ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2008) 26: 97-104

A Correlation between Symptom Severity and Unbalanced Reactive IgA Production in Japanese Cedar Pollinosis Patients

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SUMMARY To better understand the unbalanced immunoglobulin production that occurs in pollinosis, we measured the levels of IgG, IgA, and IgE reactive to either Japanese cedar pollen, Cry j 1 protein, or Cry j 2 protein in the sera of pollinosis patients. As expected, the levels of these immunoglobulins (Igs) reactive to the three antigens were significantly higher in the patients than in the controls, and the RAST scores correlated with the levels of these Igs. Only the levels of IgA reactive to the Cry j 2 protein and IgG reactive to the Japanese cedar pollen antigen did not correlate with the RAST scores. We classified the patients into mild and severe, based on the severity of their allergic symptoms, and compared their levels of Igs. As expected, the levels of IgE reactive to Japanese cedar pollen and Cry j 1 of the severe group were significantly higher than those of the mild group. It is of note that the ratio of anti-Cry j 1 IgE to anti-Japanese cedar pollen IgA was significantly higher in the patients with severe symptoms suggesting that decreased IgA production could be responsible for the severity of pollinosis.

The Japanese cedar (*Cryptomeria japonica*) is spread over most areas of Japan and causes pollinosis, a type 1 allergy, in early spring. More than 10% of the population suffers from pollinosis caused by exposure to the pollen.[1] Two major allergenic proteins of the Japanese cedar pollen are Cry j 1, which exists on the pollen surface, and Cry j 2, which is located within the pollen.[2-4] T cell responses to these allergens have been reported.[5,6] Cry j 1 is one of the major IgE-binding allergens in Japanese cedar pollen,[1] and several antigenic epitopes recognized by IgE or T cells have been identified pursuant to the development of a specific immunotherapy for pollinosis patients allergic to Cry j 1.[7-10] However, neither the levels of allergen-reactive IgG and IgA responses nor their roles in pollinosis patients have been fully investigated, although unbalanced Ig production has been suggested to be responsible for type 1 allergy.[11] Consequently, in this study we measured the levels of IgG, IgA, and IgE reactive to either Japanese cedar pollen, Cry j 1 or Cry j 2 protein in the sera of pollinosis patients to better understand

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unbalanced Ig production in allergy.

PATIENTS AND METHODS

Patients and sample collection

One hundred and twenty-three patients with pollinosis and 83 healthy donors (HDs) without allergic disease were recruited from Kurume University and the Nippon Medical University Hospital. Informed consent was obtained from all subjects. Their profiles are shown in Table 1. The RAST score, a standard marker reflecting serum IgE levels in response to Japanese cedar pollen crude extract, was measured in patients with pollinosis (n = 123), but not in the HDs. Sera were obtained from 2004 to 2005, and were frozen at -80°C until use. The diagnosis of pollinosis was based on clinical symptoms, and the RAST test scores were used as index of the IgE response specific to extracts of Japanese cedar pollen.

Measurement of Igs reactive to pollinosis antigens

The levels of Igs specific to Japanese cedar pollen antigen (HBL-SBP-1; Hayashibara Biochemical Laboratories Inc., Okayama, Japan), Cry j 1 protein (HBL-C-1; Hayashibara Biochemical Laboratories Inc.), and Cry j 2 protein (HBL-C-1; Hayashibara Biochemical Laboratories Inc.) were measured by means of a multiplex bead suspension array using the Luminex® system as reported previously.[12] In brief, 100 µl of diluted plasma were incubated with xMAP beads (Luminex Corp., Austin, TX), which were coated with Japanese cedar pollen, Cry j 1, or Cry j 2, in a 96-well filter plate (MABVN1250; Millipore Corp., Bedford, MA), at 26°C for 2 hours on a plate shaker. Two hours later, the plate was washed with PBST and incubated with 100 µl of biotin-conjugated goat anti-human IgG (BA-3080; Vector Laboratories, Burlingame, CA), biotin-conjugated goat anti-human IgA or biotin-conjugated goat anti-human IgE (AHI0509; Tago, Camarillo, CA) at 26°C for 1 hour on a plate shaker. After washing, 100 µl of streptavidin-PE were added to the wells, and the plate was incubated at 26°C for 30 minutes on a plate shaker. The bounded beads were washed three times followed by the addition of 100 µl PBS to each well. Fifty microliters of each sample were examined using the Luminex® system. The antibody levels were expressed through the fluorescence intensity, and the values were given in fluorescence intensity units (FIU) as reported previously.[12] The cutoff level was set at 10 FIU because the linear curve of FIU obtained from the sample dilution assays extended from 5 to 10,000 FIU (data not shown).

