

Epitope Analysis of Birch Pollen Allergen in Japanese Subjects

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Birch pollen is a very common cause of nasal allergy (pollinosis) not only in Scandinavia, Europe, Canada, and the northern part of the United States but also in Hokkaido, Japan. We have previously reported a positive association between the HLA-DR9 phenotype and the development of birch pollen allergy in Japanese subjects. However, there is little information about T cell epitopes of birch pollen which are presented by HLA class II molecules other than HLA-DR9. Therefore, we analyzed the difference in T cell epitope usage in patients who had HLA-DR9 versus those who did not. Seven Japanese patients with birch pollinosis were studied. Some groups of peptides representing T cell epitopes (*Betula verrucosa*; *Bet VI* peptides, p7–33, p23–46, p138–160) appeared to be shared by the majority, while another peptide (*Bet VI* p72–95) was recognized predominantly by patients who expressed HLA-DR9 and/or HLA-DQ3 molecules. Moreover, seven T cell clones and eight T cell lines were generated from two patients who did not have HLA-DR9 or HLA-DQ3. Using some of these T cell clones/lines, we investigated the relationship between HLA class II molecules and antigenic peptides. One of these T cell clones recognized antigenic peptides in the context of the HLA-DQ1 molecule. To our knowledge, this is the first indication that the epitope on *Bet VI* can be presented by the HLA-DQ molecule.

KEY WORDS: HLA; birch pollinosis; T cell epitope; *Bet VI*.

INTRODUCTION

Type I allergic diseases are caused by the production of antigen-specific IgE, which requires the help of cognate CD4⁺ T cells. Major histocompatibility complex (MHC) class II molecules are heterodimeric peptide receptors that bind antigenic peptides and present them to cognate

CD4⁺ T cells (1). Birch pollen nasal allergy (pollinosis) is a type I allergic disease and is one of the most prevalent pollinosis in Hokkaido, Japan.

Several reports have described an association between particular HLA phenotypes and nasal allergy. We have shown previously that (a) there is an association between HLA-DR9 and HLA-DQ3 phenotypes and the development of birch pollen allergy in Japanese subjects (2), (b) an HLA-DR9-specific antibody blocked proliferation of birch pollen-specific T cells (3), (c) the major antigenic fragment of Japanese birch pollen allergen (*Betula platyphylla* var. *japonica*) is a 17-kD molecule which differs in part (the amino acid at position 31 phenylalanine, which is instead isoleucine) in its amino acid sequence from that of European birch pollen allergen (*Bet VI*) (4), (d) T cells from Japanese patients with birch pollinosis responded to three types of birch pollen allergens (*Betula platyphylla* var. *japonica*, Japan; *Bet VI*, northern Europe; *Betula populifolia*, northern United States) (4), and (e) antigenic peptides recognized by a T cell clone (TCC), generated from a Japanese patient who had HLA-DR9 and HLA-DQ3, contained the HLA-DR9 peptide binding motif (5).

In this paper, we analyze differences in T cell epitope usage between patients who had HLA-DR9 and HLA-DQ3 and those who did not. Seven TCCs and eight T cell lines (TCLs) were generated from two patients who had neither HLA-DR9 nor HLA-DQ3. Using one of these TCCs, we investigated the antigenic peptide and HLA class II molecules recognized by the TCC.

METHODS

Cell Lines

EpsteinBarr virus (EBV)-transformed B lymphoblastoid cell lines (HLA-DR2/14 HLA-DQ1/7, HLA-DR2/2 HLA-DQ1/1) were used as antigen presenting cells (APC).

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Subjects

Seven Japanese patients with birch pollen nasal allergy were studied. Type I allergy to birch pollen was confirmed by clinical symptoms, eosinophilic cells in the nasal discharge, positive skin test, and positive radioallergen sorbent tests (RAST). HLA typing was carried out using a standard method at the Japanese Red Cross Blood Center (Sapporo, Japan).

Antigens

Ether-extracted *Betula platyphylla* var. *japonica* pollen was used as whole birch pollen extract and 1 g of this material was solubilized in 10 ml of 20 mM phosphate buffer at 4°C for 3 hr. After centrifugation (12,000g, 10 min), the supernatant was ultrafiltrated with an ultrafiltration membrane (YM-1; Amicon Inc., Beverly, MA). SDS-PAGE of the ultrafiltrates was performed in 15% acrylamide gels under nonreduced conditions, and proteins in the gel were blotted onto a polyvinylidene difluoride transfer membrane (Millipore Corp., Bedford, MA). After staining with Coomassie brilliant blue R-250, the 17-kD fragment was cut out and eluted, first with 40% acetonitrile at 37°C for 3 hr, then with 40% acetonitrile supplemented with 0.5% trifluoroacetic acid at 50°C for 45 min. The eluate was immediately lyophilized and preserved at -70°C until use (6, 7).

