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Identification of *Dermatophagoides farinae*-2-Derived Peptides and Class II HLA Molecules Recognized by T Cells from Atopic Individuals

Abstract

Der f 2, the group 2 allergen of *Dermatophagoides farinae*, is one of the major inhalation allergens in Japan. Using the mixture of a panel of overlapping synthetic peptides that spread over the entire Der f 2 molecule, we found that polyclonal Der f 2-specific short-term T cell lines prepared from peripheral blood mononuclear cells of 5 individuals allergic to Der f who carry most of the common HLA haplotypes seen in the Japanese population can respond mainly to 7 different peptides. Distribution of the T cell epitopes on Der f 2 was not identical with that on Der p 2. Five of 7 peptides stimulated T cells of more than 2 donors, regardless of HLA types. Inhibition patterns by anti-HLA class II mAbs were heterogeneous in proliferative responses of each cell line, three were mainly inhibited by anti-HLA-DR mAb, and the others were inhibited by anti-HLA-DQ mAb. One of these T cell lines, SM, of which the proliferative response was partially inhibited by anti-HLA-DQ mAb, was cloned. Indeed, the T cell clone SM4.6 was restricted by DQ6 molecules encoded for by an HLA-DRB1*1502-DRB5*0102-DQA1*0103-DQB1*0601 haplotype. These results indicate that patients' T cells recognize Der f 2 in association with a variety of HLA-DR or HLA-DQ as antigen-presenting molecules. Thus, although some peptides do have a more potent T cell stimulatory activity than others, the TCR ligands formed with the Der f 2 molecule are highly heterogeneous, a factor also noted in Der f 1-specific T cell lines in our previous study.

Key Words

Der f 2
T cell epitope
HLA
Peptide

Introduction

Exogenous antigens are engulfed and processed by endosomal proteases to peptides in antigen-presenting cells (APC) and are presented to T cells by appropriate MHC class II molecules present on the cell surface. The CD4⁺ helper T cells are activated upon recognition of these peptide-MHC complexes by T cell receptor molecules and exert effector functions through various biological activities

of secreted lymphokines. T cell epitopes of several major allergens have been reported [1–10]. House dust mite is the most potent allergen in Japan with more than 70% of patients affected with bronchial asthma during childhood. Two mite species of the genus *Dermatophagoides*, *Dermatophagoides farinae* (Der f) and *Dermatophagoides pteronyssinus* (Der p), are frequently responsible for atopic diseases such as bronchial asthma, allergic rhinitis, and atopic dermatitis. Immunochemical and molecular studies have

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defined at least 9 *Dermatophagoides* allergens [11]. Group 1 and group 2 allergens are the most significant cause of sensitization, including IgG and IgE antibody responses in more than 80% of patients allergic to mites [12]. Group 2 allergens were not similar to the sequence of any other known protein in contrast to group 1 allergens that have similar amino acid sequences to the thiol proteases (papain and actinidin) [13]. The importance of group 2 allergens has been substantiated [14] as the antibodies to them were detected with a higher frequency than were antibodies to other groups in the patients [15]. Der f 2 and Der p 2 share the primary structure common to group 2 allergens, and therefore have similar antigenic properties. B cell epitopes on group 2 allergens have been investigated [16, 17], and T cell epitopes of Der p 2 but not Der f 2 recognized by human T cells were reported [2–4]. We established short-term Der f 2-specific polyclonal T cell lines with the same protocol as in establishing Der f 1-specific T cell lines in our recent previous study [5]. HLA-typed Japanese patients allergic to Der f were the subjects involved in the study. We identified CD4+ T cell epitopes on Der f 2 and antigen-presenting HLA class II molecules.

Materials and Methods

PBMC Donors

Diagnosis for type I allergy to house dust mite was based on clinical symptoms, positive skin test, and positive radioallergen sorbent test results. The donors selected cover frequently observed HLA-DR haplotypes in the Japanese [18]. Thus, individuals allergic to Der f with HLA-DRB1*0901 (30.3% of the general Japanese population), DRB1*0405 (27.8%), DRB1*1502 (23.7%), DRB1*0803 (17.4%), DRB1*1302 (10.7%), and DRB1*0101 (10.4%) haplotypes were selected as peripheral blood mononuclear cell (PBMC) donors. Subjects negative for these haplotypes account for about 25% in the Japanese population.