Statistical analysis

A two-tailed Student's *t* test was used for the

Table 1 Subject profiles

Factor	Healthy donors		Patients with pollinosis	
	All	Age-matched	All	Age-matched
Number	83	32	123	48
Age	24 ± 6	26 ± 6	40 ± 13	31 ± 10
Gender (male/female)	55/28	21/11	59/64	24/24
Moderate			69	30
Severe			54	18
RAST test				
0			4	4
1			2	2
2			12	6
3			60	22
4			28	9
5			7	3
6			3	2

analysis of the significance of the antigen-reactive Ig levels. The correlation between allergen-reactive antibodies was analyzed by Pearson's correlation coefficient test. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Subject profiles

Table 1 shows the subject profiles. Eighty-three HDs and 123 pollinosis patients were enrolled in this study. The ratio of males to females was higher in the control group. However, there was no difference in the levels of allergen-reactive Igs between healthy males and females (data not shown). On the other hand, there was a significant difference in age between the patients and controls. Therefore, analyses on 48 age-matched pollinosis patients and 32 age-matched HDs were also carried out, and the results for these groups (the full groups and the age-matched groups) are shown in parallel for the following experiments.

Increased allergen-reactive Ig levels in pollinosis patients

First we used a serial dilution of samples to determine whether or not the levels of Igs reactive to Japanese cedar pollen, Cry j 1, or Cry j 2 in the sera of pollinosis patients were dose-dependent. The results showed that these levels in pollinosis patients gradually decreased with the serial dilution of samples (data not shown). Although their levels were relatively low, the antibody titers in the HDs also decreased in a dilution-dependent manner. Based on these results, 100-fold diluted sera were used for the assays in the following experiments.

We then measured the levels of Igs reactive to Japanese cedar pollen in the sera of pollinosis patients and HDs (Table 2). The levels of IgG, IgA, and IgE reactive to Japanese cedar pollen in the sera of the pollinosis patients were significantly higher than in the HDs in both the age-matched as well as the total groups. Similar results were observed with the levels of IgG, IgA, and IgE reactive to the Cry j 1 and Cry j 2 proteins. Although an increase in the levels of IgE reactive to pollinosis-related antigens is well recognized, these results indicate that allergen-reactive IgG and IgA also increase in pollinosis patients. In contrast, no elevation of Igs reactive to the pollen of hinoki, a Japanese cypress, was observed in the sera of the 123 pollinosis patients compared to the 83 HDs, and antibody levels in males and females were not significantly different either (data not shown).

Correlation between the RAST score and the levels of allergen-reactive Igs

We next determined whether or not there was any correlation between the RAST score, a standard marker reflecting serum IgE levels in response to Japanese cedar pollen crude extract, and the levels of Igs reactive to either Japanese cedar pollen, Cry j 1, or Cry j 2 (Fig. 1) in patients with pollinosis (n = 123). A significant correlation was observed between the RAST score and IgG reactive to either Cry j 1 or Cry j 2 (Fig. 1B and 1C), but no significant

Table 2 Levels of Japanese cedar pollen, Cry j 1, and Cry j 2 antibodies in pollinosis patients and HDs

