

Japanese cedar pollinosis and HLA-DP5

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Japanese cedar pollinosis is a type I allergic disease caused by Japanese cedar (*Cryptomeria japonica*) pollen. We investigated the association between the disease and HLA class II alleles by HLA-DNA typing using a PCR-SSOP method and found that the frequency of HLA-DP5 (DPA1*02022 and DPB1*0501) was significantly increased in the patients. To investigate whether the HLA-DP5 molecule is directly involved in the pathogenesis of the disease, Japanese cedar pollen antigen (CPAg)-specific T cell lines were established from 3 patients who possessed HLA-DP5 (DPA1*02022/DPB1*0501). By using these CPAg-specific T cell lines and HLA class II-expressing L-cell transfectants, we found that disease-associated HLA-DP5 restricted T cells specific for CPAg existed in the patients. Furthermore, among 38 synthesized overlapping peptides spanning the entire length of one of the major Japanese cedar pollen allergens, Cry j 1, an immunodominant peptide which induced HLA-DP5 restricted Th2 was identified. These observations suggest that the HLA-DP5 may be involved, at least in part, in the pathogenesis, by helping the IgE antibody production against CPAg.

Key words: HLA – cedar pollinosis –
T cell epitope – Cry j 1

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Japanese cedar pollinosis is a type I allergic disease caused by Japanese cedar (*Cryptomeria japonica*) pollen and is one of the most prevalent allergic diseases in Japan. IgE antibodies (Abs) to allergens are the predominant isotype of Abs responsible for inducing allergic diseases. Antibody production, including IgE synthesis by B lymphocytes, requires the help of antigen-specific CD4⁺ T cells which are activated by highly immunogenic peptides presented by HLA class II molecules.

The HLA class II molecules are composed of α and β chains of highly polymorphic glycoproteins and are encoded for by genes in the HLA class II region on the short arm of chromosome 6 {1}. In the class II region, there are at least nine HLA class II genes encoding for functional proteins on cell surfaces, i.e., HLA-DRA, -DRB1, -DRB3, -DRB4, -DRB5, -DQA1, -DQB1, -DPA1 and -DPB1 genes. Each gene except HLA-DRA shows extremely high polymorphism in the human population. The polymorphism resides primarily in the second exon which encodes for the binding sites of antigenic peptides and T cell receptors. It has been shown that the polymorphism influences peptide binding and alters the immune responses to specific antigens {2–5}. Thus, the polymorphic HLA class II molecules

play a key role in restricting Ag-specific Th cell activation. Recently, the polymorphism has been analyzed at the DNA level by using the polymerase chain reaction (PCR) in combination with sequence-specific oligonucleotide probes (SSOPs) {6} or restriction enzymes to detect the restriction fragment length polymorphism, RFLP {7, 8}.

Associations between the specific immune response to inhalant allergens and particular HLA alleles were reported. For example, high immune response to *Amb a V* from *Ambrosia artemisiifolia* (short ragweed) was associated with DRB1*1501 or DR2.2 {9}. Similarly, the Ab response toward *Lolium perenne* (perennial rye grass) pollen allergens, *Lol p I*, *Lol p II*, and *Lol p III*, was associated with DRB1*0301 or DR3 {10, 11}.

For Japanese cedar pollen, it was reported that two major allergens, Cry j 1 and Cry j 2, reacted with IgE Ab from Japanese cedar pollinosis patients {12, 13}. Recently, cDNAs coding for Cry j 1 and Cry j 2 were isolated and complete amino acid sequences were deduced from the sequencing analysis of the cDNAs {14, 15}. In this study, we investigated the HLA-linked genetic factors in Japanese cedar pollinosis by the HLA typing at the DNA level and found a statistical association between

HLA-DP5 and the disease. For further investigation of the association of this disease with HLA, we established Japanese cedar pollen antigen (CPAg)-specific T cell lines from the patients and examined an antigenic peptide within the major allergen of *Cryptomeria japonica* pollen, Cry j 1, presented by HLA-DP5.