Synthesis of Der f 2-Derived Peptides

Peptides were synthesized according to amino acid sequence of Der f 2 reported previously [19, 20], using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu, Kyoto, Japan), based on Fmoc strategy. All peptides were purified by C18 reverse-phase HPLC (Millipore, Waters, Milford, Mass.).

Generation of Antigen-Specific T Cell Lines and Clones

Der f 2-specific T cell lines were established from PBMC of 5 patients with type I allergy to Der f. T cell lines were generated by stimulating PBMC (1.5×10^5 /well) with a mixture of 17 synthetic Der f 2-derived peptides at a final concentration of 0.1 μ M each in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 μ g/ml of streptomycin, and 10% pooled, heat-inactivated normal human male plasma in 96-well flat-bottomed culture plates (Falcon, Becton Dickinson, Lincoln Park, N.J.). After 7–9 days, irradiated (3,000 cGy) autologous PBMC

(1.5×10^5 /well) pulsed with the Der f 2 peptide mixture (1 μ M each for 5 h), human rIL-2 (50 U/ml), and human rIL-4 (10 U/ml) were added only to culture wells that were carrying T cell blasts and were thus maintained for another 7 days. Aliquots of growing cultures were combined as a bulk T cell line for each patient, and reactivity against the overlapping peptides was determined. Cloning was done in Terasaki plates (Nunc, Roskilde, Denmark), by limiting dilution at 0.3 cells per well in the presence of irradiated (3,000 cGy) autologous PBMC (3×10^4 /well) pulsed with Der f 2 peptide mixture (1 μ M), human rIL-2 (50 U/ml), and human rIL-4 (10 U/ml), in the same medium described above. Growing microcultures (approximately 5% of all culture wells) were then expanded at weekly intervals first in a 96-well plate, and then in a 24-well plate by feeding irradiated feeder cells (1.5×10^6 /well) pulsed with Der f 2 peptide mixture (1 μ M), in the presence of rIL-2 and rIL-4.

Antigen-Induced Proliferative Responses of the T cells

Antigen-induced proliferation of the T cell lines was assayed by culturing the T cells (3×10^4 /well) in 96-well flat-bottomed culture plates in the presence of the soluble Der f 2 peptide mixture (1 μ M each) or respective overlapping peptides (1 μ M), and 3,000 cGy-irradiated autologous PBMC (1.5×10^5 /well). Cells were cultured for 72 h, in the presence of 1 μ Ci/well of [^{3} H]TdR during the final 16-hour period, and the incorporated radioactivity was measured by liquid scintillation counting. To determine restriction molecules for antigen presentation, the T cell lines were cultured with irradiated autologous PBMC, with or without anti-HLA class II monoclonal antibodies (mAbs) HU-4 (anti-HLA-DRB1+DRB5 IgG2a, monomorphic) [21, 22], L243 (anti-HLA-DRB1+DRB4 IgG2a, monomorphic, reactivity to DRB3 and DRB5 yet to be determined) [23], PLM16 (anti-HLA-DRB3) [24], HU-11 (anti-HLA-DQ4+DQ5+DQ6 IgG2a) [25], HU-18 (anti-HLA-DQ7+DQ8+DQ9 IgG2a) [26], HU-46 (anti-HLA-DQ4 IgG2a) [25], MAB1764 (anti-HLA-DQ IgG2a)+1a3 (anti-HLA-DQ IgG2a, monomorphic) [27], and B7/21 (anti-HLA-DP IgG1, monomorphic) [28]. Percent inhibition was calculated using the following formula: $[1 - (\text{value obtained with a peptide and an mAb} - \text{that with medium alone}) / (\text{value obtained with a peptide and without an mAb} - \text{that with medium alone})] \times 100$. Other experiments were done in which PBMC of two donors with one haplotype-shared and one donor with irrelevant HLA haplotypes were used as APC. In this experiment, allogeneic PBMC (3×10^5 /well) were cultured in plates with peptides (1 μ M each) in the culture medium, for 2 h at 37°C, then excess peptides and nonadherent cells were removed by gently washing the plates 3 times with RPMI 1640 media containing 3% human serum. The remaining adherent cells were irradiated at 3,000 cGy and used as APC. Crude extract of Der f which contains 10.6 μ g of Der f 2/mg of total protein was kindly provided by Torii Pharmaceuticals (Tokyo, Japan).