Antigen	SBP		Cry j 1		Cry j 2	
Age	Matched	Non-matched	Matched	Non-matched	Matched	Non-matched
IgG HDs	452 ± 543	394 ± 592	568 ± 1031	695 ± 1441	0 ± 284	0 ± 499
IgG Pts	949 ± 1316	946 ± 1215	1,737 ± 1,427	1,767 ± 1,528	354 ± 627	411 ± 811
IgA HDs	17 ± 17	20 ± 20	19 ± 23	21 ± 26	0 ± 7	0 ± 9
IgA Pts	45 ± 60	46 ± 55	43 ± 58	66 ± 122	5 ± 21	12 ± 51
IgE HDs	17 ± 43	14 ± 34	55 ± 117	54 ± 113	14 ± 30	9 ± 23
IgE Pts	107 ± 185	75 ± 142	266 ± 504	228 ± 393	65 ± 93	61 ± 106

correlation was observed in the case of IgG reactive to Japanese cedar-pollen (Fig. 1A). In regard to IgA, a significant correlation was also observed between the RAST score and IgA reactive to either Japanese cedar pollen or Cry j 1 (Fig. 1D and 1E), but no significant correlation was observed in the case of IgA reactive to Cry j 2 (Fig. 1F). On the other hand, the RAST score showed a positive correlation with the levels of IgE reactive to all of the three antigens (Fig. 1G, 1H, and 1I).

Levels of reactive IgE in patients with severe symptoms

We next compared the levels of allergen-reactive Igs of pollinosis patients with mild symptoms to those with severe symptoms to better understand the immunological balance in this disease (Ta-