Materials and methods

Patients

A total of 72 unrelated Japanese patients with Japanese cedar pollinosis diagnosed by typical clinical history, positive skin test, and positive radioimmunosorbent test (RAST) were subjected to this study (26 men and 46 women; mean age, 44.1 years, ranging 15-65 years old). All patients gave informed consent. As the control subjects, 525 healthy unrelated Japanese were used.

HLA DNA typing

DNA was extracted from the peripheral granulocytes of each subject following the protocol of the 11th International Histocompatibility Workshop {16}. HLA class II genotypes (DRB1, DRB3, DRB4, DRB5, DQA1, DQB1, DPA1, DPB1) were determined by PCR/SSOP analysis, as described previously {6, 16-18}. The allele designations are those recommended from the WHO Nomenclature Committee for HLA {19}.

Statistical analysis

Frequencies of HLA-DRB1, DRB3, DRB4, DRB5, DQA1, DQB1, DPA1, and DPB1 alleles in 72 patients with Japanese cedar pollinosis were compared with those in 525 healthy unrelated Japanese controls. Strength of the statistical association between the disease and HLA alleles was expressed by relative risk (R.R.) as given by Woolf {20} and the statistical significance was examined by chi-square (χ^2) test with Yates' correction. The corrected p value (p_c) was calculated by multiplying the p value (p) by the number of alleles tested for each locus (DRB1=29, DRB3=4, DRB4=1, DRB5=3, DQA1=14, DQB1=15, DPA1=6, DPB1=15).

Antigen preparations

Crude antigen of *Cryptomeria japonica* pollen (CPAg) was prepared as described {12, 21}. Briefly, dry pollen was defatted with ether and stirred in 0.125M ammonium bicarbonate. Solid ammonium sulfate was added to the extract to 100% saturation. The precipitate was dissolved in 0.05M Tris-HCl

(pH 7.8), and dialyzed against the same buffer. The sample was applied to a DEAE-cellulose column and eluted with the same buffer. The eluted fraction was dialyzed against phosphate buffered saline (PBS). This fraction contains Cry j 1 and Cry j 2, two major allergens of *Cryptomeria japonica* pollen {13}.

The fraction was further applied to CM-cellulose column equilibrated with 10mM sodium acetate, pH 5.0, and the Cry j 1 antigen bound to the column was eluted with the buffer containing 0.5M NaCl and 10mM Tris-HCl, pH 8.0. The purified Cry j 1 was then dialyzed against PBS for overnight and was stored at -70°C prior to use {14}. Recombinant Cry j 2 was prepared as described previously {15}.

Synthetic peptides

Synthetic peptides based on the amino acid sequence deduced from the Cry j 1 cDNA sequence, were synthesized using 396MPS multiple peptide synthesizer (Advanced ChemTech, Louisville, Kentucky), using a standard F-moc chemistry program. Peptides were cleaved from the resin with trifluoroacetic acid (TFA) containing 1% ethanedithiol, 3% anisole, and 3% ethylmethylsulfide. The cleaved peptides were washed with ether, and were frozen dry in 10% acetic acid. Purity of the cleaved peptides was monitored by reversed-phase HPLC (gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile, UV detection at 214nm) using a C18 column (Millipore Corp., Milford, MA). Peptides were dissolved in autoclaved double-distilled H₂O at 1mM and stored at -20°C.

Transfectants

L-DP5, containing one pair of HLA class II α and β genes (DPA1*02022 and DPB1*0501) were cotransfected with PAG60 harboring a neomycin-resistant gene into a mouse L fibroblast cell line, Ltk⁻, was kindly provided by Dr. H. Inoko, Tokai University, Isehara, Japan {22}. As a control cell line, Ltk⁻ transfectant with the neomycin-resistant gene alone was established (L-Neo) {23}. Stable expression of HLA molecules on L-DPS was confirmed by FACS analysis, as described previously {23}.

