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Letter to the Editor

Identification of dominant T-cell epitopes in the major Japanese cypress pollen allergen Cha o 3

Dear Editor,

Many kinds of pollen from Cupressaceae/Taxodiaceae-family trees cause pollinosis worldwide.¹ In Japan, pollens of *Cryptomeria japonica* (Japanese cedar) and *Chamaecyparis obtusa* (Japanese cypress) are the most common causes of seasonal allergic diseases.^{2,3} Recently, we identified and reported the novel major Japanese cypress pollen allergen Cha o 3⁴ and the homologous Japanese cedar pollen allergen Cry j 4,⁵ both of which belong to the cellulase family. The Cha o 3 content (approximately 75 µg/g cypress pollen) of Japanese cypress pollen was higher than that of Cry j 4 in Japanese cedar pollen.⁵ High homology (84% amino acid sequence identity) and cross-reactivity at the T-cell level were observed between Cha o 3 and Cry j 4.⁵ However, simultaneously, Cha o 3-specific T-cell clones (TCCs) that did not respond to Cry j 4 stimulation were also established, suggesting the existence of epitopes that are unique to Cha o 3.⁵ However, T-cell epitopes of Cha o 3, especially in Japanese cypress pollinosis patients, remain to be identified.

To identify T-cell epitopes of Cha o 3, 57 overlapping peptides (20-mer peptides, each overlapping by 10 amino acids, extending from Leu29 to the C-terminal Leu556 of the mature Cha o 3 protein) were designed and chemically synthesized (Supplementary Table 1). Cha o 3-specific T-cell lines (TCLs) were established via PBMCs from a total of 20 Japanese cedar/cypress pollinosis patients (Supplementary Table 3), and peptide-specific T-cell responses were evaluated using the T-cell proliferation assay. A stimulation index (SI) ≥ 2 was defined as a positive T-cell response to peptides, consistent with a previous report.⁶ All twenty of the Cha o 3-specific TCLs showed strong T-cell responses against positive control wells treated with Cha o 3 (mean SI $\pm$ SD: 74.5 $\pm$ 61.7), indicating the validity of our evaluation system (Fig. 1A). A frequent peptide-specific T-cell response was observed against several peptides. Notably, positive T-cell responses against the Cha o 3 #38, #39, and #52 peptides were obtained in 40%, 50%, and 55% of the patients, respectively (Fig. 1A). To take into account the magnitude of T-cell activation, we re-analyzed the results of the epitope mapping by calculating "positivity indices" according to the methodologies of previous reports.^{6,7} A criterion value was set using a "positivity index ≥ 100 (SI = 2 $\times$ 50% frequency)" to define the dominant T-cell epitope. The results of this reanalysis identified

Cha o 3 #39, #52, #24, #38, and #20 as dominant T-cell epitopes (Fig. 1B). The human leukocyte antigen (HLA)–DRB1 allele frequency of the 17 subjects who provided consent for HLA typing in our study was significantly correlated ($r = 0.783$ and $p = 0.0015$ by Pearson's correlation coefficient test, data not shown) with that of the general Japanese population reported previously,⁸ indicating that the dominant T-cell epitopes identified in our panel of subjects should reflect those that are allergenic in the Japanese population (Supplementary Table 2). Concurrently, we found several epitopes, separate from the five dominant T-cell epitopes, that induced strong T-cell responses; for example, Cha o 3 #13 in Subject Nos. 2 and 14, and Cha o 3 #42 in Subject Nos. 12, 17 and 18. These peptide-specific T cells may play a key role in Cha o 3 sensitization in these particular patients. Strong responders were limited in the present study; features shared among the affected patients (for example, a common HLA haplotype) will need to be investigated in future experiments.

The limitations of this study include the lack of cytokine measurements for the established Cha o 3-specific TCLs. Although almost all established allergen-specific TCLs have been classified as Th2 or Th0 in previous similar work,^{6,9,10} further experiments will be needed to confirm the Th character of the novel TCLs.

We also were intrigued to see whether the dominant T-cell epitopes of Cha o 3 were shared with those of Cry j 4. Four of five of the T-cell epitopes identified in the present study exhibited high sequence identity between the two proteins, with Cha o 3 #39, #52, #24, and #38 exhibiting 95%, 90%, 100%, and 95% identity to the respective Cry j 4 peptides (Fig. 2A). In addition, we found the possibility of T-cell cross-reactivity for Cha o 3 #52, given that the Cha o 3 #52-specific TCL responded equivalently to the corresponding segment of Cry j 4, which has deletion of two amino acids at the C-terminus (Fig. 2B). These findings suggested that the Cha o 3 #24 and #52 epitopes may be the sources of the previously reported⁵ T-cell cross-reactivity between Cha o 3 and Cry j 4, and may contribute to Th2 activation during both the Japanese cedar and cypress pollen dispersion seasons, similar to epitopes of another major Japanese cypress allergen, Cha o 1.¹⁰

