

Epitope analysis of HLA-DR-restricted helper T-cell responses to *Der p* II, a major allergen molecule of *Dermatophagoides pteronyssinus*

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T-cell epitopes of *Der p* II, a major allergen of *Dermatophagoides pteronyssinus*, were analyzed by using human T-cell clones. We tested 38 cloned T cells from two Japanese patients with allergic rhinitis, and identified at least two peptides (K33-T47 and I58-C73) as helper T-cell epitopes. The former epitope was shown to be restricted by HLA-DRB1*1502, and the latter by HLA-DRB1*0405, both of which are typical Japanese HLA-DR alleles, suggesting that those T-cell epitopes might be important for the onset of house-dust mite allergy in the Japanese population. We prepared 15 analog peptides of the HLA-DRB1*1502-restricted 15-mer peptide. Of those 15 residues, five (F35, L37, A39, F41, and E42) were critical for the epitope activity, and three residues (F35, A39, and E42) seemed to be included in anchor motifs for HLA-DRB1*1502. The epitope peptide was also recognized by HLA-DRB1*1502-positive healthy donors; however, only allergic T cells showed Th2 functions. Antigen-presenting cells of nonallergic donors were able to activate allergic T cells to express Th2 function. This seemed to suggest that antigen recognition of T cells, as well as additional unknown factors which promote Th2, rather than Th1, responses, might be important for the onset of house-dust mite allergy.

**M. Okano¹, T. Nagano¹,
M. Nakada¹, Y. Masuda¹, K. Kino²,
H. Yasueda³, Y. Nose⁴,
Y. Nishimura⁵, N. Ohta⁶**

¹Department of Otorhinolaryngology, Okayama University Medical School, Okayama; ²Meiji Institute of Health Science, Odawara; ³Department of Clinical Research, National Sagami Hospital, Sagami; ⁴Hyogo Prefectural Blood Transfusion Center, Kobe; ⁵Division of Immunogenetics, Kumamoto University Graduate School of Medical Sciences, Kumamoto; ⁶Department of Medical Zoology, Nagoya City University Medical School, Nagoya, Japan

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Nobuo Ohta, MD
Department of Medical Zoology
Nagoya City University Medical School
Kawasumi, Mizuhochi, Mizuhoku
Nagoya 467
Japan

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Allergic hypersensitivity is triggered by the production of allergen-specific IgE antibodies. *Dermatophagoides*, a main genus of house-dust mite, is thought to be one of the most important sources of environmental allergens (1), and several major target glycoproteins of human IgE responses have been isolated from this genus (2–4). Allergen-specific IgE production is under the strict control of allergen-specific CD4⁺ T cells through the orchestration of regulatory cytokine production (5). Induction of the CD4⁺ T-cell response requires molecular interactions among a T-cell receptor (TCR), a class II major histocompatibility complex (MHC) molecule, and an epitope peptide composed of 13–25 amino acids with critical binding motifs (6, 7). Accumulation of analytic data on class II-binding peptides, as well as the crystallography of the HLA molecules, has provided a clue for pre-

dicting probable epitope peptides from the primary sequences of antigen molecules (8–11). Molecular cloning of several major mite allergens has been completed (12, 13), and studies have been conducted to analyze the molecular basis of MHC-restricted T-cell responses to the mite allergens.

HLA-DR molecules are the dominant restriction elements for CD4⁺ T cells. This genetic region is highly polymorphic, and differences in the gene frequency are observed in respective ethnic groups. In the Japanese population, HLA-DRB1*1502, DRB1*0405, and DRB1*1302 are common, but are virtually absent in Caucasians (14). An HLA-DRB1*1101-restricted epitope of *Der p* II (15) has been reported in Caucasians; however, this DR allele is, in turn, very rare in Japanese. It is likely that functional T-cell epitopes of major allergens in a given ethnic group are determined to some extent

by the characteristic polymorphism of the HLA system in the population. Such a concept is not inconsistent with our previous population study which showed the presence of a particular T-cell epitope(s) of *Der p* II commonly recognized by the Japanese population (16).