HLA Typing

HLA class II (DR, DQ) alleles were determined by hybridization of HLA-DR, DQ genes amplified by polymerase chain reaction with sequence-specific oligonucleotide probes distributed at the 11th International Histocompatibility Workshop, as described elsewhere [1]. The nomenclature of the HLA-DR, DQ alleles was according to the WHO Nomenclature Committee for factors of the HLA system [29].

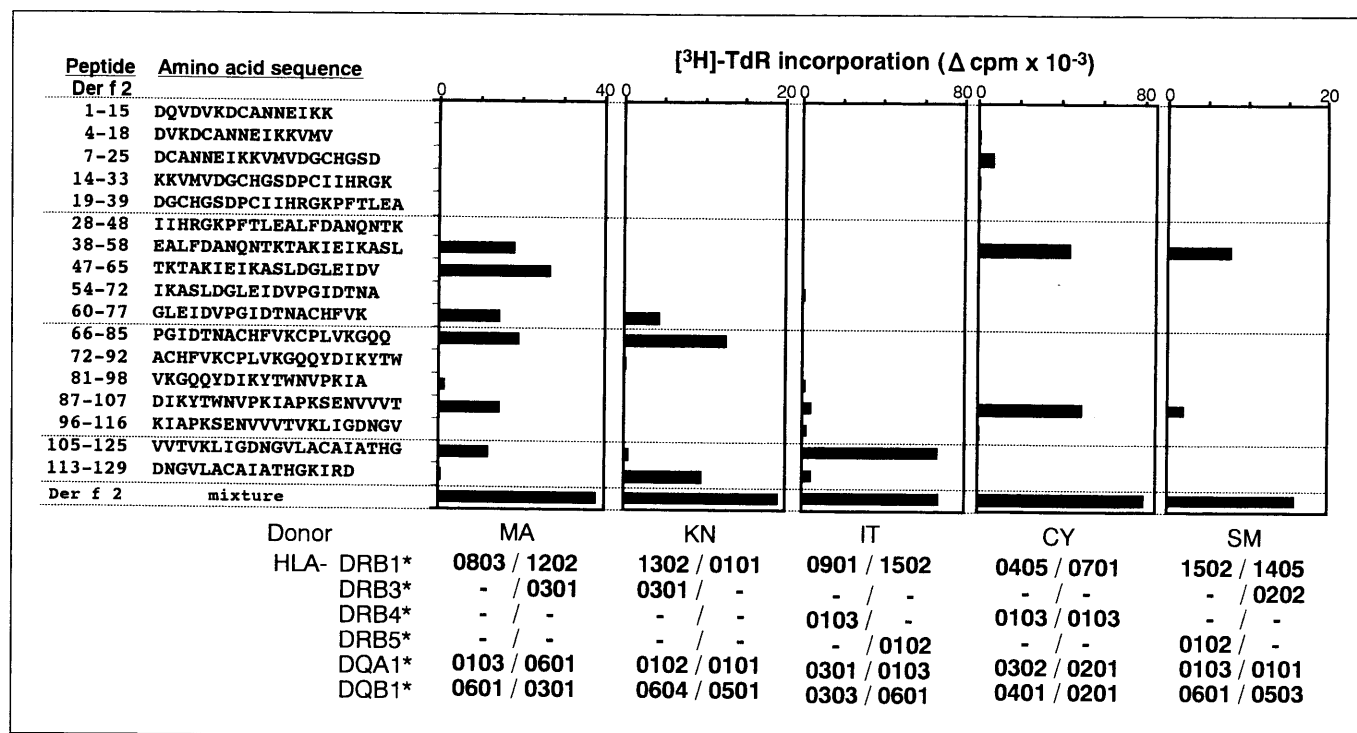


Fig. 1. Proliferative responses of five short-term T cell lines to 11- to 15-residue-overlapped synthetic peptides derived from Der f 2. Peptides are designated by indicating amino acid residue numbers of the amino- and carboxy-termini. Values shown are the mean cpm of duplicate cultures after subtraction of mean cpm obtained from culture without peptides. The standard error of duplicate culture was <20%. Cultures without peptides showed 15–1,134 cpm (MA, IT, CY, and SM) and 4,425 cpm for KN.