On the other hand, Cha o 3 #20 and the corresponding peptide Cry j 4 #20 exhibit a lower sequence identity (75%; Fig. 2A) than was observed with the other four dominant peptides. None of the three Cha o 3 #20-specific TCCs responded to Cry j 4 #20 peptide

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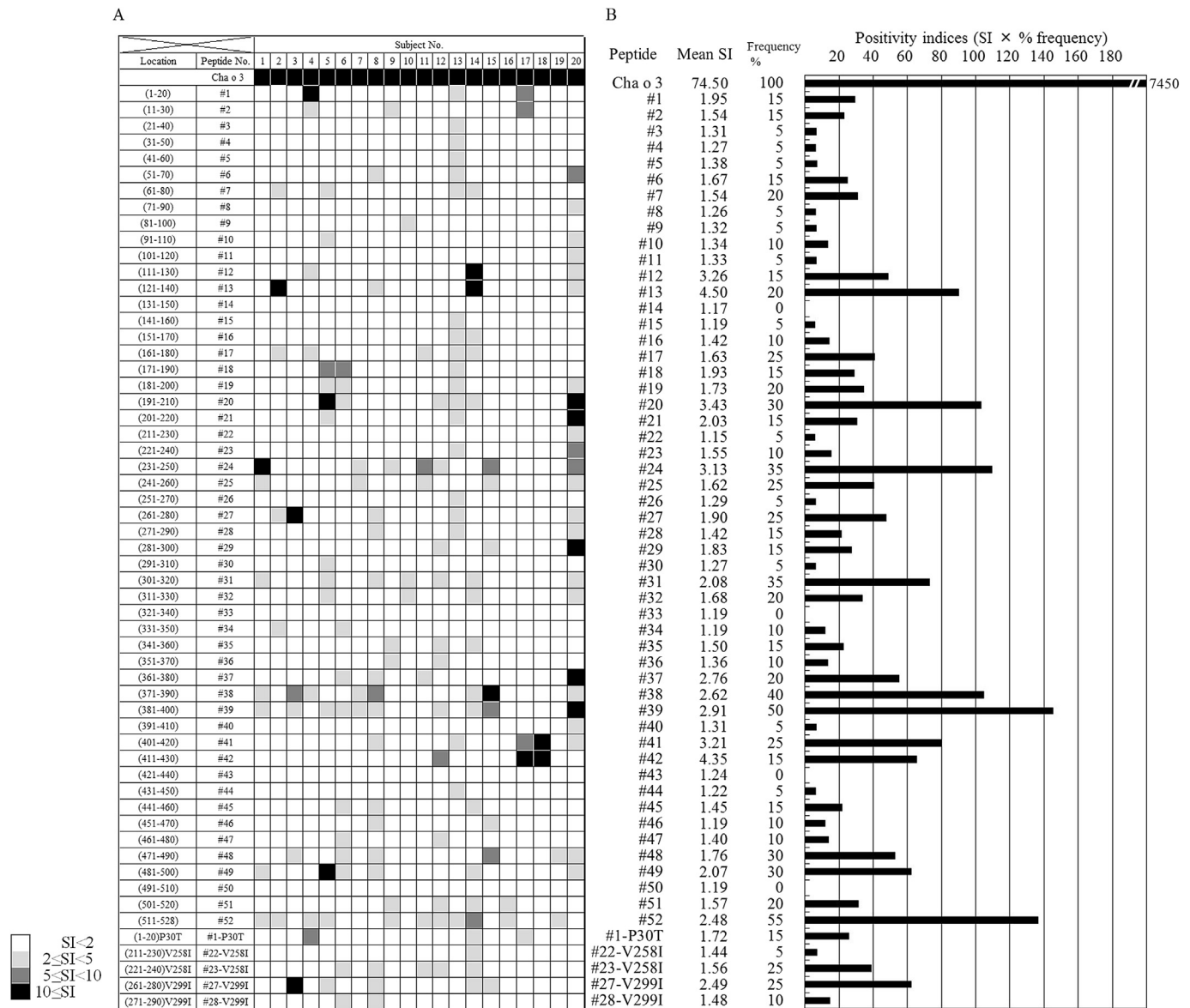


Fig. 1. T-cell epitope mapping of Cha o 3 and its dominant T-cell epitopes identified by positivity indices. PBMCs from 20 Japanese cedar and Japanese cypress pollinosis patients were employed for Cha o 3-specific TCL generation for the epitope mapping analysis. The resulting 20 TCLs were stimulated with each of the overlapping peptides constituting a total of 57 peptides spanning the mature Cha o 3 protein (Supplementary Table 1); each experiment was performed in duplicate. SI ≥ 2 was defined as a positive response (A). Positivity indices were calculated as the product of the mean SI and the positive rate % (B).