Peptides

Peptides were synthesized according to the amino acid sequence of *Bet VI* (8), using a multiple peptide synthesizer (AMS 422; Abimed, Langelfeld, Germany) based on the Fmoc solid-phase method. The purity of these peptides was confirmed to be more than 90% by reverse-phase high-performance liquid chromatography and mass spectrometry (JMS-SX102; Nippon Denshi, Japan).

Lymphocyte Proliferation Assay

Peripheral blood mononuclear cells (PBMC; 10^5) were cultured in triplicates in 96-well plates (200 μ l) in the presence of either whole birch pollen extract (25 μ g/ml), overlapping peptide (20 μ M), or purified 17-kD fragments of birch pollen extracts (50 μ g/ml), for 5 days at 37°C, in a 5% CO₂, humidified atmosphere. After pulsing for 18 hr with [³H]thymidine (TdR; Amersham; 0.5 μ Ci per well), cells were harvested on glass-fiber filters and the incorporated radioactivity was measured by liquid scintillation counting. The proliferative re-

sponse is shown as the stimulation index (SI) and the cutoff for the positive response was set at a SI of 2.0. The SI was calculated as follows: counts per minute (cpm) observed with peptide/cpm observed without peptide.

Antigen-Specific T Cell Clones and Lines

TCCs and TCLs specific for the 17-kD fragment of *Betula platyphylla* var. *japonica* were established from the PBMC of two patients who did not express HLA-DR9 or HLA-DQ3. TCCs and TCLs were generated by stimulating PBMC (1×10^5 /well) with either whole birch pollen extract (25 μ g/ml) or purified 17-kD fragments of birch pollen extracts (50 μ g/ml). Cells were maintained in minimum essential medium α (GIBCO, Grand Island, NY), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2×10^{-5} M 2ME, and 10% heat-inactivated normal human male serum in 24-well flat-bottom culture plates (Falcon; Becton Dickinson, Lincoln Park, NJ). Secondary cultures were set up by the addition of mitomycin C-treated autologous PBMC (2×10^5 /well) with either whole birch pollen extract (25 μ g/ml) or purified 17-kD fragments of birch pollen extracts (50 μ g/ml) to the primary 7-day cultures.

The specificity of TCLs was determined using the [³H]TdR incorporation assay described above. Cloning was performed in 96-well round-bottom culture plates (Falcon; Becton Dickinson) by limiting dilution at 0.3 cell/well in the presence of 10^5 irradiated (3000 rad) allogeneic PBMC cells as feeder cells; human r-IL-2 (50 U/ml) and 0.5% (v/v) PHA (Difco, Detroit, MI) were added to the previously described medium. Growing microcultures were then expanded by weekly stimulation with mitomycin C-treated autologous PBMC (2×10^5 /well) with either whole birch pollen extract (25 μ g/ml) or purified 17-kD fragments of birch pollen extracts (50 μ g/ml).

The specificity and MHC restriction of the established TCCs were confirmed as follows: briefly, 10^4 T cell blasts were incubated in triplicate cultures in 96-well round-bottom culture plates in the presence of mitomycin C-treated autologous PBMC or an HLA-matched allogeneic EBV-transformed B lymphoblastoid cell line (2×10^4 /well) with either whole birch pollen extract, purified 17-kD fragments of birch pollen extracts, or various peptides for 48 hr at 37°C, in a 5% CO₂, humidified atmosphere. The cells were pulsed and harvested as described above.

Blocking assays were performed to determine which HLA molecules were involved in antigen presentation. TCCs (1×10^4 /well) were cultured with mitomycin

C-treated autologous PBMC (2×10^4 /well) and antigenic peptide (0.1 and 10 μ M) in the presence or absence of various mouse monoclonal antibodies specific for HLA molecules [anti-HLA-DR, L243 (9); anti-HLA-DQ, SPV-L3 (10); and anti-HLA class I, W6/32 (11)]. All antibodies were used at final concentrations of 50, 20, and 10 (μ g/ml).

