

Non-IgE,-IgG4 Antibody to Japanese Cedar Pollen Allergens: Comparison of Its Prevalence and Titters between Pollinosis Patients and Non-Patients

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ABSTRACT

Background: IgG antibody to allergens in the serum of pollinosis patients is not routinely measured, because there are no simple methods for assaying small amounts of specific IgG in the serum ; so far only IgE and IgG4 antibodies have been assayed. In this study, we used a reverse-sandwich ELISA for measuring specific non-IgE,-IgG4 (probably, mainly IgG1) to Japanese cedar pollen allergens, and compared the antibody-positive rates and geometric mean antibody titers between pollinosis patients and non-patients (healthy individuals).

Methods: Antibodies to two major allergens of Japanese cedar, Cry j 1 and Cry j 2, were assayed by the following methods : specific non-IgE,-IgG4 was measured by a reverse-sandwich ELISA ; specific IgE and IgG4 were measured by indirect ELISAs.

Results: We detected specific non-IgE,-IgG4 in both the patients and non-patients. In comparison to the IgE antibody, which was detected in a small proportion of the non-patients with low titers, the non-IgE,-IgG4 antibody was present in a higher proportion with higher titers among both the patients and non-patients.

Conclusions: Healthy individuals had specific non-IgE,-IgG4 to Japanese cedar allergens in a higher proportion than the specific IgE. The non-IgE,-IgG4 antibody assay may be useful in studies on the prevalence of allergen-specific antibody responders and may help in clarifying the natural history of pollinosis.

KEY WORDS

IgG4 antibody in non-patients, Japanese cedar pollinosis, non-IgE, reverse-sandwich ELISA

INTRODUCTION

In contrast to IgE antibody, IgG antibody is not usually measured in the serum of pollinosis patients, because its detection has little diagnostic value and there are no simple methods for assaying small amounts of specific IgG in the serum. Furthermore, it has not been assayed in the serum of healthy individuals (non-patients).

In this study, we assayed specific IgE and IgG4 as well as non-IgE,-IgG4 in the serum of two groups, Japanese cedar pollinosis patients and non-patients. For the non-IgE,-IgG4 antibody assay, we used a

reverse-sandwich ELISA which detects small amounts of high-affinity divalent antibody without nonspecific background reactions.^{1,2} For a functionally monovalent IgG4 antibody,³⁻⁵ we used a conventional indirect ELISA. Then we compared the two groups in terms of the prevalence and geometric mean titers of the IgE, IgG4 and non-IgE,-IgG4 antibodies. Furthermore, we compared the allele frequencies in several single nucleotide polymorphisms (SNPs) which are reportedly related to atopy or asthma.

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Table 1 Anti-Cry j 1 antibody titers of a purified IgG4 fraction

	Total IgG4 ($\mu\text{g/ml}$)	Antibody Titers determined by	
		Indirect IgG4 ELISA	Reverse-Sandwich ELISA
Original Serum	313	1537	153
Purified IgG4	6.3	25	< 1

METHODS

SUBJECTS

Medical students and workers at the Jikei University Hospital in Tokyo voluntarily participated with informed consent; this study was approved by the Ethics Committee for Biomedical Research at the Jikei University School of Medicine. There were 82 Japanese cedar pollinosis patients (age 28.7 ± 8.5 years) who were diagnosed pollinosis using specific IgE antibody, and 57 non-patients (age 29.6 ± 10.6 years) who considered themselves as having no pollinosis symptoms and never attended an allergy clinic. Patients receiving hyposensitization therapy to Japanese cedar pollen were excluded from this study. Blood samples were collected after the cedar pollinosis season of February to April in 2000 and 2001. Sera and white blood cells were separately stored at -70°C .

ANTIBODY TITRATION

The two major Japanese cedar (*Cryptomeria japonica*) pollen allergens, Cry j 1 and Cry j 2, were purified as previously described,^{6,7} and used as antigen in the following antibody assays.