stimulation, although these TCCs were strongly activated by Cha o 3 #20 peptide treatment (Fig. 2C). Therefore, Cha o 3 #20 appears to constitute a T-cell epitope that is unique to Cha o 3. However, this study has potential limitations. Peptide-specific T cells were generated from only one patient. Those T cells were established only with Cha o 3, and not with Cry j 4. Furthermore, the cross-reactivity of Cha o 3 #38 and #39 remains unclear. To gain information about the T-cell cross-reactivity between Cha o 3 and Cry j 4, additional studies using antigen-specific T cells from multiple patients will be needed.

In summary, our data detected five dominant T-cell epitopes of the Japanese cypress pollen allergen Cha o 3 in Japanese people. Our findings are expected to facilitate the development of a novel T-cell epitope peptide-based allergen immunotherapy for Japanese cypress pollinosis. In addition, our preliminary data suggested the possible existence of dominant Cha o 3 T-cell epitopes that are both shared and distinct from those of Cry j 4. We hope that acceleration of further investigations regarding Japanese cypress pollinosis and the corresponding allergens will yield a suitable novel treatment for Japanese cypress pollinosis patients.

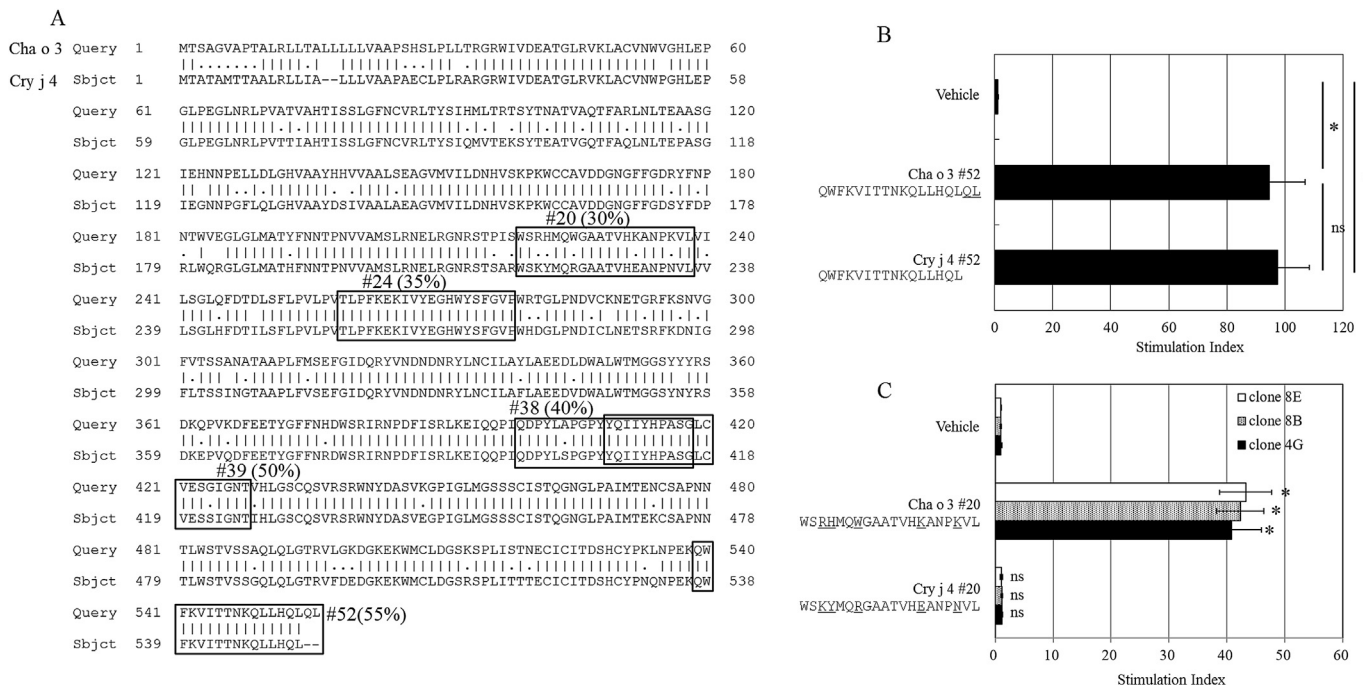


Fig. 2. Cross-reactivity of dominant Cha o 3 T-cell epitopes. (A) Comparison of dominant T-cell epitopes between Cha o 3 and Cry j 4. The boxes represent the locations of dominant T-cell epitope peptides. (%) shows the positive rate in T-cell epitope mapping (Fig. 1B). Amino acid sequences were aligned using GENETYX, ver. 12.0.4. Numbers indicate amino acid positions. Corresponding amino acid residues are linked by vertical lines. (B) Evaluation of the T-cell response to Cry j 4 #52 peptide using a Cha o 3 #52-specific TCL established from subject No.2's PBMCs. (C) T-cell response to the Cry j 4 #20 peptide using Cha o 3 #20-specific TCCs established from subject No.20's PBMCs. Proliferation was measured with a T-cell proliferation assay, as described in the Supplementary Methods. Statistical significance versus the vehicle-treated control or between indicated pairs was evaluated using the two-tailed non-paired Student's *t*-test. **p* < 0.05; ns, not significant. Data are presented as the mean $\pm$ SD from triplicate independent experiments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2019.07.008>.

Conflict of interest

TO and ES are employees of Taiho Pharmaceutical and TU is a board member of Taiho Pharmaceutical. AY has no conflict of interest.

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Supplementary Methods

Subjects