In this study, we analyzed helper T-cell epitopes of a major allergen molecule of *D. pteronyssinus* restricted by HLA-DRB1*1502 or DRB1*0405. Those HLA-DR alleles are common in the Japanese population, and the epitope peptides seemed to be commonly recognized in the Japanese population. The allergen tested in the present study is *Der p* II, which has been known to induce specific IgE and vigorous CD4⁺ T-cell responses in allergic subjects (17). After analysis of the T-cell epitopes, we discuss and compare the biologic roles of these epitopes in house-dust-mite allergy in respective ethnic groups.

Material and methods

Subjects

Two Japanese patients with allergic rhinitis, as well as a healthy individual, were studied. Those patients showed strong skin scratch tests to crude extract of *D. pteronyssinus* and *Der p* II, and their IgE antibodies were 17 or more Pharmacia RAST units/ml (Pharmacia AB, Uppsala, Sweden). HLA-DR types of all tested subjects were determined by the method of DNA typing (18), as summarized in Table 1.

Antigens

Crude *D. pteronyssinus* antigen (Dp-c) and *Der p* II were extracted by the method described elsewhere

(19, 20). PPD (Nihon BCG Co., Tokyo, Japan) and soluble schistosome egg antigen (SEA) (21) were used as positive and negative controls, respectively. A malarial antigen, MSP1 (22), was used to establish CD4⁺ T-cell clones of unrelated specificity from a healthy donor. Protein concentration was measured by the method of Lowry et al. (23), and a single lot of each antigen was used throughout the present study. The optimal antigen concentration was determined in our preliminary study: 10 µg/ml for Dp-c and SEA, 5 µg/ml for *Der p* II and MSP1, and 2 µg/ml for PPD (data not shown).

Peptides

On the basis of the primary sequence of *Der p* II reported by Chua et al. (13) (Fig. 1), four parts were tentatively predicted to contain possible T-cell epitopes (10, 11); D4-P19, E38-E42, D64-G67, and K109-G112. Peptides were synthesized as 15–16-mer, each of which included the predicted motif. The purity of those peptides was confirmed to be more than 90% by high-performance liquid chromatography. For the second peptide, K33-T47, we prepared overlapping synthetic 15-mer peptides from K33 with an interval of five residues. This part was also analyzed by using analog peptides with sequential substitution of a single amino acid for alanine. When the original residue was alanine, it was replaced with tyrosine. All synthetic peptides were tested at the concentration of 5 µg/ml.

T-cell clones

By the micromanipulation method described elsewhere (24), T-cell clones from two patients and a

Table 1. HLA-DRB1 allorestricted responses of human CD4⁺ T-cell clones

APC (HLA-DRB1)	Clone: BP-001 <i>Der p</i> II*	NP-15C4 <i>Der p</i> II*	NOP-1 <i>Der p</i> II*	NO-MSP1 MSP1*
Y.B. (0406/1502)	15 800 (18.6)**	900 (1.6)	9800 (9.3)	34 200 (55.2)
T.N. (0405/0901)	2200 (1.4)	6400 (15.3)**	1100 (0.7)	600 (0.6)
N.O. (1302/1502)	15 900 (16.1)	600 (0.9)	13 200 (9.4)**	22 900 (33.7)**
K.I. (1302/1502)	8600 (9.5)	500 (0.5)	7800 (7.1)	15 600 (7.1)
H.M. (1101/1502)	10 000 (11.2)	NT	NT	9700 (13.2)
I.N. (0901/1502)	9800 (13.1)	1100 (2.8)	18 300 (8.3)	10 500 (14.0)
K.Y. (0405/1502)	7800 (6.5)	5500 (19.7)	11 000 (6.7)	20 700 (21.8)
T.E. (0405/1302)	800 (0.8)	4800 (6.7)	3600 (1.9)	NT
K.H. (0406/0901)	200 (0.3)	300 (0.6)	3100 (1.3)	1100 (2.4)
M.I. (0802/0901)	600 (1.3)	500 (0.6)	900 (0.8)	800 (1.5)
H.K. (1010/1501)	1100 (2.2)	NT	NT	1200 (2.2)
T.K. (0802/0901)	800 (0.7)	800 (4.0)	2600 (2.7)	700 (0.8)
K.B. (0802/1101)	200 (0.6)	300 (0.8)	1300 (1.4)	1000 (1.7)

* Antigens used for stimulation of respective T-cell clones are shown. Results are shown in mean cpm of triplicate culture with stimulation index in parentheses.