Generation of T cell lines

CPAg-specific T cell lines were generated as follows: PBMC from HLA-DP5 (DPA1*02022/DPB1*0501) positive donors were isolated from heparinized venous blood by density gradient centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Swe-

den). PBMC (5×10^6) were cultured in 6-well, flat bottom plate in the presence of crude CPAg ($5 \mu\text{g/ml}$) in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% pooled male serum, 2% L-glutamine (Life Technologies), and 1% penicillin-streptomycin (Life Technologies) at 37°C in a humidified atmosphere containing 5% CO_2 . After 7 days, viable lymphocytes were collected by density gradient centrifugation over Ficoll-Paque and 1×10^6 lymphocytes were cultured with irradiated ($3,000\text{-rad}$) autologous PBMC (5×10^6) pulsed with $40 \mu\text{g}$ CPAg in the presence of rIL-2 (Boehringer Mannheim Biochemica, Germany) (100U/ml) in 24-well flat bottom plate for a week. Each T cell line was analyzed on the day 7 for its specificity to CPAg. Then, each T cell line was restimulated every 7 days in culture flask with $40 \mu\text{g}$ of CPAg-pulsed and irradiated allogeneic PBMC which shared HLA-DRB1*0405-DRB4*01-DQA1*0302-DQB1*0401-DPA1*02022-DPB1*0501 haplotype. These T cell lines were cultured for 4 weeks before assay.

Peptide-specific T cell line

A Peptide-specific T cell line was generated from a CPAg-specific T cell line. CPAg-specific T cell line was cultured for 3 weeks with $40 \mu\text{M}$ peptide-pulsed and irradiated allogeneic PBMC which shared HLA-DRB1*0405-DRB4*01-DQA1*0302-DQB1*0401-DPA1*02022-DPB1*0501 haplotype in culture flask.

T cell proliferation assay

Ag specific proliferation of the T cell line was assayed by culturing the cells ($4 \times 10^4/\text{well}$) in 96-well flat-bottom culture plates in the presence or absence of crude CPAg ($5 \mu\text{g/ml}$), synthetic peptide ($10 \mu\text{M}$), native Cry j 1 ($5 \mu\text{g/ml}$), or recombinant Cry j 2 ($5 \mu\text{g/ml}$), using autologous or haplotype-shared allogeneic irradiated PBMC ($5 \times 10^4/\text{well}$) or mitomycin C (MMC) treated L-cell transfectant ($3.5 \times 10^4/\text{well}$) in a total volume of $200 \mu\text{l}$. Cells were cultured for 72h in the presence of $1 \mu\text{Ci/well}$ of ^3H -methyl thymidine (ICN Biomedicals, Costa Mesa, CA) for the last 12h. Cells were harvested on glass fiber filters (Wallac, Turku, Finland), and the incorporated radioactivity was quantified by a liquid scintillation counter (Wallac). Results were expressed as the mean cpm for triplicate cultures.

Cytokine assay

The peptide-specific T cell line ($4 \times 10^4/\text{well}$) was incubated with irradiated and haplotype-shared allo-

Table 1.

Association between Japanese cedar pollinosis and HLA-DP alleles

HLA-DP alleles	% patients (n=72)	% controls (n=525)	Relative Risk	χ^2	p_c
DPA1* 01	55.6	61.5	0.78	0.95	
0201	26.4	31.0	0.80	0.65	
02021	0	1.1			
02022	83.3	64.6	2.74	10.05	<0.01
0301	0	0			
0401	0	0			
DPB1* 0201	33.3	39.8	0.76	1.12	
0202	9.7	5.3	1.91	2.21	
0301	8.3	7.8	1.07	0.02	
0401	9.7	11.4	0.83	0.19	
0402	9.7	16.4	0.55	2.13	
0501	79.2	60.6	2.42	9.37	<0.04
0601	2.8	1.5	1.85	0.08	
0801	0	0.4			
0901	13.9	20.6	0.62	1.78	
1001	0	0.2			
1301	5.6	3.2	1.76	0.44	
1401	2.8	3.8	0.72	0.01	
1601	0	0.8			
1701	0	0			
1901	0	1.1			