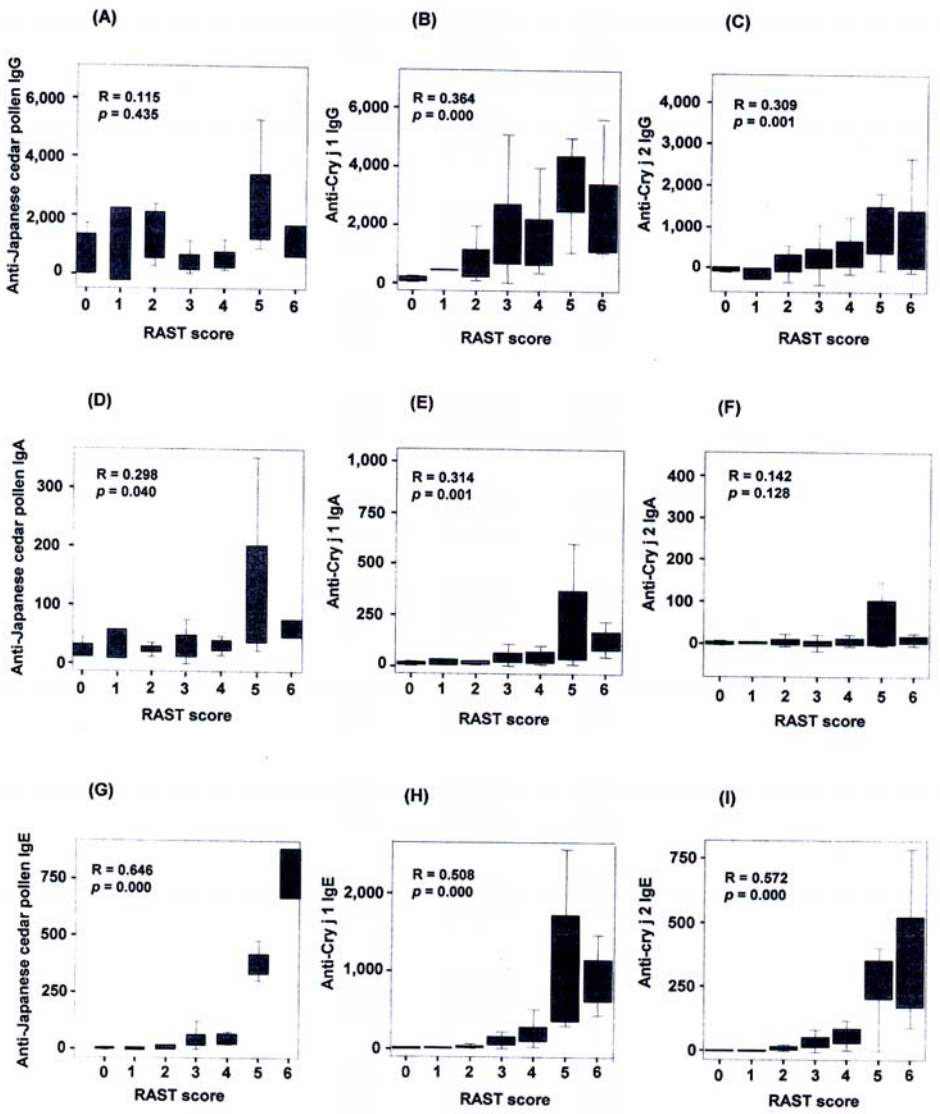

Fig. 1 Correlation between allergen-reactive Igs and the RAST score of pollinosis patients. Values represent the FIU of the antigen-specific Igs. The diluted samples (x100) were measured for the levels of anti-Japanese cedar pollen (A, D, and G), anti-Cry j 1 (B, E, and H), and anti-Cry j 2 (C, F, and I) antibodies. Student's *t* test was used for statistical analysis among the RAST scores, and R indicates Pearson's correlation coefficient.

ble 3). Those of the HDs served as control. No significant difference in the levels of either IgG or IgA reactive to any of the three antigens was observed between the patients with mild and those with severe symptoms. In contrast the levels of IgE reactive to Japanese cedar pollen and Cry j 1, but not to Cryj 2, were significantly higher in patients with severe symptoms than in those with mild symptoms.