** Proliferative responses in presence of autologous APC.

APC: antigen presenting cells; NT: not tested; cpm: count per minute.

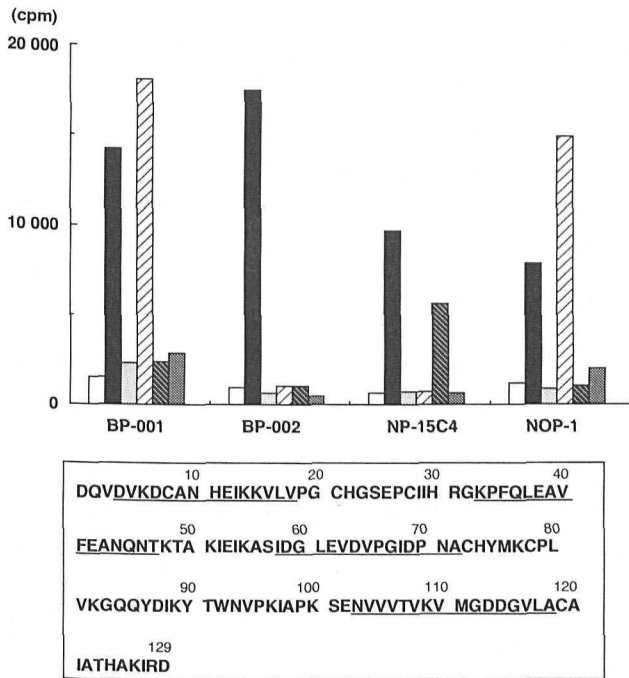


Fig. 1. Peptide-driven proliferation of *Der p II*-reactive human CD4⁺ T-cell clones obtained from allergic or healthy donors. BP-001, BP-002, and NP-15C4 were from allergic patients, and NOP-1 from healthy donor. Responses to six antigens are shown; open bar: PPD; solid bar: whole *Der p II*; light-dotted bar: peptide D4-V18; light oblique-lined bar: peptide K33-T47; dark oblique-lined bar: peptide I58-A72; and dark-dotted bar: peptide N103-A118. Primary sequence of *Der p II* is shown in lower box. Peptides tested in present study are underlined.

healthy subject, and an MSP1-specific clone from the healthy donor were established *in vitro*. Surface markers of established cells were tested by flow cytometry by using anti-CD3, CD4, CD8 (Beckton Dickinson, Mountain View, CA, USA), and anti-TCR $\alpha\beta$ (T cell Diagnostics, Cambridge, MA, USA) monoclonal antibodies (mAb).

Functional analysis of T-cell clones

T-cell function was tested by antigen-driven proliferation and cytokine production. We determined the antigen specificity of the T cells by proliferative response and/or cytokine production in response to appropriate antigens. Proliferation was tested by the method described elsewhere (25), and a tentative cutoff of positive response was set at a stimulation index (SI) of 5.0. SI was calculated as follows: mean cpm of test culture/mean cpm of antigen-free culture. HLA-restriction in T-cell response was tested with a panel of allogeneic antigen-presenting cells (APC) or HLA-DR-transfected mouse L cells (26). We measured antigen-driven production of interleukin-4 (IL-4), IL-5, and interferon-gamma (IFN- γ) *in vitro*. T-cell clones were incubated in RPMI 1640 supplemented with 10% heat-

inactivated pooled human male sera (HS) at the concentration of 1×10^6 /ml with 5×10^6 /ml of autologous or HLA-DR-sharing allogeneic APC in 24-well plates (Corning, Corning, CA, USA). After a 48-h incubation in the presence or absence of antigen, supernatants were collected. IL-4 was assessed by an EIA kit (R & D Corp., Minneapolis, MN, USA), IL-5 by the enzyme-linked immunosorbent assay (27), and IFN- γ by an RIA kit (Centcor, Malvern, MA, USA), according to manufacturers' instructions.