RESULTS

T Cell Responses to Overlapping Synthetic Peptides

To identify the T cell epitopes of the 17-kD antigenic fragment (*Bet VI*) recognized by Japanese patients, we synthesized overlapping peptides based on the amino acid sequence of *Bet VI*. Using these peptides, we investigated the T cell responses of seven Japanese patients with birch pollinosis. Proliferative responses are shown as the stimulation index (SI) and the cutoff for a positive response was set at a SI of 2.0. Five or fewer consecutive peptides were grouped based on SIs of 2.0 or above to represent a T cell epitope. As shown in Fig. 1, about 11 groups of peptides were recognized by T cells from patients. The data showed that five to seven (all) patients reacted to some of the groups (*Bet VI* p7–33, p23–46, p138–160). However, one of the groups (*Bet VI* p72–95) was recognized predominantly by patients who expressed HLA-DR9 or HLA-DQ3 molecules.

Betula platyphylla var. japonica-Specific T Cell Clones and Lines from Two Patients Who Expressed Neither HLA-DR9 nor HLA-DQ3

Seven TCCs (2M, 2R, 2W, 3F, 17216, 2J, 21) and eight TCLs (2A, 2F, C313, 2U, 2T, 2Z, 26, 2X) were generated from two patients (patients 5 and 6) who expressed neither HLA-DR9 nor HLA-DQ3. The clones and lines proliferated specifically in response to whole birch pollen extracts or the purified 17-kD fragment of birch pollen extracts when presented with autologous PBMC. Epitope specificity was then determined by the use of overlapping peptides.

Figure 2 shows which of the TCCs and TCLs responded to the seven pools of overlapping peptides. Pool 1 peptides were found to stimulate the majority of TCCs and TCLs (13/15), indicating that this pool possibly contained the T cell epitope critical for development of birch pollinosis in these two patients. One of the TCCs, 2W (which originated from patient 6), which responded only to pool 1 peptides, was typed as CD3⁺, CD4⁺, CD8[−], and TCR $\alpha\beta$ ⁺ by flow cytometry and chosen for

subsequent studies. The other seven TCCs had the same peptide/MHC specificity as TCC 2W. Therefore we show the data on TCC 2W as representative.

The Core Peptide Sequence Recognized by T Cell Clone 2W

TCC 2W recognized the peptide fragments p13–27 and p16–30. To identify the core peptide sequence recognized by 2W, truncated peptides were synthesized and used for testing. As shown in Fig. 3, the proliferative response of 2W decreased when the peptide was truncated either from the N terminus to alanine at position 17 (A17) or from the C terminus to isoleucine at position 24 (I24). Therefore, the core peptide sequence recognized by 2W incorporates the amino acid sequence p17–24 (ARLFKAFI). It has been observed previously that TCC does not proliferate maximally with an 8- or a 9-mer core peptide, and one or two additional residues at both the N and the C-termini are required for a full response (6). Therefore the 12-mer amino acid sequence p15–26 (PAARLFKAFILD) was used for the subsequent study.

Proliferative Responses of T Cell Clone 2W to Substituted Peptides

To identify the important amino acid residues for T cell activation on the antigenic amino acid sequence p15–26 (PAARLFKAFILD), a number of peptides were generated from this sequence containing amino acid substitutions. These peptides were tested for their ability to induce specifically the proliferation of the T cell clone, 2W. These amino acid substitutions were carried out as follows. Hydrophilic residues (lysine, arginine, asparatic acid) were substituted with the small hydrophobic alanine, and hydrophobic residues (alanine, leucine, isoleucine, phenylalanine, proline) were substituted with the small hydrophilic serine. As shown in Fig. 4, R18A (indicates the substitution of R, arginine at position 18, with A, alanine), L19S, K21A, and F23S significantly diminished the proliferative response of TCC 2W, while F20S showed a lesser effect. Therefore it is likely that at least five residues (18R, arginine; 19L, leucine; 20F, phenylalanine; 21K, lysine; 23F, phenylalanine) are important for either binding to the MHC class II molecule or recognition by the T cell receptor. Moreover, chemically conservative amino acid substitutions were carried out as follows: alanine and proline were substituted with glycine, and leucine and isoleucine were substituted for each other. Likewise, phenylalanine and tyrosine, lysine and arginine, and asparatic acid and glutamic acid were