genic PBMC ($5 \times 10^4/\text{well}$) in the presence or absence of Cry j 1 peptide ($10 \mu\text{M}$) in 96-well flat-bottom culture plate in a final volume of $200 \mu\text{l}$. Supernatants were collected after 24h, 48h, and 72h of incubation and stored at -20°C . IL-2, IL-4, and IFN- γ levels in the supernatants were measured using sandwich ELISA kits purchased from Genzyme, Cambridge, MA.

Results

Association between HLA and Japanese cedar pollinosis

To investigate HLA-linked genetic factors involved in the pathogenesis of Japanese cedar pollinosis, 72 patients and 525 healthy controls were examined for HLA class II alleles by DNA typing using the PCR-SSOP method. The frequencies of HLA class II alleles in 72 patients were compared with findings in 525 healthy unrelated controls. As shown in Table 1, significant positive associations between HLA-DP alleles and Japanese cedar pollinosis were observed with DPA1*02022 (83.3% vs. 64.6%, $\text{RR}=2.74$, $\chi^2=10.05$, $p<0.002$, $p_c<0.01$) and DPB1*0501 (79.2% vs. 60.6%, $\text{RR}=2.42$, $\chi^2=9.37$, $p<0.003$, $p_c<0.04$). In both patients and control subjects, most of DPB1*0501 positive subjects possessed DPA1*02022 (patients; 94.7%, control; 93.3%) and these alleles together encode for an HLA-DP5 molecule. In contrast, no significant difference in frequencies of HLA-DR and DQ alleles between patients and control subjects was observed

(data not shown). Therefore, these results suggest that the susceptible gene might be in linkage disequilibrium with the HLA-DPA1*02022 and DPB1*0501 or it might be HLA-DPA1*02022 and DPB1*0501 themselves.

Generation of CPAg-specific T cell lines from patients

To investigate whether the HLA-DP5 molecule contributes to the immune response to CPAg, CPAg-specific T cell lines were established from three patients by stimulating PBMC with crude CPAg. These patients had not received immunotherapy and possessed HLA-DP5 (DPA1*02022/DPB1*0501). T cell lines were obtained from all 3 patients tested.