Inhibition of T-cell proliferation by anti-HLA mAb

Inhibitory effects of anti-HLA class II mAb on antigen-driven T-cell proliferation were tested. We used HU-4 as anti-HLA-DR and HU-11 as anti-HLA-DQ (28), both of which were in culture-supernatant form. Each mAb was added to the T-cell culture at the concentration of 5–20% (v/v), and inhibition of ³H-labeled thymidine-uptake was compared with mAb-free culture.

Competitive inhibition of T-cell proliferation by pretreatment of APC with HLA-DR-binding peptides

Analog peptides of the HLA-DRB1*1502-restricted peptide were cocultured at the concentration of 50 μ M overnight with DRB1*1502-positive APC in 10% HS RPMI 1640, and then the APC were added to an HLA-DRB1*1502-restricted T-cell clone specific for MSP1. MSP1-driven T-cell proliferation was compared between the APC with and without pretreatment with those peptides.

Statistical analysis

Statistical analysis was done by Student's *t*-test, and significance was determined with $P < 0.05$.

Results

Der p II peptide-reactive T-cell clones

Peripheral blood mononuclear cells of both the patients and a healthy subject showed vigorous proliferation *in vitro* to PPD, Dp-c, and *Der p II*, but not to SEA. On the other hand, established bulk lines of T cells responded only to *Der p II*, suggesting fine specificities to *Der p II* (data not shown). We isolated 48 cloned cells from the three subjects, all of which were CD3⁺4⁺8[−] and TCR $\alpha\beta$ ⁺. We prepared four synthetic *Der p II* peptides which were predicted to be possible T-cell epitopes (Fig. 1). Three out of 12 clones derived from patient T.N. were shown to be reactive to the peptide I58-C73, while seven out of 26 clones from patient

Y.B. were reactive to the peptide K33-T47. Three of 10 clones obtained from a healthy subject also responded to K33-T47. Specificities of other clones were not determined in our present study. Responding patterns of those T-cell clones to synthetic peptides were not changed at an antigen concentration of 1–80 µg/ml in our preliminary experiments (data not shown). Representative results indicating the respective fine specificities of *Der p* II-reactive T-cell clones are shown in Fig. 1.

T-cell responses to overlapping synthetic peptides

Since T-cell clones of patient Y.B. and a healthy subject recognized the same peptide, the more refined epitope was determined by testing overlapping synthetic peptides. Both of the cloned T cells showed the strongest proliferation to the peptide of K33-T47; however, those cells showed undetectable or significantly reduced responses to other ones (Fig. 2).

HLA-DR-restriction of Der p II- or MSP1-driven T-cell responses

The proliferative response of all *Der p* II-reactive T-cell clones tested was completely inhibited by addition of HU-4 at a concentration of 5% (21 430 cpm at 0% mAb vs 2190 cpm at 5% mAb; BP-001 clone), but not by HU-11 mAb even at a concentration of 20%, suggesting that those T-cell clones were HLA-DR-restricted. HU-4 did not inhibit mitogen-induced proliferative responses at the concentration of 20% (data not shown). Three proliferative clones, BP-001, NP-15C4, and NOP-1 were tested for interactions with APC of allogeneic panel cells. BP-001 and NOP-1 were reactive only in the presence of HLA-DRB1*1502-sharing APC, while NP-15C4 responded only in the presence of HLA-

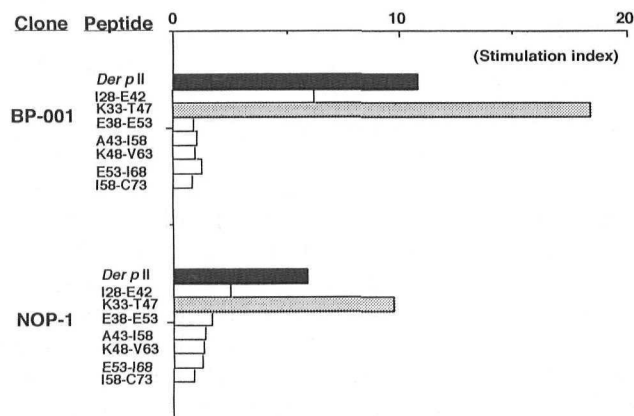


Fig. 2. *Der p* II overlapping peptide-driven proliferation of two CD4⁺ human T-cell clones. Peptide K33-T47 induced strongest proliferative responses for both T-cell clones.