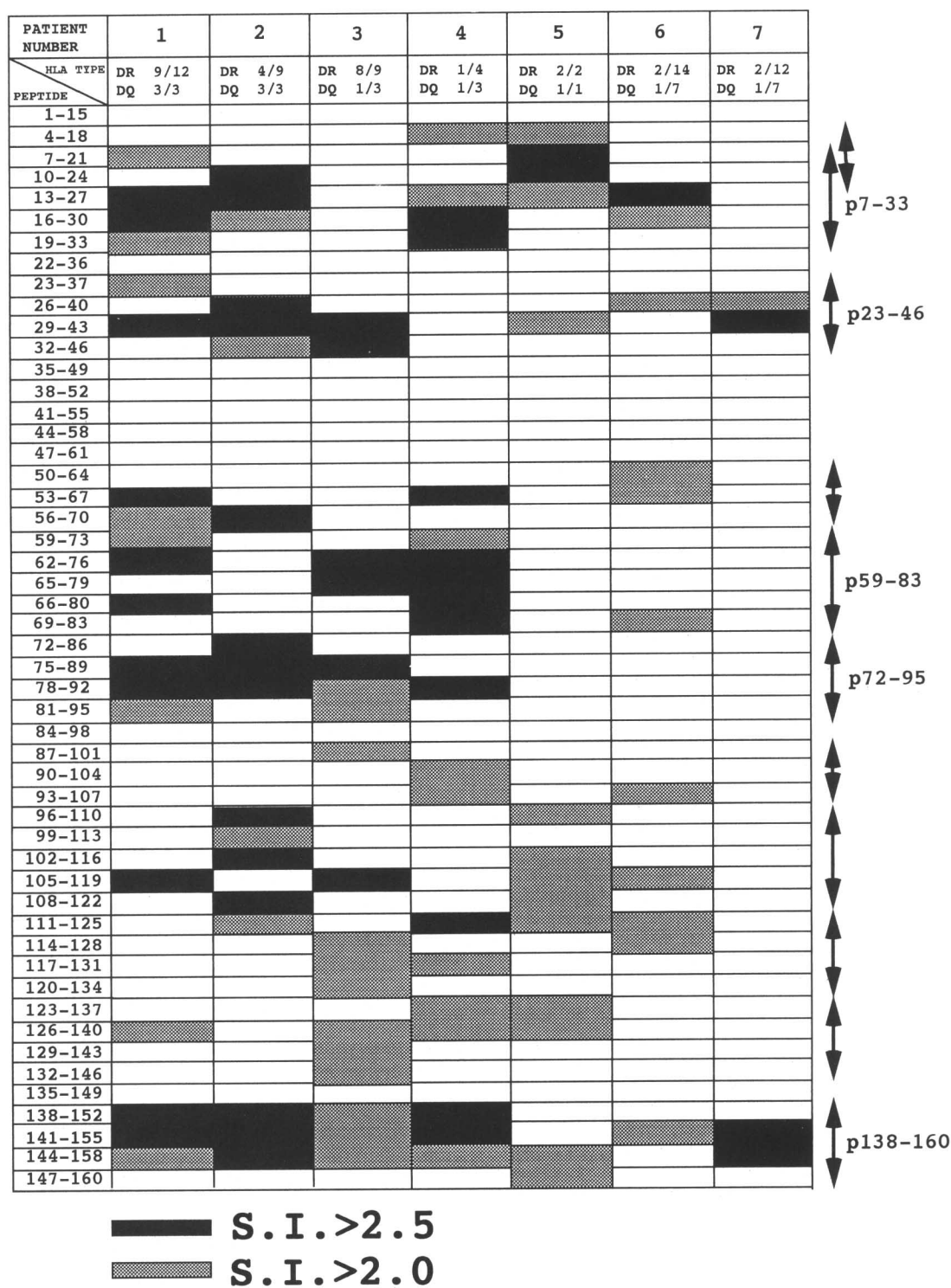


Fig. 1. Proliferative responses of lymphocytes from patients with birch pollinosis to *Bet VI* overlapping peptides. Seven Japanese patients with birch pollinosis were studied. The first column indicates the amino acid position of the synthetic peptide. The patient numbers and HLA phenotypes are shown in the first two rows. Proliferative responses are shown as the stimulation index (SI) calculated as follows: cpm observed with peptide/cpm observed without peptide. Black boxes indicate peptides which induce a proliferative response greater than a SI of 2.5. Gray boxes show peptides which induce a proliferative response greater than a SI of 2.0. Arrows indicate the 11 groups of peptides which represent T cell epitopes.