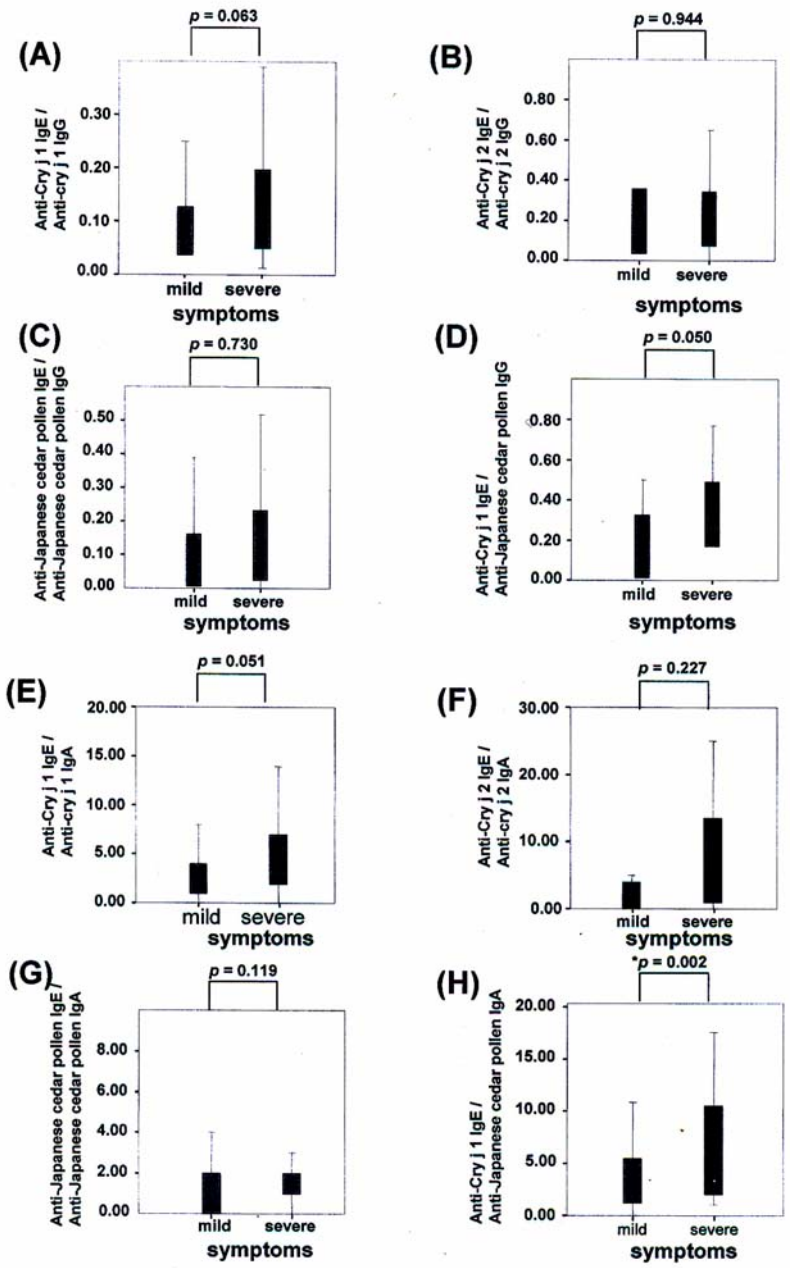

Fig. 2 Ratios of allergen-reactive IgE/IgG or IgE/IgA in pollinosis patients with mild or severe symptoms**.** Age-matched samples were provided (21 patients with mild symptoms, 15 patients with severe symptoms, and 26 HDs). The diluted samples (x100) were measured for their antibody levels and the IgE/IgG (A, B, C, and D) and IgE/IgA (E, F, G, and H) indices were calculated. Student's *t* test was used for statistical analysis.

The mean levels of IgG and IgA reactive to either Japanese cedar pollen or Cry j 1 were approximately 1.3 times higher in the sera of patients with severe symptoms than in those with mild symptoms. In contrast, the mean levels of IgE reactive to either Japanese cedar pollen or Cry j 1 were approximately 3 times higher in the sera of patients with severe symptoms than in those with mild symptoms.

In summary, although all three types of Igs increased in pollinosis patients (Table 2), the increase was more prominent in IgE than in IgG or IgA, especially for IgE reactive to either Japanese cedar pollen or Cry j 1.

A new marker representing the IgE/IgA balance

Besides measuring allergen-reactive IgE and

Table 3 Antibody levels in healthy donors and patients with pollinosis

Antigen	Ig	Healthy donors	Patients with pollinosis	
			Mild symptoms	Severe symptoms
Japanese cedar pollen	IgG	394 ± 592	824 ± 796	1100 ± 1593
	IgA	20 ± 20	40 ± 46	54 ± 64
	IgE	14 ± 34	40 ± 59	121 ± 195
Cry j 1	IgG	695 ± 1441	1639 ± 1511	1930 ± 1549
	IgA	21 ± 26	58 ± 95	77 ± 151
	IgE	54 ± 113	128 ± 188	355 ± 530
Cry j 2	IgG	0 ± 499	445 ± 968	367 ± 556
	IgA	0 ± 9	6 ± 26	18 ± 70
	IgE	9 ± 23	44 ± 71	82 ± 137

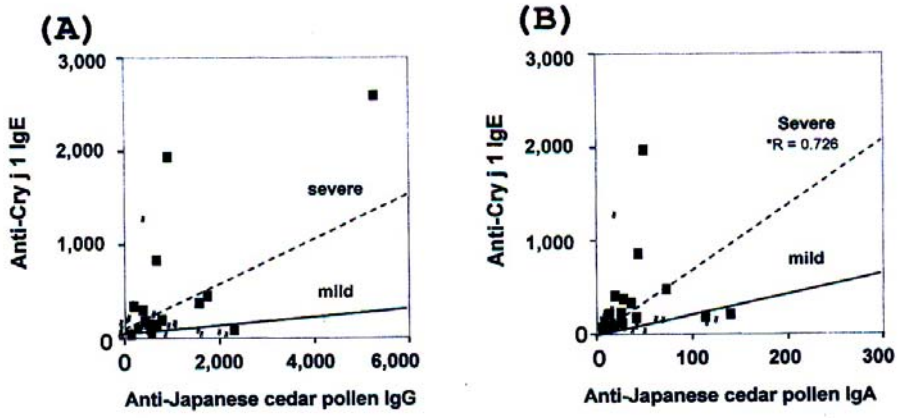

Fig. 3 Relationship of allergen-reactive IgE *vs* IgG and IgE *vs* IgA in pollinosis patients with mild or severe symptoms. The same samples as in Fig. 2 were used in this figure. Values represent the FIU of the antigen-specific antibodies. A, the relationship of IgG to IgE in sera from patients with mild or severe symptoms. B, the relationship of IgA to IgE in sera from patients with mild or severe symptoms. Open circles indicate the data for patients with mild symptoms, and closed squares the data for those with severe symptoms. R indicates Pearson's correlation coefficient. *$p < 0.050$.

