-specific mAb to Cry j 1 (Table 5). Binding between mAb and EP-1, -2, -3 and -5 was significantly decreased after heat treatment. However, after heating of Cry j 1 at 56° for 1 hr or at 100° for 5 min, mAb S46, which recognized EP-4, still bound to Cry j 1. These findings indicate that EP-1, -2, -3 and -5 are heat-labile, whereas EP-4 is heat-stable (Table 5).

Table 6 shows the reactivity of IgE antibodies with heated Cry j 1. The sera with IgE to EP-1 did not react with heated Cry j 1, whereas the sera with IgE to EP-4 and -5 still bound to heated Cry j 1. These findings agree with those in Table 5.

DISCUSSION

In humans, Cry j 1 is a major allergen of CJ pollen.[10,11] In a previous study, we found that more than 90% of 145 patients with IgE to crude CJ extract had IgE to purified Cry j 1.[15] A histamine-release assay with patients' leucocytes demonstrated that several ng/ml of Cry j 1 caused maximum histamine release.[15]

Naturally occurring pollinosis to CJ has also been detected in Japanese monkeys.[6] Previously, no IgE-mediated clinical

Table 5. Binding of anti-Cry j 1 mAb to heat-treated Cry j 1

	mAb to:				
Treatment	EP-1 S14	EP-2 S32	EP-3 S131	EP-4 S46	EP-5 S95
37° 1 hr	98(±1·0)*	101(±4·8)	96(±2·0)	105(±5·2)	103(±1·5)
56° 1 hr	17(±0·3)	9(±0·3)	10(±0·3)	136(±4·7)	32(±0·7)
100° 5 min	0	0	0	80(±2·5)	13(±1·2)

*The values are expressed as percentage of the binding of mAb to heat-treated Cry j 1 (absorbance) compared with that to untreated Cry j 1. The values are mean (±SD).

Table 6. Binding of human and monkey IgE antibodies to heat-treated Cry j 1

	Sera with IgE to EP-1			Sera with IgE to EP-4, -5		
Treatment	19	20	21	B-2	B-3	B-4
37° 1 hr	99(±1·7)*	98(±1·1)	99(±3·1)	98(±5·1)	99(±1·2)	102(±2·0)
56° 1 hr	55(±0·8)	69(±2·1)	69(±2·4)	94(±3·1)	67(±0·6)	78(±1·2)
100° 5 min	0	0	0	50(±0·9)	29(±2·0)	34(±0·3)

*The values are expressed as percentage of the binding of IgE to heat–treated Cry j 1 (FU) compared with that to untreated Cry j 1. The values are mean (±SD).

disease, including pollinosis, had been described in non-human primates, although its existence had been surmised. We evaluated the prevalence of IgE antibodies to CJ allergens in sera of 276 Japanese monkeys from nine monkey troops without regard to pollinosis symptoms. It was found that 45 monkeys (16%) had CJ-specific IgE.[8] Moreover, all 45 monkeys had specific IgE to at least one of the two major allergens, Cry j 1 and Cry j 2, and 23 monkeys had IgE to the both allergens. These findings suggest that, as in human patients, Cry j 1 is an important allergen in monkeys.

Using mAb specific to Cry j 1, we identified five independent epitopes (EP-1 to EP-5) on the Cry j 1 molecule (Table 1). EP-1, -2, -3 and -5 are heat-labile, whereas EP-4 is heat-stable. Changes in the three-dimensional configuration induced by heat denaturing (100°, 5 min) of the allergen may result in the complete loss of antigenicity by the conformational epitopes. Most of the epitopes on Cry j 1 recognized by mAb may be conformational.

We then investigated which epitopes are the most immunodominant for inducing IgE antibodies in human and monkeys. The reactivity of IgE antibody to each epitope was assessed by the ability of the respective mAb to inhibit the binding of IgE to Cry j 1. In the human patients, the binding of IgE in all the sera tested (21 patients) was inhibited by mAb to EP-1. We assume that EP-1 is the most important allergenic epitope present.

In the monkeys with the pollinosis, although the mAb to EP-5 blocked the binding of IgE in all the sera tested to Cry j 1, the major epitopes varied among the troops of monkeys. It was speculated that the Japanese monkey originated from the rhesus monkey (*Macaca mulatta*) that lived on the Asian continent 400 000 to 500 000 years ago.[24] At present, Japanese monkeys are distributed over all the islands of Japan except Okinawa and Hokkaido islands. The total monkey population is estimated by census as 20 000–70 000, and is comprised of several hundred troops.[25] Analysis of the correlation between the geographic and genetic distances in these troops showed that the gene constitutions of two troops living more than 100 km apart on the island can be regarded as independent. The four troops in our study live more than 100 km apart.[24] We speculate that genetic variability in the individual troops may lead to the variable IgE responses to the different epitopes. In the future, the relationship between genetic background and the IgE response in monkeys should be investigated.

Finally, monkeys with CJ pollinosis had symptoms similar to those of human patients. Some, but not all, monkeys had IgE antibody responses to the major allergen of CJ pollen that were similar to those seen in human patients. We believe that monkeys with pollinosis can serve as a suitable animal model for developing such anti-allergy therapies as desensitization treatments.

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