	POOL 1	POOL 2	POOL 3	POOL 4	POOL 5	POOL 6	POOL 7
peptide	p 1-15	p 19-33	p 35-49	p 56-70	p 78-92	p 102-116	p 126-140
	p 4-18	p 22-36	p 38-52	p 59-73	p 81-95	p 105-119	p 129-143
	p 7-21	p 23-37	p 41-55	p 62-76	p 84-98	p 108-122	p 132-146
	p 10-24	p 26-40	p 44-58	p 65-79	p 87-101	p 111-125	p 135-149
	p 13-27	p 29-43	p 47-61	p 66-80	p 90-104	p 114-128	p 138-152
	p 16-30	p 32-46	p 50-64	p 72-86	p 93-107	p 117-131	p 141-155
			p 53-67	p 75-89	p 96-110	p 120-134	p 144-158
TCC or TCL					p 99-113 p 102-116	p 123-137	p 147-160
TCC 2M							
TCC 2R							
TCC 2W							
TCC 3F							
TCC 17216							
TCC 2J							
TCC 21							
TCL 2A							
TCL 2F							
TCL C313							
TCL 2U							
TCL 2T							
TCL 2Z							
TCL 26							
TCL 2X							

Fig. 2. Pooled peptides recognized by TCCs and TCLs. Peptide pools and their amino acid positions are shown in the first rows. The first column indicates T cell clones and T cell lines. Gray boxes show pools of peptides recognized by TCCs and TCLs.

substituted for each other. As shown in Fig. 4, only the K21R substitution diminished the proliferative response of TCC 2W, while R18K, L19I, F20Y, and F23Y showed almost no effect. The result indicates that 21K is critical for T cell recognition.

HLA Class II Restriction of T Cell Clone 2W

As shown in Fig. 5, 2W proliferated in response to antigenic p15-26 peptide presented by mitomycin C-treated autologous (HLA-DR2/14 HLA-DQ1/7) and allogeneic (HLA-DR2/2 HLA-DQ1/1) EBV-transformed B lymphoblastoid cell lines. In the antibody blocking experiment, only the anti-HLA-DQ monoclonal antibody inhibited TCC 2W proliferation in the presence of antigenic peptide p15-26 and the mitomycin C-treated autologous EBV-transformed B lymphoblastoid cell line. Therefore 2W recognized antigenic peptide p15-26 in the context of the HLA-DQ1 molecule.

DISCUSSION

Several groups (including our own) have already reported a relationship between HLA class II molecules and the development of birch pollen allergy (1, 12, 13). Interestingly, various HLA haplotypes appear to be involved. This, however, may be a result of the different ethnic backgrounds studied. For example, in Japanese subjects, HLA-DR9 and/or HLA-DQ3 phenotypes are associated with birch pollen allergy (2). In contrast, in Austrian (12) and Danish (13) subjects, HLA-DRB3*0101/1301 and HLA-DRB3*0101 alleles are, respectively, associated predominantly with this allergy. On the other hand, Ebner and his colleagues have reported that no association between HLA class II molecules and particular epitopes of *Bet VI* could be observed (14).

In this paper, we initially analyzed the response of peripheral T cells, from patients who had HLA-DR9 and/or HLA-DQ3 and those who did not, to *Bet VI* peptide. These responses were segregated into 11 groups