DRB1*0405-sharing ones (Table 1). The MSP1-specific CD4⁺ T-cell clone was also restricted by HLA-DRB1*1502. HLA-DRB1*1502 restriction for the peptide K33-T47 was confirmed by testing HLA-DR-transfected mouse L cells because only L cells transfected with HLA-DRA and DRB1*1502 showed strong proliferation of BP-001 clone, as compared with HLA-DQ- or control DNA-transfected L cells (12 483 cpm vs 2551 and 2371 cpm, respectively).

Proliferative responses of T-cell clones to analog peptides of K33-T47

Analog peptides of K33-T47 were prepared, and were tested for induction of proliferative responses of BP-001 and NOP-1 clones. Of 15 analog peptides, stimulatory activities of five peptides (35F-A, 37L-A, 39A-Y, 41F-A, and 42E-A) were significantly diminished compared with the wild type peptide (Fig. 3).

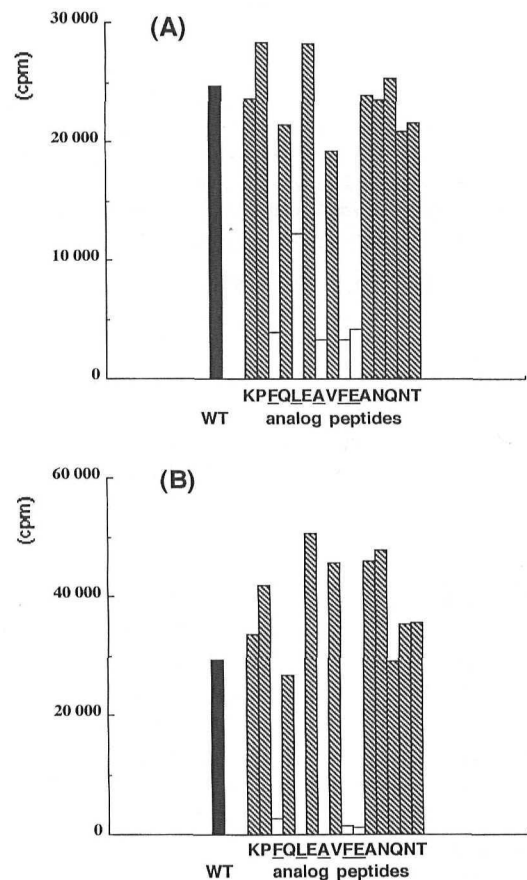


Fig. 3. Proliferative responses of A) NOP-1 and B) BP-001 clones to K33-T47 peptide (WT), as well as to its analog peptides. Fifteen analog peptides with single amino acid substitution by alanine were tested. Proliferative responses of both T-cell clones to five peptides shown as open columns (F35, L37, F41, and E42) were significantly reduced compared with responses to WT ($P < 0.05$).

Cytokine production by *Der p II*-reactive T-cell clones

We tested seven T-cell clones from patients, all of which produced elevated levels of IL-4 in response to *Der p II*, whereas IFN- γ production was weak or only marginal, suggesting that these T cells had Th2 function. Such a profile of cytokine production was not affected when nonallergic allogeneic APC were used instead of autologous (Table 2). On the other hand, cloned T cells from a healthy subject failed to produce detectable IL-4, although this T-cell clone had the same specificity as the allergic Th2 clone BP-001.

Competitive inhibition of HLA-DRB1*1502-restricted T-cell proliferation by pretreatment of APC with K33-T47 and the analog peptides

Since the K33-T47 peptide was thought to be HLA-DRB1*1502-restricted, APC with this HLA-DR type were pretreated with this peptide, and effects on T cells with the same HLA-DR allorestriction were tested. Of five analog peptides which lost stimulatory activities against the *Der p II*-specific clones, three (35F-A, 39A-Y, and 42E-A) did not show competitive inhibition against HLA-DRB1*1502-restricted T-cell responses (Fig. 4).