Results and Discussion

Der f 2 Epitopes Recognized by Polyclonal

Der f 2-Reactive T Cell Lines

Short-term Der f 2-specific T cell lines were prepared from PBMC of five atopic donors with type I allergy to Der f, using Der f 2-derived overlapping peptides. These donors carry common HLA haplotypes seen in the Japanese population. Use of such donors minimizes the possibility of deviation from mother distribution, in determining T cell epitopes [5]. These cell lines were tested for proliferative responses to a panel of overlapping synthetic peptides corresponding to the amino acid sequence of Der f 2. As shown in figure 1, seven major T cell epitopes were identified in these 5 donors (Der f 2 p38–58, 47–65, 60–77, 66–85, 87–107, 105–125, 113–129). T cell line established from donor MA showed the most heterogeneous pattern regarding the specificity. However, regardless of the HLA phenotypes, five peptides were commonly recognized by more than two different T cell lines, all of which were recognized by MA line (Der f 2 p38–58 and p87–107 by CY and SM, p60–77

and p66–85 by KN, p105–125 by IT). CY and SM reacted with similar patterns, despite the different HLA phenotypes. All these lines, when tested for reactivity to crude Der f extract, showed proliferative responses ($SI \geq 2.4$, fig. 2). When tested with an irrelevant T cell clone BC33.5 [30], the crude Der f extract did not induce proliferation, indicating that the extract does not have nonspecific stimulatory activity for T cells. Thus, these T cell lines recognize not only synthetic peptides, but also naturally processed Der f 2 peptide fragments.

Antigen-Presenting HLA Molecules

Proliferation inhibition experiments using anti-HLA class II mAbs were carried out to determine antigen-presenting molecules, as shown in figure 3. The T cell line MA was inhibited mainly by PLM16 (anti-DRB3 mAb; 47% inhibition), CY was inhibited by L243 (anti-DRB1+4 mAb; 54% inhibition) but not by HU4 (anti-DRB1+5), and KN was inhibited by HU4 (anti-DRB1+5; 36% inhibition), PLM16 (anti-DRB3; 48% inhibition), L243 (anti-DRB1+4; 57% inhibition) and B7/21 (anti-DP; 35% inhibition). Un-