Specific non-IgE-IgG4 : Reverse-sandwich ELISA^{1,2} was used. In this method, one combining site of an antibody molecule attaches to the antigen on the solid phase and, after washing, another site catches the biotin-conjugated antigen added in the liquid phase, then streptavidin-conjugated β -galactosidase (Rosche) is reacted with resulting biotin-antigen-antibody complex. Since divalent IgE antibody is reactive in this assay, it is removed prior to testing; that is, for absorption of IgE, sera are mixed with Sepharose 4B gel (Pharmacia) conjugated with anti-human IgE (Biosource International, USA). In this test, two serum dilutions of 1 : 5 and 1 : 25 were employed. 4-methylumbelliferyl- β -D-galactoside (4MUG) was used as the enzyme reaction substrate, and the fluorescence intensity of the reaction product, 4MU, was measured on a Fluoroskan II reader (Flow Laboratories, USA). Antibody concentrations of test sera were determined from an antibody titration curve made with a reference serum using arbitrary antibody units.

Specific IgG4 : Conventional indirect ELISA was used. Two serum dilutions of 1 : 10 and 1 : 100 were

employed. Peroxidase-conjugated anti-human IgG4 mouse monoclonal antibody (Yamasa, Japan) and orthophenylenediamine- H_2O_2 were used.

Total IgG4 : Sandwich ELISA was used. Unconjugated anti-human IgG4 mouse monoclonal antibody (Yamasa) was immobilized on the solid phase. An IgG4 preparation with a known concentration derived from human myeloma plasma (Athens Research and Technology, Inc, USA) was used as a standard.

Specific IgE : Fluorometric indirect ELISA,¹ β -galactosidase-conjugated anti-human IgE (Pharmacia), and 4MUG were used.

Total IgE : Fluorometric sandwich ELISA¹ was used. Anti-human IgE monoclonal antibody (Chemicon International, USA) was immobilized on the solid phase. Human sera with known concentrations of IgE (Pharmacia RIST kit) were used as a standard. β -galactosidase-conjugated anti-human IgE and 4MUG were also used.

PURIFICATION OF IgG4

Streptavidin-conjugated agarose (Oncogene Science, USA) was mixed with biotinylated anti-IgG4 monoclonal antibody (Yamasa), followed by mixing with a serum with high IgG4 antibody titers. After washing the gel, an IgG4 fraction was eluted at pH 3. This fraction was used to determine IgG4 antibody reactivity in the reverse-sandwich ELISA.

DETERMINATION OF ALLELES IN SINGLE NUCLEOTIDE POLYMORPHISMS

SNP alleles in three genes which were reported to be related to asthma or atopy were determined in each subject. Of the 82 patients only 59 samples were acceptable for the DNA test. The alleles of interleukine (IL)-4 promoter C-590T were determined according to the methods described by Noguchi *et al.*,⁸ and those of IL-4 receptor α chain Ile50Val according to the methods described by Mitsuyasu *et al.*⁹ The alleles of IL-13 Gln110Arg (Heinzmann *et al.*)¹⁰ were determined by sequencing of the polymerase chain reaction (PCR) products. Primers for PCR which we designed were: 5'-CGTGAGGACTGAATGAGACAGTCC-3' (forward) and 5'-GGTCGGCTAGGCTGAAGACG-3' (reverse).