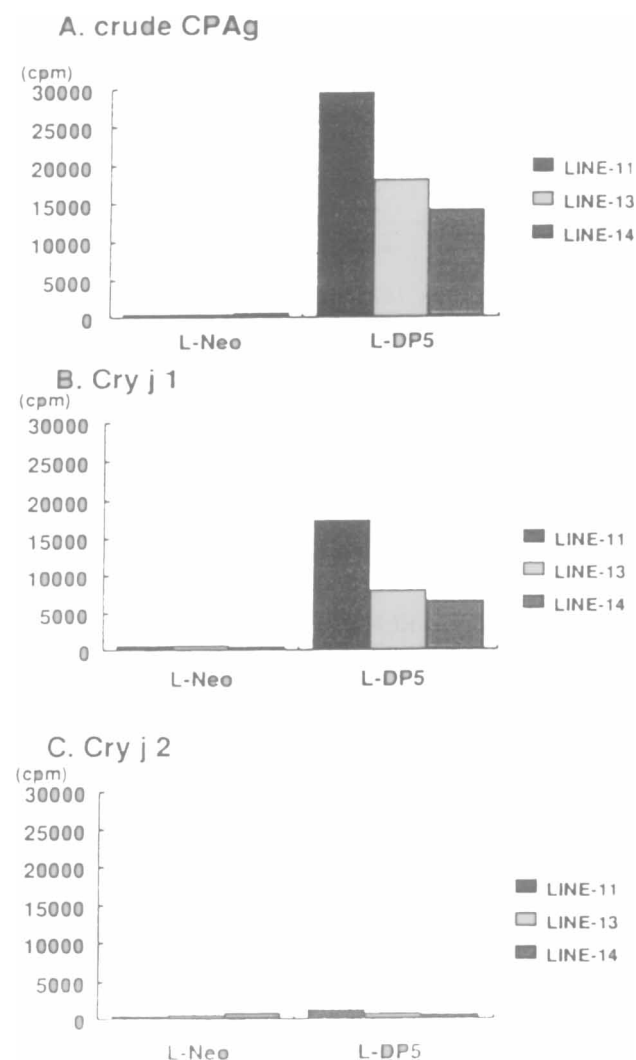


Figure 1. T cell response to Japanese cedar pollen allergens. 4×10^4 CPAg-specific T cell lines were cultured in the presence of crude CPAg (10 μ g/ml) (A), native Cry j 1 (5 μ g/ml) (B) or recombinant Cry j 2 (5 μ g/ml) (C), by using MMC-treated L-cell transfectant as APC in a final volume of 200 μ l. ^{3} H]thymidine incorporation was measured after 72h. Results are expressed as mean cpm of triplicate cultures. SD was <10%.

These T cell lines were designated LINE-11, LINE-13 and LINE-14 (HLA class II alleles of each patient were as follows, LINE-11; DRB1* 0901/0405 - DRB4*01- DQA1* 0302/- -DQB1* 03032/0401 - DPA1*02022/- -DPB1* 0501/-, LINE 13; DRB1* 1401/0405 -DRB3* 0202 -DRB4* 01 -DQA1* 0104/0302 -DQB1*05031/0401 -DPA1*01/02022 -DPB1* 0201/0501, and LINE-14; DRB1* 08032/0405 -DRB4*01- DQA1*0103/0302 -DQB1*0601/0401 -DPA1*02022/- -DPB1*0501/-, respectively). All T cell lines specifically responded to crude CPAg in the presence of allogeneic PBMC sharing the DRB1*0405-DRB4*01-DQA1*0302-DQB1*0401-DPA1* 02022-DPB1* 0501 (DR4-DR53-DQ4-DP5) haplotype (data not shown).

HLA-DP5 restricted and CPAg-specific T cells exist in patients

To investigate whether or not the statistically associated HLA-DP5 restricted and CPAg-specific T cells exist in the patients, T cell proliferation with crude CPAg of T cell lines was examined by using MMC-treated L-cell transfectant expressing HLA-DP5 (DPA1*02022/DPB1*0501) molecule alone as APC. As shown in Figure 1-A, specific T cell response to crude CPAg was observed in the presence of L-DP5 as APC while it was not observed in the presence of L-Neo as APC, indicating that the disease-associated HLA-DP5-restricted and CPAg-specific T cells existed in the patients.

We also examined the T cell response to native Cry j 1 (Fig. 1-B), and recombinant Cry j 2 (Fig. 1-C) by using L-cell transfectants as APC. In the case of the response to Cry j 1, each T cell line responded to Cry j 1 in the presence of L-DP5, while none of them responded in the presence of L-Neo. In the case of the response to Cry j 2, none of the T cell lines responded in the presence of L-DP5 or L-Neo. These results suggested that the antigenic peptide which induced T cell responses restricted by HLA-DP5 might exist in Cry j 1.

T cell epitope on Cry j 1

To determine the antigenic site within the major cedar pollen antigen, Cry j 1, we synthesized overlapping peptides that span the entire Cry j 1 molecule (353 amino acid long). Each peptide was 20 amino acids in length and overlapping with neighbors by 11 amino acid long. Total of 38 peptides were synthesized. Using these peptides, T cell proliferative response to each synthetic peptide was examined in the presence of L-DP5 as APC. As shown in Figure 2, all of tested T cell lines responded only to Cry j 1 peptide-No. 24 (208-227). These results indicated