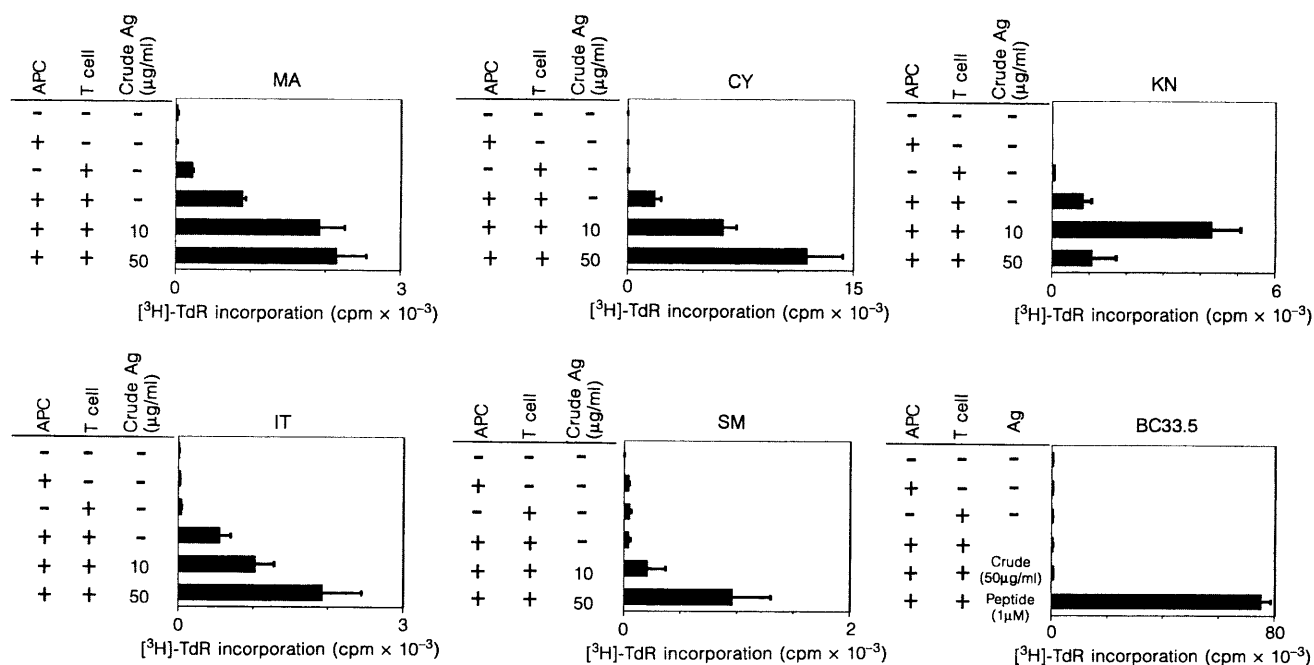


Fig. 2. Proliferative responses of the bulk T cell lines and an irrelevant T cell clone BC33.5 [specific to BCGa; see Ref. No. 30] to crude Der f antigen. Antigen-induced proliferation of T cells was assayed by culturing T cells (3×10^4 /well) in 96-well flat-bottomed culture plates in the presence of the crude Der f antigen (10 or 50 $\mu\text{g/ml}$) and 3,000 cGy-irradiated autologous PBMC (1.5×10^5 /well) for five bulk lines. The crude antigen (50 $\mu\text{g/ml}$) or BCGa peptide (1 μM of BCGap84-100; EEYLILSARDVLAVVSK) was tested for BC33.5. Results are expressed as the geometric means of triplicate determinations $\pm$ standard error.

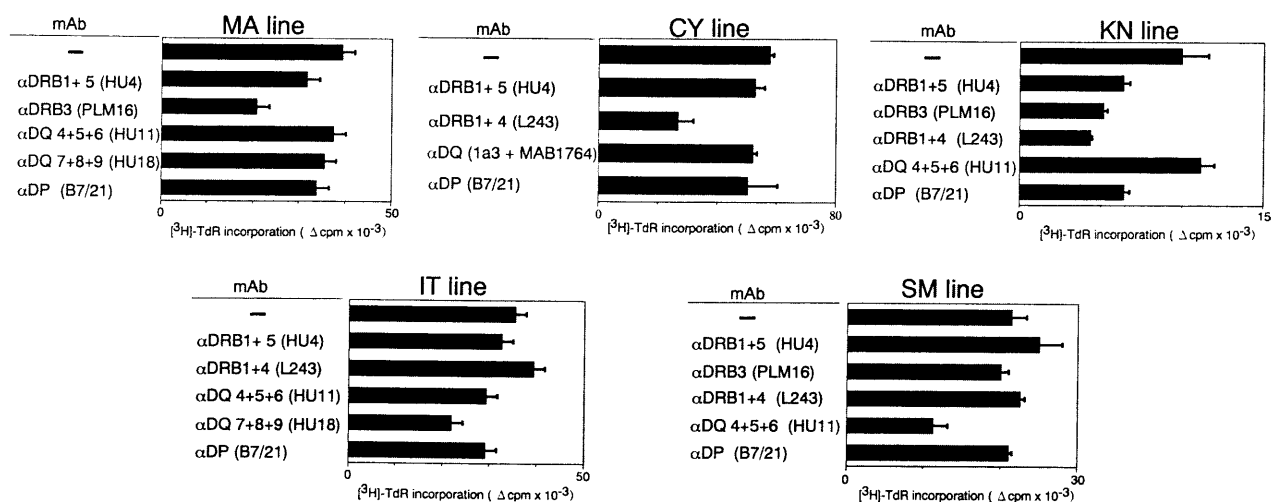


Fig. 3. Proliferation inhibition of five bulk T cell lines using anti-HLA class II mAbs. HU4 (anti-HLA-DRB1+DRB5), PLM16 (anti-HLA-DRB3), L243 (anti-HLA-DRB1+DRB4), HU11 (anti-HLA-DQ4+DQ5+DQ6), HU18 (anti-HLA-DQ7+DQ8+DQ9), HU46 (anti-HLA-DQ4), 1a3+MAB1764 (anti-DQ) and B7/21 (anti-HLA-DP) were cocultured with T cells (3×10^4 /well) and irradiated autologous PBMC (1.5×10^5 /well), with or without Der f 2 peptides (1 μM each). Results are expressed as the geometric means of triplicate determinations $\pm$ standard error. All mAbs in ascites forms were tested at a final dilution of 1:200.