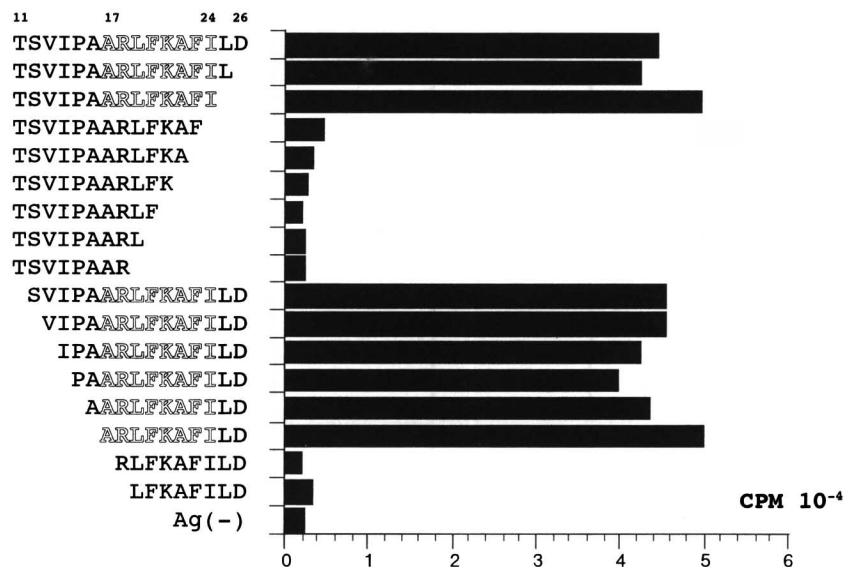


Fig. 3. The core peptide sequence recognized by T cell clone 2W. Bars show proliferative responses (cpm). The common sequence among the peptides which induced a high response of the T cell clone is indicated by outlined letters. This sequence (ARLFKAFI) corresponds to the core peptide sequence recognized by TCC 2W. The single-letter code for amino acids is used. Ag(—) indicates the proliferative response of the antigen-free culture.

and represented 11 epitopes. Patients expressing HLA-DR9 and/or HLA-DQ3 preferentially responded to *Bet VI* peptides p72–95. We have previously generated a TCC (clone H36) from a Japanese patient who had HLA-DR9 and HLA-DQ3. This clone recognizes the p80–87 amino acid sequence (FKYNYSVI), which belongs to this group of peptides (p72–95), in the context of the HLA-DR9 molecule (6).

We have also reported naturally processed peptides bound to HLA-DR9/53 molecules containing a proposed HLA-DR9 binding motif (15) as well as anchor residues in a naturally processed HLA-DR53 ligand (16). Fujisao *et al.* have reported a similar motif for HLA-DR9 (17). All groups of peptides reported in this paper were found to contain the HLA-DR9 binding motif. These data indicate that the HLA-DR9 phenotype may associate with the development of birch pollen allergy in Japanese subjects.

Our groups of peptides, representing T cell epitopes, are identical to those of Ebner and co-workers (18, 19), except for *Bet VI* p59–83. Therefore the group of peptides *Bet VI* p59–83 may be unique to Japanese subjects.

Ebner and his colleagues have employed the term “immunodominant” or “major” for epitopes which are recognized by most TCCs and TCLs generated from patients, and they designated *Bet VI* p77–92 or *Bet VI* p144–154 as an “immunodominant” or “major” epitope

(19, 20). Presumably, in Japanese subjects, *Bet VI* p77–92, which contains the HLA-DR9 peptide binding motif, is recognized predominantly by subjects who expressed HLA-DR9 and/or HLA-DQ3 molecules. If *Bet VI* p77–92 is “immunodominant” in Japanese subjects, individuals who do not have HLA-DR9 might not present this fragment to T cells and therefore may not develop birch pollen allergy.

To obtain further details of epitopes used in Japanese patients with birch pollinosis, we generated seven TCCs and eight TCLs from two patients who had neither HLA-DR9 nor HLA-DQ3 (HLA-DR2/14 HLA-DQ1/7, HLA-DR2/12 HLA-DQ1/7). Of these, TCC 2W was used to identify the amino acids important for T cell activation. The epitope was determined to contain the core peptide sequence p17–24 (ARLFKAFI). Other T cell clones recognizing pool 1 peptides gave similar results.

Amino acid substitutions on p15–26 (PAARLFKAF-ILD) revealed that at least five residues (18R, 19L, 20F, 21K, 23F) are important either for binding to MHC class II molecule or for recognition by the T cell receptor. Since the MHC binding motif can generally accommodate conservative amino acid exchange, chemically conservative amino acid substitution shows that one of these residues (21K) may be critical for T cell recognition. Furthermore, this activation required presentation of p15–26 peptide by autologous (HLA-DR2/14 HLA-