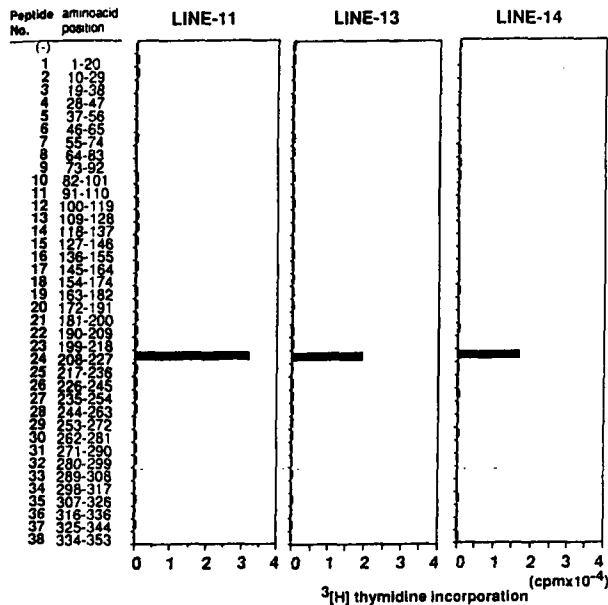


Figure 2. T cell response to synthetic peptides derived from Cry j 1. 4×10^4 CPAg-specific T cell lines were cultured in the presence or absence of synthetic peptides ($10 \mu\text{M}$) with MMC-treated L-DP5 ($3.5 \times 10^4/\text{well}$) in a final volume of $200 \mu\text{l}$. ^3H thymidine incorporation was measured after 72h. Results are expressed as mean cpm of triplicate cultures. SD was $<10\%$.

that this peptide was an immunodominant peptide that induced T cell response to CPAg in the context of HLA-DP5.

Minimal epitope in the immunodominant peptide

To determine the minimal antigenic peptide that induces T cell response, we synthesized 13 amino acid long truncated peptides that shifted from the N-terminal to C-terminal with overlapping by one amino acid residue each. A peptide-specific T cell line that reacted to Cry j 1 208-227 was generated from a CPAg-specific T cell line, LINE-11, by stimulating with Cry j 1 208-227 peptide in the presence of irradiated PBMC. Using this peptide-specific T

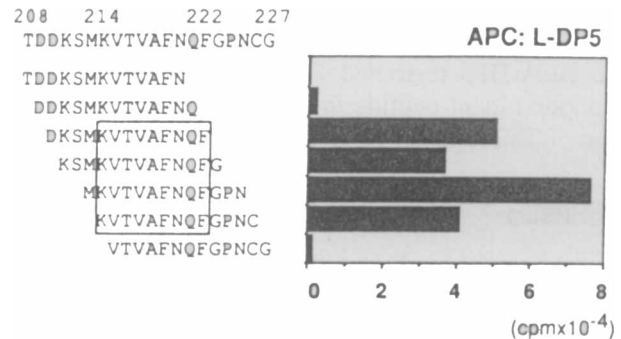


Figure 3. Determination of minimal T cell epitope in the immunodominant antigenic site. Cry j 1 208-227 peptide-specific T cell line was cultured with indicated seven synthetic peptides in the presence of MMC-treated L-DP5 as APC. Results are expressed as mean cpm of triplicate cultures. SD was $<10\%$. Square shows minimal amino acid sequence for T cell response.

cell line, T cell proliferation to each peptide was examined in the presence of L-DP5 as APC. As shown in Figure 3, T cell response was observed in four peptides. These four peptides contain a common 9 amino acid sequence, Cry j 1 214-222 (KVTVAFNQF).

Thus, the minimal antigenic site of the immunodominant peptide for the HLA-DP5 restricted T cells was suggested to be Cry j 1 214-222.