Non-IgE & IgG4 Antibody to Pollen Allergens

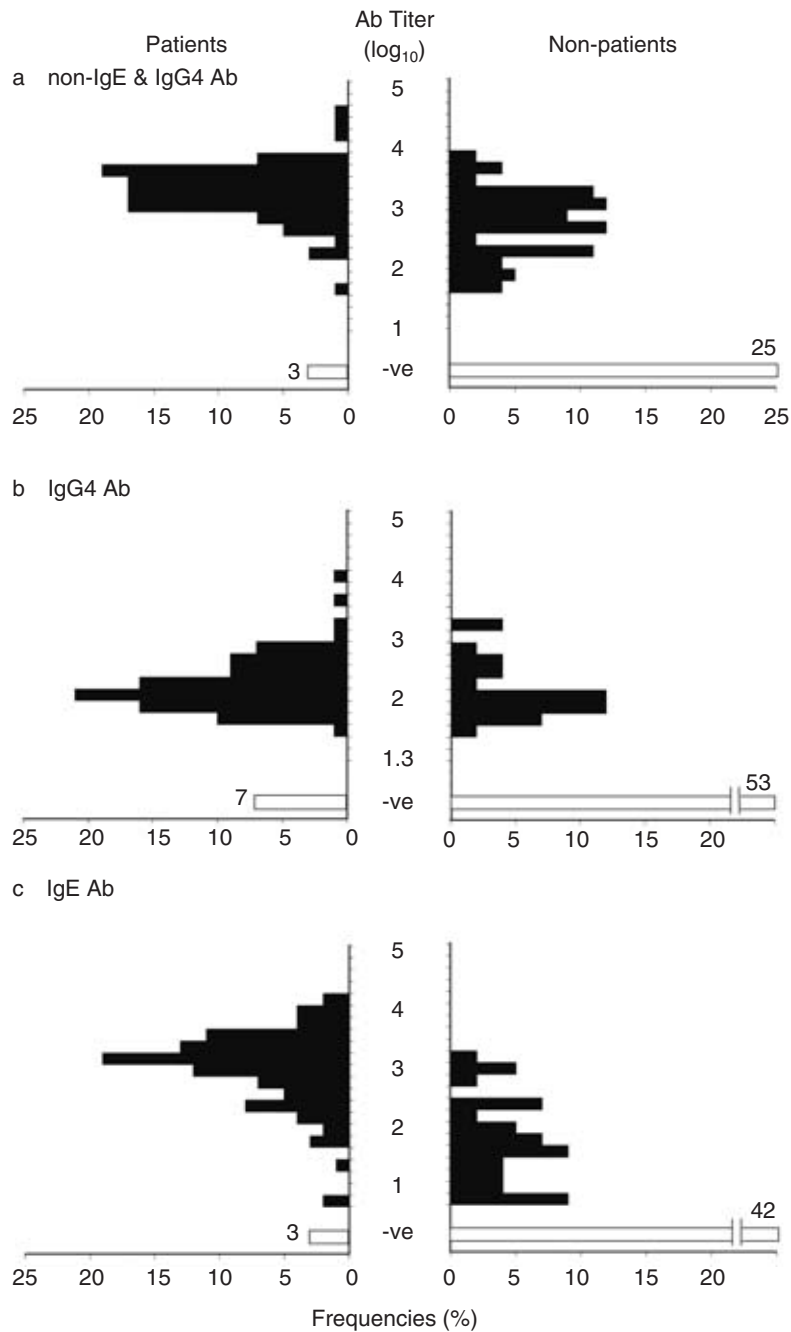


Fig. 1 Frequency distribution of antibody titers in different immunoglobulins among both patients and non-patients. Different kinds of anti-Cry j 1 antibodies in sera from both the pollinosis patients and non-patients were titrated. Frequencies at different antibody titers were shown. — ve, antibody negative.

STATISTIAL METHODS

Student's *t*-test was employed for differences in geometric mean antibody titers. χ^2 -test and Fisher's exact probability test were used for differences in antibody prevalence. χ^2 -test was used for differences in allele frequencies.

RESULTS

NON-REACTIVITY OF IgG4 IN THE REVERSE-SANDWICH ELISA

Schuurman *et al.*⁵ reported that IgG4 antibody is bispecific, that is, it binds to two different epitopes ; this means that the reverse-sandwich ELISA is unable to

Table 2 Prevalence and geometric mean titers of anti-Cry j 1 antibodies belonging to different immunoglobulin classes among pollinosis patients and non-patients

Antibodies		Patients (A)	Non-Patients (B)	A/B	P
non-IgE & IgG4	Prevalence	97%	75%		< 0.0001#
	Titer	1396	376	3.7	< 0.001
IgG4	Prevalence	92%	47%		< 0.0001 *
	Titer	118	80	1.5	N.S **
IgE	Prevalence	97%	58%		< 0.0001#
	Titer	855	51	16.8	< 0.001
Total IgE Conc. (IU/ml)		136	44		< 0.001