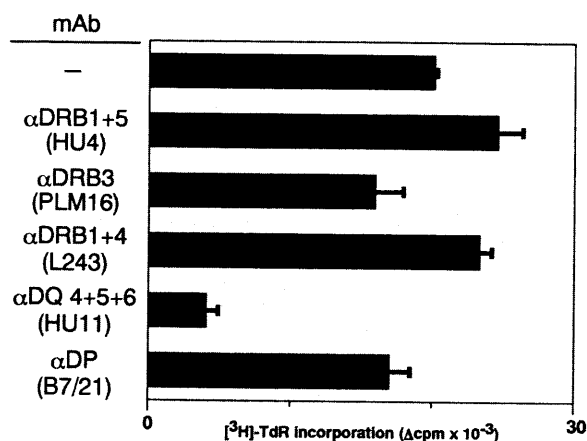


Fig. 4. Proliferation inhibition of a clone SM4.6. The clone SM4.6, generated from the SM line, was cultured in the presence of Der f 2 peptide (p38–58, 1 μM) and irradiated autologous PBMC, with or without anti-HLA class II mAbs. Results are expressed as the geometric means of triplicate determinations ± standard error. All mAbs were tested at a final dilution of 1:200.

like these three lines, IT and SM lines were not inhibited by mAbs to HLA-DR products. Thus, the addition of anti-DQ mAb HU18 (anti-DQ7+8+9) or HU11 (anti-DQ4+5+6) inhibited the proliferative responses of two cell lines of the donors IT (38% inhibition) or SM (47% inhibition), respectively. It is conceivable that DR molecules are major antigen-presenting molecules for donors MA, KN and CY, and DQ molecules for donors IT and SM. It is also important to note that CY and SM, with a similar antigen specificity, showed different HLA restriction patterns.

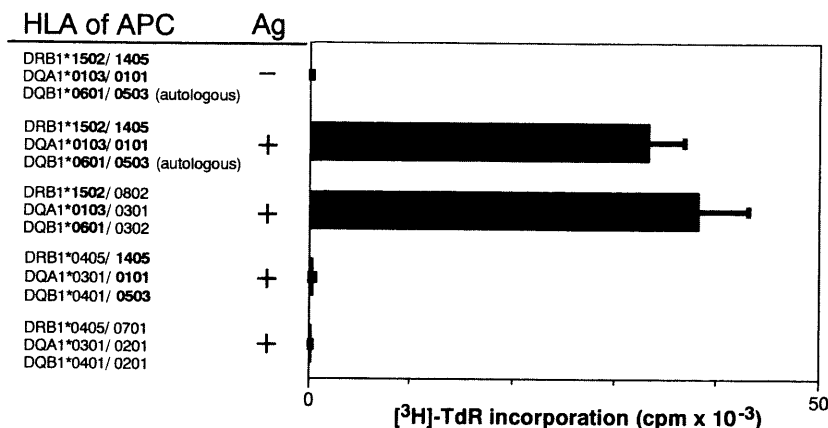
One of these T cell lines, SM, the proliferative response of which was partially inhibited only by HU11, was cloned by limiting dilution, and was investigated regarding the restriction molecules. An IL-4-producing (data not shown) and Der f 2 p38–58-specific clone SM4.6, generated from T cell line SM, was cultured in the presence of Der f 2 p38–58 and autologous PBMC with anti-HLA class II mAbs. As shown in figure 4, HU11 inhibited fully (80% inhibition) the peptide-driven proliferation of SM4.6 ($p < 0.001$). Furthermore, as shown in figure 5, SM4.6 proliferated in response to Der f 2 p38–58 presented by allogeneic PBMC which share the *HLA-DRB1*1502-DRB5*0102-DQA1*0103-DQB1*0601* haplotype alone with the donor SM. In contrast, allogeneic PBMC with another haplotype shared, and with irrelevant haplotypes did not present Der f 2 p38–58 to SM4.6. These observations indicate that the T cell clone SM4.6 recognizes Der f 2 p38–58 in the context of DQ6

(DQA1*0103/DQB1*0601), which is likely to be the major antigen-presenting molecule for the SM line. It is unclear why inhibition of a peptide-driven response of clone SM4.6 by HU11 was complete, whereas that of the SM line was only partial. HU11 might stimulate proliferative responses of nonspecific autoreactivity present in the SM line [5].