Discussion

Although only three individuals were tested, we identified T-cell epitopes of *Der p II* which were restricted by the Japanese-specific HLA-DRB1 alleles. Those *Der p II* peptides induced strong IL-4 and IL-5 production in allergic patients, suggesting their important role in the onset of mite allergy. In the present study, all bulk T cells induced by the

Table 2. *Der p II*-driven cytokine production by human T-cell clones obtained from allergic or healthy donors

Clone	Donor	Specificity	IL-4 (pg/ml)	IL-5 (pg/ml)	IFN- γ (U/ml)
Exp. 1					
BP-001	Allergic	K33-T47	1700	1460	<10
BP-007	Allergic	K33-T47	650	450	<10
BP-012	Allergic	K33-T47	480	ND	16
BP-002	Allergic	ND	980	ND	<10
BP-009	Allergic	ND	450	ND	<10
NP-15C4	Allergic	I58-C73	350	ND	<10
NP-46	Allergic	I58-C73	220	ND	22
NOP-1	Nonallergic	K33-T47	<10	<10	78
Exp. 2*					
BP-001	Allergic	K33-T47	1340	510	<10

* Cytokine production was tested in presence of HLA-DR-sharing allogeneic APC obtained from donor of NOP-1 clone.
ND: not determined.

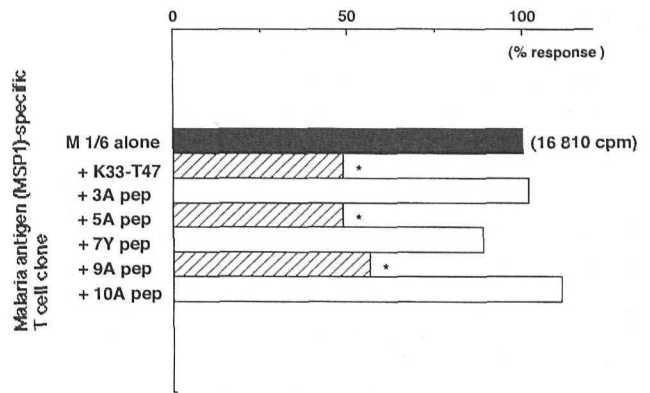


Fig. 4. Inhibition effects on MSP1-specific T-cell responsiveness by pretreatment of APC with peptide K33-T47 or its analog peptides. Of five analog peptides which lost stimulatory activities against BP-001 clone, three (open bars) did not inhibit antigen-driven proliferation of the MSP1-specific, HLA-DRB1*1502-restricted T cells, probably because these three peptides were critical binding motifs for HLA-DR15. *Significant inhibition compared with culture without *Der p II* peptides ($P < 0.05$).

stimulation of *Der p II* peptides showed allorestriction by HLA-DRB1, but we did not observe restriction by other HLA class II isotypes, as was reported in Caucasian patients (15, 29). Our data were derived from relatively few T-cell clones, and it is probable that T cells with HLA-DQ- or -DP-restriction also could be observed in the Japanese patients. Although four peptides were prepared from *Der p II* on the basis of a hypothesis of T-cell epitope prediction, one of the motif K109-G112 was not fully studied because of its solubility problems (6). In our previous population study, the peptide seemed to be less important (16). On the other hand, this part was reported to be important in the Caucasian population (15, 30).

HLA-DRB1*1502 and DRB1*0405 are virtually absent in the Caucasian population, while they are common alleles in Japan; up to 40% of the Japanese population carry one or the other of those alleles. Allospecificities of class II MHC are closely related to molecular diversities of the antigen-binding sites of MHC (8, 31). Therefore, it is possible that the present T-cell epitopes restricted by such typical Japanese HLA-DR alleles are clinically important only for Japanese patients. Our previous observation suggested that the peptide K33-T47 is commonly recognized by T cells of Japanese patients with allergic rhinitis (16). In the report by Verhoef et al., the present *Der p II* peptides were not preferentially recognized by Caucasian T cells (15). Conversely, a T-cell epitope, I29-E42, restricted by HLA-DR1 in Caucasians (30) was less stimulatory for T cells of Japanese allergic subjects. We must test many more T-cell epitopes before we can determine whether clinically important T-cell epi-

topes are so strictly determined by the HLA polymorphism.