Fisher's exact probability test, * Chi-square test, ** N.S., non-significant

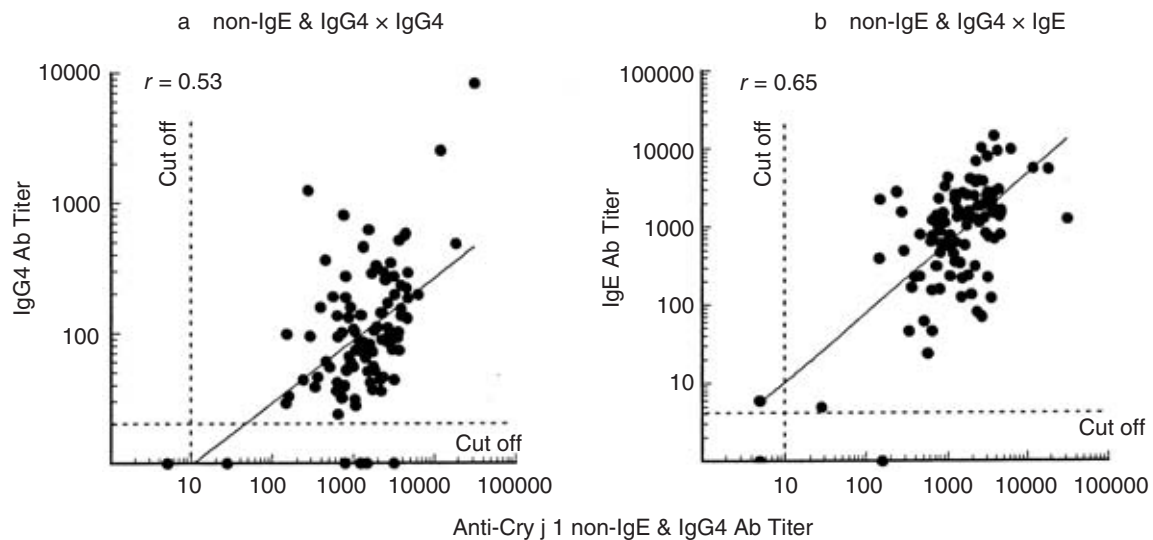


Fig. 2 Correlation between antibody titers in different immunoglobulins. Anti-Cry j 1 titers of each pollinosis patient in different immunoglobulin classes were plotted in the graphs.

detect IgG4 antibody. To confirm their results, we performed the following experiment. We determined anti-Cry j 1 antibody titers in both serum and IgG4 fraction. As shown in Table 1, the antibody activity in the IgG4 fraction was detected by the indirect IgG4 ELISA method, but not by the reverse-sandwich ELISA method. Further, the ratio of the IgG4 antibody titers in the original serum versus the titers of the purified fraction, 61 (1537/25), was comparable to the ratio of total IgG4 concentrations, 50 (313/6.3). Therefore, we concluded that the reverse-sandwich ELISA is not capable to detect IgG4 antibody, and the antibody activities determined by this ELISA belong to IgG1, 2, 3 plus IgA (IgE is absorbed prior testing). Hereafter, we will refer to the antibody detected by the reverse-sandwich ELISA as non-IgE,-IgG4 antibody.

COMPARISON OF THE PREVALENCE AND GEOMETRIC MEAN TITERS OF ANTI-CRY J 1 ANTIBODIES BETWEEN POLLINOSIS-PATIENT AND NON-PATIENT GROUPS

Figure 1 shows the frequency distribution of anti-Cry j 1 titers in different immunoglobulin classes in the two groups (pollinosis patients and non-patients). Table 2 shows antibody-positive rates in the two groups in respective immunoglobulins and geometric mean titers of antibody-positive sera. Among the pollinosis patients, the prevalence of Cry j 1 antibody was more than 90% in all immunoglobulin classes. Among the non-patients, the prevalence of non-IgE,-IgG4 (75%) was higher than that of IgG4 and IgE (47% and 51%, respectively). The mean antibody titers among non-patients were closer to those among patients in non-IgE,-IgG4 antibody than in IgE antibody.