Cytokine production by peptide-specific T cell line

To investigate whether the T cell response to the immunodominant peptide restricted by HLA-DP5 molecule would contribute to IgE Ab synthesis, cytokine productions of culture supernatant (SN) by the peptide-specific T cell line was examined. The culture SN was collected at the time of 24, 48 and 72 h after culture initiation, and the amount of cytokines was measured by ELISA. Simultaneously, ^3H -thymidine incorporation was measured at the 72 h culture. As shown in Table 2, stimulation of T cell line by the peptide resulted in the production of

Table 2.
Cytokine production by peptide-specific T cell Line

Specificity of T cell line	HLA restriction		peptide ($10 \mu\text{M}$)	culture time (h)			
				24	48	72	
Cry j 1 208-227	DP5	IL-2	(-)	<150	<150	<150	(pg/ml)
			(+)	<150	260	<150	
		IFN- γ	(-)	<100	<100	<100	
			(+)	300	<100	<100	
		IL-4	(-)	<45	<45	<45	
			(+)	8,400	6,200	1,800	
		^3H -thymidine incorporation	(-)			1,709	(c.p.m.)
			(+)			64,673	

IL-4, while low or undetectable production of IL-2 or IFN- γ was observed. These results indicated that the HLA-DP5 restricted T cell response to the immunodominant peptide induced a cytokine production of Th2 type.

Discussion

In the present study, we demonstrated that the frequencies of HLA-DPA1*02022 and DPB1*0501 alleles, which together encode for HLA-DP5 molecule, were significantly increased in patients with Japanese cedar pollinosis. In the healthy Japanese population, DPA1*02022 and DPB1*0501 are in weak linkage disequilibrium with DRB1*0405, DQA1*0302 and DQB1*0401 {24}. The frequencies of DRB1*0405, DQA1*0302 and DQB1*0401, however, were not increased in the patients, indicating that one of the susceptible genes to this disease was mapped around the HLA-DP loci.

There are at least two explanations for the statistical association between HLA alleles and disease. First, HLA alleles themselves are involved in the pathogenesis of the disease in question. Second, HLA is merely a genetic marker for the disease, and a gene in strong linkage disequilibrium with the associated HLA allele is responsible for the disease.

Although the function of HLA-DP molecule has not been fully elucidated, several autoimmune diseases such as Graves' disease and Takayasu arteritis were associated with specific DP alleles {17, 25-27}. It has been reported that HLA-DP molecules can present foreign and self peptides to activate T cells {28-30} and may also induce rejection of transplanted tissues {31} or provoke acute graft-vs-host disease in bone marrow transplantation {32}. These observations suggest that the HLA-DP molecules, like the other HLA class II molecules (DR and DQ), play an important role in regulating immune response and disease susceptibility.

We could clearly demonstrate that there were CD4⁺ T cells which were restricted by HLA-DP5 and specific for CPAG in the patients with cedar pollinosis. There is a possibility that peptides restricted by molecules other than HLA-DP5 may induce T cell responses. We have, in fact, found peptides derived from Cry j 1 and Cry j 2 that are restricted by DR4 or DR53 (data not shown). Thus, it is possible that other alleles in addition to HLA-DP5 could be involved in the pathogenesis of cedar pollinosis, even though there is no statistical association of the disease with DR4 or DR53.

CD4⁺ T lymphocytes can be subdivided into T helper 1 (Th1), T helper 2 (Th2), and intermediate T helper 0 (Th0) cells, based on the cytokine production in mice and also in humans {33-35}. It was re-

ported that the majority of allergen-specific T cell clones or T cell lines from atopic patients produced Th2-type cytokines {36-39}. This is consistent with our study that T cells from patients recognizing a Cry j 1 derived-peptide/HLA-DP5 complex produced Th2-type cytokines. Because the synthesis of IgE antibody by B lymphocytes is induced by IL-4 and inhibited by IFN- γ {40}, these Th2 cells are suggested to be responsible for induction of IgE antibody.

Therefore, HLA-DP5 may not be merely a genetic marker but may be involved, at least in part, in the pathogenesis of the disease, by activating T cells, which produce IL-4 but not IFN- γ to help IgE antibody production against CPAG.

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