In the current study, we stimulated PBMC with overlapping peptides to establish Der f 2-specific T cell lines, and confirmed their reactivity to natural Der f 2 protein by using crude Der f extract. Although a disadvantage of this strategy might be that certain peptide-derived T cell clones are not reactive with natural protein [31], the use of crude extract is free from possible changes in the three-dimensional structure of antigens, which can be caused by the preparation of recombinant protein molecules or by purification [32], eventually affecting antigen processing in APC. Several investigators reported a variety of T cell epitopes on Der p 2 [2–4, 13, 33]. Van Neerven et al. [2] identified 10 different T cell epitopes (p1–15, p11–24, p20–33, p29–42, p38–51, p47–60, p56–69, p92–105, p101–114 and p116–129) by analysis of short-term Der p 2-specific T cell lines from five donors. O'Brien et al. [3] reported that p111–129 was recognized by 66.7% of patients, and that each of the other peptides was recognized by 8–25% of individuals. O'Hehir et al. [14] described that T cell epitopes located in two regions (11–50 and 61–104) might be immunodominant. Moreover, four distinct T cell epitopes were identified by clonal analysis, and were located within residues 16–31, 22–40, 82–100, and 111–129 [4]. All of these cited studies show that Der p 2 contains multiple T cell epitopes that may differ among individuals. Three isoforms of Der f 2 have been identified [19], which differ mutually at residues 76, 88, 111 and 125. The synthetic peptides used in this study were derived from one of these sequences, pFL1. Of four positions, residues 76 and 125 might be involved in the heterogeneity of T cell responses observed in this study, because some T cell lines proliferate whereas others do not against peptides containing these residues. These points need further investigation.

With regard to antigen-presenting HLA molecules, it was also reported that the magnitude of T cell-proliferative responses to Der p 2 was associated positively with DQ7 and negatively with DQ2 [3]. Other investigators reported that the addition of anti-DR mAb inhibited antigen-specific proliferation of all T cell clones, whereas anti-HLA-DP and anti-HLA-DQ had no effect [2]. In the present study, we found that DQ6 (DQA1*0103/DQB1*0601), one of the major HLA-DQ type found in the Japanese, yet uncommon in Caucasians, functioned as an antigen-presenting molecule for Der f 2, of which the primary structure is highly homologous to Der p 2 (87% in amino acid sequence). Of 17

Fig. 5. Antigen presentation to SM4.6 by autologous and allogeneic PBMC. Autologous or allogeneic PBMC (3×10^5 /well) were cultured in a 96-well plate with the peptide (p38–58, 4 μ M) in the culture medium, for 2 h at 37°C, then excess peptides and nonadherent cells were removed by gently washing the plate 3 times with RPMI 1640 media containing 3% human serum. The remaining adherent cells were irradiated at 3,000 cGy and used as APC. Then T cells (3×10^4 /well) were added in the culture plate. Shared HLA haplotypes are indicated with bold lettering. Results are expressed as the geometric means of triplicate determinations $\pm$ standard error.



Der f2-derived peptides fragments tested in the current study, only p81–98 and p87–107 have an amino acid sequence identical with those of Der p 2. Both fragments are reportedly stimulatory for T cells from Caucasians [2, 4], whereas p81–98 were not for T cells from Japanese patients shown in the current study. It is therefore conceivable that individuals from different ethnic groups and carrying distinct HLA phenotypes have different T cell epitopes. Another 15 fragments have different sequences from Der p 2, indicating that responses observed in the current study were not Der p 2-primed.

T cells seem to be target cells affected by successful desensitization [34], and several studies have indicated that a shift in the production of Th2 to Th1 cytokines occurs in successful immunotherapy [35–37]. However, HLA types are apparently distinct in different ethnic groups, and as demonstrated in the current study, they indeed function as Der f2-presenting molecules. In addition, the T cell epi-

topes on Der p 2 noted in Caucasians differed from those on Der f2 in Japanese populations, albeit their primary structure was highly homologous [18, 19, 38, 39]. These observations indicate that peptides for the desensitization of T cells need to be designed while keeping in mind that different ethnic groups will have different requirements.

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