Figure 2 shows the correlation between pollinosis individuals' antibody titers in different immunoglobu-

Table 3 Prevalence and geometric mean titers of anti-Cry j 2 antibodies belonging to different immunoglobulin classes among pollinosis patients and non-patients

Antibodies		Patients (A)	Non-Patients (B)	A/B	P
non-IgE & IgG4	Prevalence	84%	49%		< 0.0001 *
	Titer	262	77	3.4	< 0.01
IgG4	Prevalence	98%	48%		< 0.0001 *
	Titer	194	54	3.6	< 0.01
IgE	Prevalence	90%	32%		< 0.0001#
	Titer	963	75	12.8	< 0.001

* Chi-square test, # Fisher's exact probability test

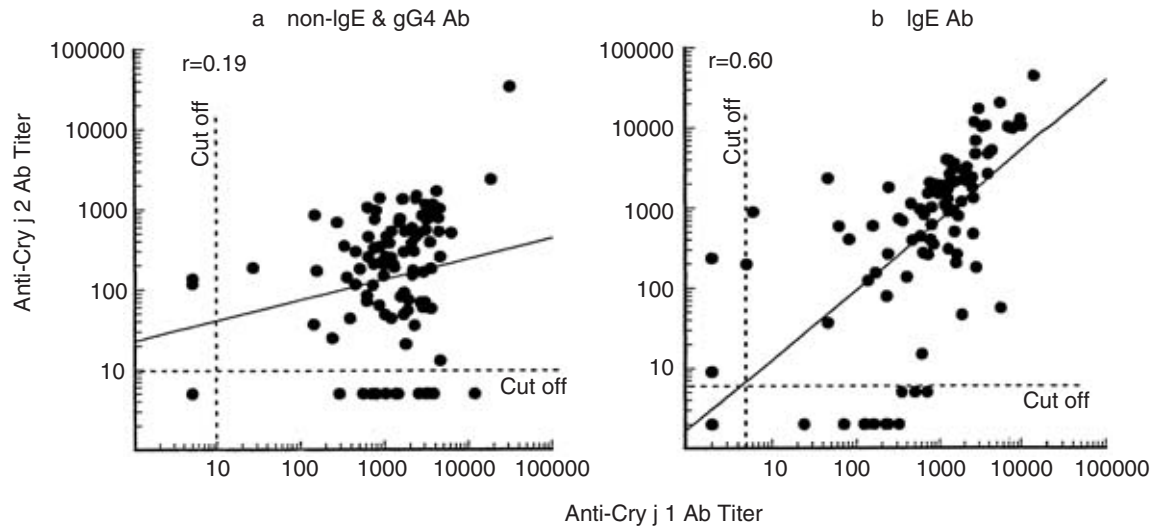


Fig. 3 Correlation between anti-Cry j 1 and anti-Cry j 2 titers. Anti-Cry j 1 and anti-Cry j 2 titers of each pollinosis patient were plotted in the graphs.

lins. Correlation between non-IgE,-IgG4 and IgG4 titers is shown in Figure 2a ($r = 0.53$), and correlation between non-IgE,-IgG4 and IgE titers is shown in Figure 2b ($r = 0.65$).

ANTI-CRY J 2 ANTIBODY TITERS AND THEIR CORRELATION WITH ANTI-CRY J 1 ANTIBODY TITERS

Table 3 shows the prevalence and geometric mean antibody titers of anti-Cry j 2 antibodies. The prevalence of non-IgE,-IgG4 and IgE antibodies among the patients (84% and 90%, respectively) was slightly lower than that of Cry j 1 antibodies. The prevalence of non-IgE,-IgG4 and IgE antibodies among non-patients (49% and 32%, respectively) was also lower. The prevalence of IgG4 antibody both among the patients and non-patients was similar to that of Cry j 1 antibody. The mean antibody titers among non-patients were closer to those among patients in non-IgE,-IgG4 antibody than in IgE antibody, results which were similar to the results of anti-Cry j 1 anti-

bodies.

Figure 3 shows the correlation between antibody titers to two major allergens in the pollinosis individuals. Correlation in non-IgE,-IgG4 titers ($r = 0.19$ Fig. 3a) was much weaker than that in IgE titers ($r = 0.60$, Fig. 2b).