The precise binding elements of each peptide were still not fully determined in our study. Accumulated results of studies have been uncovering HLA-DR allele-specific anchor motifs of epitope peptides, and this information is available for HLA-DR1, DR4, DR8, and others (9, 32, 33). Detailed motif analysis was reported for DRB1*0405 (33). When the hypothesis is applied to I58-C73, the DRB1*0405-restricted *Der p* II peptide, I57xxL60xV62, seems to be fitted for the binding motif of DRB1*0405-binding peptides, although the third anchor (V62) does not show such a high affinity.

In the case of HLA-DRB1*1502, the critical anchor motifs are still not fixed. Testing analog peptides of K33-T47 showed at least five amino acids to be responsible for stimulatory activities against T cells. Of those five analog peptides tested, two competitively inhibited the binding of MSP1 to HLA-DRB1*1502 molecules. On the other hand, three peptides, 35F-A, 39A-Y, and 42E-A, did not show such inhibitory effects, and this indicated that those three analog peptides lost the binding elements to HLA-DRB1*1502. We tested only alanine-substituted analog peptides, and it is likely that the molecular basis of each binding motif could be uncovered when the full set of substitution of amino acid is tested. Our observation suggested that at least those three residues might be critical as HLA-DRB1*1502-binding anchors, while 37L and 41F seemed to be TCR-binding elements. In many cases, the first anchor residues bound to HLA-DR were aromatic or hydrophobic amino acids, and this is consistent with the present observation. The observation that the peptide I28-E42 gave a weak but significant response of BP-001 clone might be explained by the possibility that all five motifs were shared with the peptide K33-T47 (Fig. 2), although other binding motifs could exist in the latter peptide.

If the three residues are anchor elements for the DRB1*1502 gene products, such motifs do not fit, so far, to any other known ones specific for particular HLA-DR allotypes (9). Moreover, the peptide K33-T47 does not express the "super motif" showing promiscuous binding to many different HLA-DR allospecificities (34). Moreover, the epitope peptide, K33-T47, is probably recognized preferentially by individuals with HLA-DRB1*1502. It is well known that this HLA-DR allotype is quite common and specific in the Japanese population; therefore, we emphasize the important role of the epitope in the pathogenesis of house-dust mite allergy in the Japanese population.

We obtained CD4⁺ T-cell clones from both healthy and allergic donors. These T cells probably recognized the same epitope of *Der p* II; however,

functional profiles were different. It was suggested that healthy subjects with HLA-DRB1*1502 might be able to respond to the allergen epitope, although these responses were less functional in allergic hypersensitivity because they seemed to be Th1-like. HLA-DRB1*1502 is one of the most common alleles in the Japanese population. This indicated that many Japanese people were able to recognize the allergen epitope; however, induction of the typical Th2 response to the epitope seemed to be controlled not only by the recognition phase, but also by other host factors. The APC of a healthy donor induced peptide-driven IL-4 production by the BP-001 clone, suggesting that APC do not have a critical role in the T-cell response after the functional category is established. Therefore, it is possible that TCR diversity strongly influences the differential categorization into Th1 or Th2. The slight difference in response of two T-cell clones, BP-001 and NOP-1, to the 37L-A analog peptide might suggest such a difference in TCR usage.

With the molecular information of helper T-cell epitopes, it seems to be possible to control allergic responses by preparing partially antagonistic peptides (35–37) or peptides inducing functional profiles of responding T cells (38). Several analog peptides in the present study, which showed clear alteration in stimulatory activities for T cells, could be candidates for prophylactic reagents for allergic hypersensitivity *in vivo*. Studies are in progress to analyze the functional alteration of *Der p* II-reactive Th2 T cells by various analog peptides in the hope of developing immunotherapy.

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