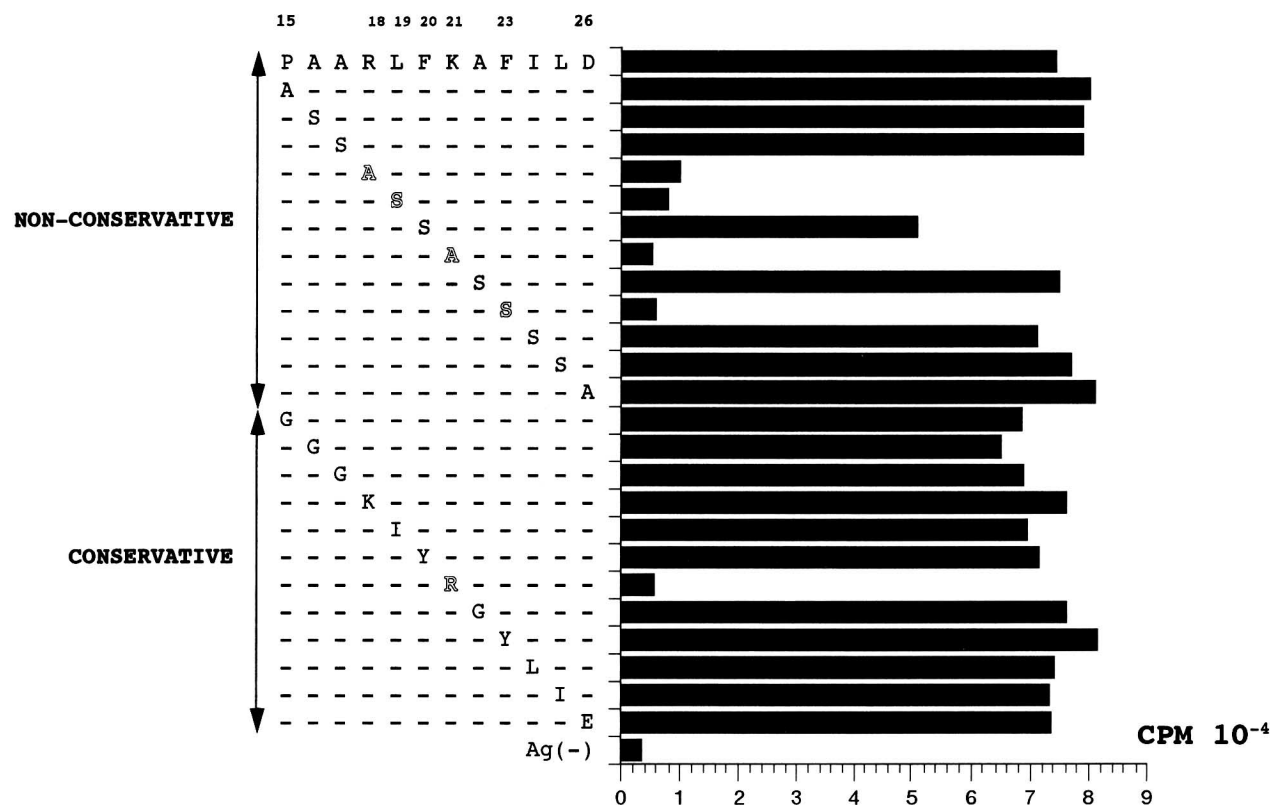


Fig. 4. Effect of amino acid substitutions of peptides on T cell clone 2W stimulation. Bars show proliferative responses (cpm). The upper amino acid sequence shows the wild-type peptide. Single letters show the site of substitution (nonconservative and conservative) and dashes indicate amino acids identical to the wild-type peptide. The single-letter code for amino acids is used. The outlined letters show that substituted peptides diminished the proliferative response of 2W. Ag(—) indicates the proliferative response of the antigen-free culture.

DQ1/7) or allogeneic (HLA-DR2/2 HLA-DQ1/1) EBV-transformed B lymphoblastoid cell lines and could be blocked only by an anti-HLA-DQ monoclonal antibody. Therefore TCC 2W recognized the antigenic peptide p15–26 in the context of the HLA-DQ1 molecule. Indeed, the amino acid sequence p15–26 (PAARLFKAFILD) contains the HLA-DQ1 peptide binding motif (leucine at position 1, phenylalanine at position 5) (21). This is the first indication that the epitope on *Bet VI* is presented by the HLA-DQ molecule. According to the HLA-DQ1 peptide binding motif, of these five residues (18R, 19L, 20F, 21K, 23F), we postulate that 19L and 23F are critical for binding to MHC class II, while 18R, 20F, and 21K are at the TCR contact sites. It is well-known that in some cases substitution of the amino acid at the TCR contact site on the peptide converts the agonist peptides to partial agonists or antagonists (22). Our nonstimulatory peptide K21R may work as either a TCR antagonist or a competitor for MHC binding against the wild-type agonist. Similarly R18K and F20Y may possibly function as partial agonists which alter the nature of T cell activation (i.e., cytokine profiles).