SINGLE NUCLEOTIDE POLYMORPHISMS RELATED TO ATOPY

We carried out case-control studies on the effects of SNPs in three genes related to IgE production : IL-4 promoter C-590T, IL-4 receptor α -chain Ile50Val, and IL-13 Gln110Arg. As shown in Table 4, we found no significant difference in the frequency of the alleles in the three SNP systems between the patients and non-patients groups.

We also compared the geometric mean antibody titers between different alleles in the three SNPs among the patients as well as those among the non-patients. Figure 4 shows antibody (IgE and non-IgE,-IgG4) titers for each subject with different alleles at

Table 4 Gene frequencies of atopy-related SNPs

	IL-4RA Ile50Val				IL-4 promoter C-590T				IL-13 Gln110Arg			
	Ile/Ile	Ile/Val	Val/Val	Frq* (Val)	C/C	C/T	T/T	Frq* (T)	Gln/Gln	Gln/Arg	Arg/Arg	Frq* (Arg)
Patients	11	20	28	64%	2	27	31	74%	5	29	25	67%
Non-Patients	9	24	24	63%	5	27	25	68%	3	29	25	69%
Odds Ratio (95%CI)	1.06 (0.61–1.80)				1.38 (0.78–2.43)				0.9 (0.52–1.56)			

*Frq, Allele frequency

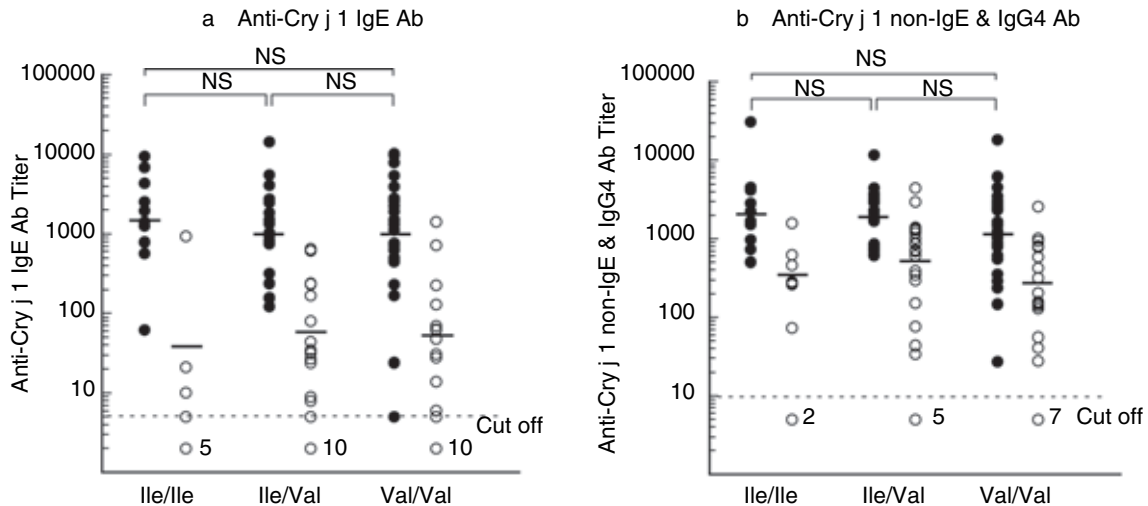


Fig. 4 Antibody titer distribution of each subject with different alleles of IL-4RA Ile50Val. There were no significant differences (NS) in antibody titers of the patients (●) between different alleles ($P = 0.16-0.85$). Also there were no differences in antibody titers of the non-patients (○) between the alleles.

IL-4RA Ile50Val. There were no significant titer differences between the alleles among the patients, and also no differences between the alleles among the non-patients. Similar results were obtained for the other two SNPs, IL-4 promoter C-590T and IL-13 Gln 110Arg (data not shown).

DISCUSSION

The indirect ELISA test for IgG4 has long been used in addition to the indirect test for IgE in pollinosis patients' serum. In this ELISA test, a low serum dilution of 1 : 10 can be used without nonspecific reactions. Aalberse *et al.*³ reported that the IgG4 titers of pollinosis patients rose after hyposensitization therapy. Aalberse's group^{4,5} has also claimed that IgG4 antibody is functionally monospecific : the two combining sites of the molecule react with different epitopes. We reconfirmed that the reverse-sandwich ELISA which detects antibodies with two identical combining sites does not detect IgG4 antibody.