the RAST score, we examined the immunological balance of allergen-reactive IgE, IgG, and IgA in pollinosis patients in relation to the severity of their symptoms in age-matched samples. Therefore, we had to establish a new marker that could represent not only IgE but also IgG or IgA. Among 8 combinations examined, only the ratio of anti-Cry j 1 IgE/anti-Japanese cedar pollen IgA was significantly different between patients with mild symptoms (n=21) and those with severe symptoms (n = 15) (p = 0.002, Fig. 1H). The median ratio in patients with mild and severe symptoms was 2.0 and 6.3, respectively. That of the negative control, HDs (n = 26), was zero. The mean ratio ± standard deviation in patients with mild and severe symptoms was 3.5 ± 3.7 and 7.0 ± 5.5, respectively, when the highest and the lowest value in each group were not counted. That of the HDs was 1.6 ± 2.5.

In contrast, none of the other combinations showed significant levels of difference (Fig. 2A-2G), though the ratios of anti-Cry j 1 IgE/anti-Japanese cedar pollen IgG (p = 0.050, Fig. 2D) and anti-Cry j 1 IgE/anti-Cryj 1 IgA (p = 0.051, Fig. 2E) were slightly different between patients with mild symptoms and those with severe symptoms.

We further investigated the correlation between anti-Cry j 1 IgE and anti-Japanese cedar pollen IgG or IgA in the age-matched samples (Fig. 3), and found that the balance between Cry j 1-reactive IgE and Japanese cedar pollen-reactive IgA preferentially shifted to Cry j 1-reactive IgE in pollinosis patients with severe symptoms (Fig. 3B), but such a shift was not observed in patients with mild symptoms (Fig. 3B). A similar shift was observed to some extent between Cry j 1-reactive IgE and Japanese cedar pollen-reactive IgG in pollinosis patients with severe symptoms (Fig. 3A), but not in patients with mild symptoms (Fig. 3A).

DISCUSSION

Studies on the disease severity of pollinosis have generally focused on allergen-specific IgE as a biomarker, whereas there is a large number of reports suggesting that allergen-specific IgG or IgA should also be considered to better understand the immunological mechanisms involved in the disease severity. Therefore in this study we determined the levels of not only IgE but also IgG and IgA reactive to either Japanese cedar pollen, Cry j 1 protein, or Cry j 2 protein and found that they were significantly elevated in pollinosis patients compared to the controls. To our knowledge, there has been no study of either IgG or IgA levels in patients allergic to Japanese cedar pollen or Cry j proteins, which are major allergens of Japanese cedar pollen.

This study used the sera from pollinosis patients to measure the levels of allergen-specific IgA, although ideally the levels of allergen-specific IgA would have been determined using nasal fluids from pollinosis patients, but such samples were not available.

In an attempt to determine the most practical and indicative ratio as a new biomarker, we found that the ratio of anti-Cry j 1 IgE to anti-Japanese cedar pollen IgA was significantly higher in patients with severe symptoms than in those with mild symptoms.

IgA represents the most prominent class of Igs in mucosal secretions.[14] Allergen-specific IgA in mucosal secretions might have an influence on local allergic manifestations and perhaps protect against allergy.[15] Therefore, several attempts have been made to induce protective allergen-specific IgA responses by oral immunotherapy as specific immunotherapy represents a curative approach towards type I allergy.[13] Some studies have reported that such immunotherapies were clinically effective,[16,17] whereas another found them ineffective.[18] Our study suggested that decreased IgA production could be responsible for the severity of pollinosis. Therefore, measurement of serum levels of IgA and IgE reactive to pollen antigens could be useful to evaluate the efficacy of specific immunotherapy in individual patients.

ACKNOWLEDGEMENTS

The study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (No. 18689025 to N. Komatsu).

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