Recently, Faber and his colleagues have shown the secondary structure and tertiary fold of *Bet VI* (23). They revealed three α -helices (*Bet VI* p15–31, p128–139, p144–159) which were involved in forming the conformation of T cell epitopes (23, 24).

We assume that because patients who do not have HLA-DR9 or HLA-DQ3 fail to present the proposed “immunodominant” epitope containing the HLA-DR9 binding motif, the amino acid sequence p15–26 (PAARLFKAFILD) containing the HLA-DQ1 binding motif will be presented instead and cause the development of birch pollen allergy.

In Italy, Cirila *et al.* tested the clinical efficacy and safety of local nasal immunotherapy using capsules containing birch (*Betula pendula*) in powder form (25). To develop specific and effective immunotherapy using peptides containing T cell epitopes, it would be necessary to analyze other T cell epitopes recognized by most patients (i.e., p23–46 and p138–160), their HLA class II restriction, and the effect of analogue peptides on proliferation and cytokine profiles. In addition, IgE B cell

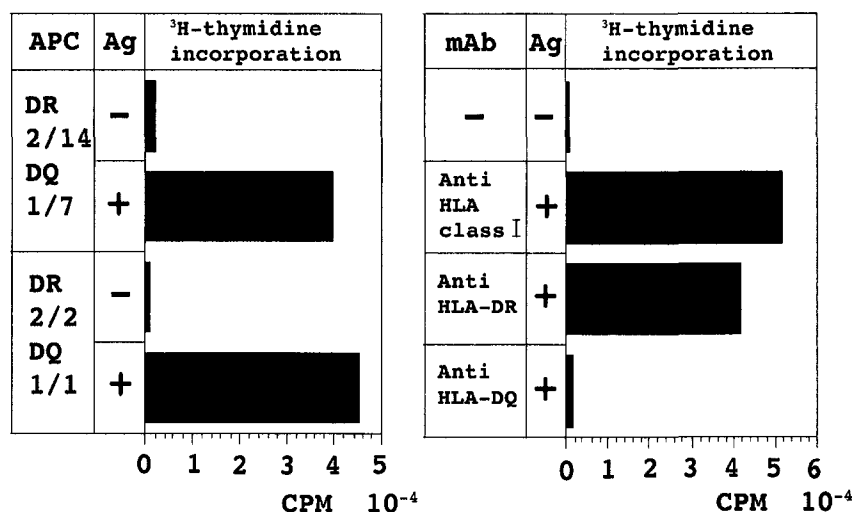


Fig. 5. T cell clone 2W recognizes antigenic peptide in the context of the HLA-DQ1 molecule. Bars show proliferative responses (cpm). T cell clone 2W proliferated in response to the antigenic peptide (p15–26) presented by an autologous (HLA-DR2/14 HLA-DQ1/7) and an allogeneic (HLA-DR2/2 HLA-DQ1/1) EBV-transformed B lymphoblastoid cell line. In the antibody blocking experiment, only SPV-L3 (anti-HLA-DQ monoclonal antibody) inhibited the proliferative response of TCC 2W. The concentration of monoclonal antibody was 50 μ g/ml.

epitopes must be considered for secure peptide immunotherapy to avoid anaphylaxis.

CONCLUSIONS

(1) In Japanese subjects with birch pollen allergy, about 11 groups of peptides representing T cell epitopes were found within the *Bet VI* protein. Of these, some epitopes within *Bet VI* p7–33, p23–46, and p138–160 were used by five to seven (all) patients. In contrast, the epitope in *Bet VI* p72–95 was recognized predominantly by patients who expressed HLA-DR9 and/or HLA-DQ3 molecules.

(2) We generated seven T cell clones and eight T cell lines from two patients who had neither HLA-DR9 nor HLA-DQ3. Thirteen of fifteen T cell clones and T cell lines recognized pool 1 peptides (*Bet VI* p7–21, p10–24, p13–27, p16–30). This indicates that epitopes residing in these peptides were possibly involved in the development of birch pollinosis in these two patients.

(3) T cell clone 2W recognized two peptides (*Bet VI* p13–27, p16–30) and the core peptide sequence was identified as being p17–24 (ARLFKAFI).

(4) T cell clone 2W recognized the antigenic peptide in the context of the HLA-DQ1 molecule and the amino acid sequence of this peptide contained the HLA-DQ1 peptide binding motif.

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