In contrast to IgG4, detection of IgG1 antibody to allergens is not easy, since only a minute amount of

allergen-specific antibodies exist among a vast amount of non-relevant antibody molecules ; the signal-to-noise ratio is probably lower in IgG1 than in IgG4 and IgE antibodies. In the indirect ELISA test for IgG1 antibody, nonspecific reactions do occur at a very low serum dilution of 1 : 10. However, in the reverse-sandwich ELISA, specific antibodies are separated from irrelevant molecules, thus nonspecific reactions are eliminated. Therefore, in this study, we employed the indirect ELISA for detection of IgG4 antibody and the reverse-sandwich ELISA for non-IgE, IgG4 antibodies which may consist of IgG1,2,3 and IgA ; probably IgG1 antibody is in the majority, although we could not determine separately the antibody activities of each subclass by the current techniques.

It is of note that the prevalence of non-IgE,-IgG4 antibody to Cry j 1 in non-patients (75%) was higher than that of IgG4 antibody (47%) (Table 2). We reported¹¹ in 1989 that the prevalence of non-IgE,-IgG4 antibody was higher among forestry workers than among urban residents, whereas the IgG4 antibody

prevalence was nearly the same between the two groups. We think that the reverse-sandwich ELISA with the antigen may be useful for population-based, seroepidemiologic studies of Japanese cedar pollinosis.

When we compared differences in antibody titers between the patients and non-patients, the difference in IgE titers was larger than that in non-IgE, IgG4 (Table 2,3), indicating that IgE antibody production to the allergen in the non-patients is less stimulated than IgG antibody production. To investigate whether there is a difference in the frequencies of the SNP alleles in IgE-related genes between the patient and non-patient groups, we determined the allele frequency for each subject. We found no difference in the frequencies (Table 4). On the other hand, Nakamura *et al.*¹² reported a difference in IL-4 R α Ile50Val. Further studies are needed for understanding the multiple SNP-allele background of IgE responsiveness.

With regard to the correlation between anti-Cry j 1 and anti-Cry j 2 titers in non-IgE, IgG4 and IgE assays in pollinosis individuals, the correlation was higher in IgE ($r = 0.60$) than in non-IgE, IgG4 ($r = 0.19$) (Fig. 3). Although we do not know the exact reasons for the difference, one possibility may be that there are more B cell clones committed for IgG production than for IgE production. Another reason may be that the immunologic memory of non-IgE, IgG4 antibody is longer than that of IgE antibody. Ogawa *et al.*¹³ determined anti-Cry j 1 non-IgE, IgG4 and IgE titers in the sera collected every month for three years from four pollinosis patients. They found that the antibody titers rose after the pollen season each year, then gradually fell until the next season, and that the extent of the rise/fall was more prominent in IgE antibody than in non-IgE, IgG4 antibody, suggesting a shorter memory of IgE antibody.

The first report on Japanese cedar pollinosis was published in 1964 by Horiguchi and Saito.¹⁴ Since then the number of the pollinosis patients in Japan has been increasing. The cedar trees which were extensively planted after World War II in mountains throughout Japan have started to pollinate, thus the extent of pollen exposure has been increasing. In addition, the lifestyles have changed greatly in a tendency toward greater susceptibility for allergy. Recently, Shima¹⁵ reported that the positive rate for specific IgE (RAST ≥ 2) in a cohort of 175 school children increased from 38% in 1997 (age 6–7 years) to 59% in 2001 (age 11–12 years). Currently, the pattern of the pollinosis development in society may still be changing, but the natural history of this disease¹⁶ has not been completely elucidated.

In order to further understand the natural history and seroepidemiology of Japanese cedar pollinosis, in future studies, antibodies of not only IgE and IgG4 but also non-IgE, IgG4 subclasses need to be titrated

in the sera from patients and non-patients of different age groups, patients with different symptom grades, patients receiving immunotherapy, and patients with spontaneous remission.

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