A total of 20 Japanese cedar and cypress pollinosis patients (from 20 to 60 years old) were selected from among the employees of Taiho Pharmaceutical Co., Ltd., in 2011. Positive scores (scores ≥ 2) by Japanese cedar and Japanese cypress ImmunoCAP test and clinical symptoms of seasonal allergic rhinitis suffered every spring were confirmed in the patients (Supplementary Table 3). In addition, subjects who tested positive to Cha o 3 stimulation (SI [stimulation index] ≥ 2) in a lymphocyte stimulation test were enrolled for further experiments. All studies were approved by the Research Ethics Review Committee of Taiho Pharmaceutical Co., Ltd. (Approval No. S09-002). Written informed consent was obtained from each subject.

Antigens

A panel of 57 overlapping peptides was purchased from a custom service (PepTrack Peptide Libraries) of JPT Peptide Technologies GmbH (Berlin, Germany). Peptides for TCL/TCC assays were synthesized and analyzed by Toray Research Center, Inc. (Tokyo, Japan). Cha o 3 was isolated and purified from Japanese cypress pollen as described previously¹. An additional five peptides (#1-P30T, #22-V258I, #23-V258I, #27-V299I, and #28-V299I) also were prepared to evaluate T cell recognition of peptides harboring substitutions (relative to the consensus Cha o 3 protein)².

Lymphocyte stimulation test

Between 30 to 50 mL of whole blood was drawn from each subject by venipuncture using sodium heparin as the anticoagulant. PBMCs were separated from whole blood by Ficoll-Paque density gradient centrifugation. PBMCs (2×10^5 cells/well) were stimulated with 10 $\mu\text{g/mL}$ of Cha o 3. PBMCs also were treated with Dulbecco's phosphate buffered saline (-) (D-PBS[-], Wako Pure Chemical Industries, Osaka, Japan) for negative controls. Incorporation of [^3H]-thymidine (PerkinElmer, Waltham, MA, USA) was assessed according to a standard method, as described previously¹. The stimulation index (SI) was calculated as the quotient of (mean cpm in triplicate cultures stimulated with antigen)/(mean cpm in negative control triplicate cultures). For all experiments in the present study, autologous complete medium (RPMI-1640 [Sigma-Aldrich, St. Louis, MO, USA] supplemented with heat-inactivated 10% autologous plasma and penicillin-streptomycin mixed solution [Nacalai Tesque, Kyoto, Japan]) was used.

Epitope mapping of Cha o 3

The generation of TCLs to determine Cha o 3 T cell epitopes was performed as described in the previous report³, with several modifications. Briefly, PBMCs (4×10^6 cells/well) in 2 mL of autologous complete medium were stimulated with 20 $\mu\text{g/mL}$ of Cha o 3 in a 24-well flat-bottomed plate for 7 days. After culturing for 7 days, 1 mL of supernatant was removed from each well, and 1 mL of fresh autologous complete medium containing 40 U/mL of recombinant human IL-2 (PeproTech, Rocky Hill, NJ, USA) was added for medium exchange (final IL-2 concentration: 20 U/mL). Medium exchange was performed every day for 10-12 days. Finally, TCLs were stimulated with 2 μM of overlapping peptides or 10 $\mu\text{g/mL}$ of Cha o 3 and antigen specific proliferation was measured.

Generation of Cha o 3 epitope peptide-specific TCLs and TCCs

PBMCs (1.5×10^5 cells/well) were stimulated with 1 μ M Cha o 3-derived peptide in a 96-well flat-bottomed plate for 7 days. Six days after PBMC seeding, autologous PBMCs were stimulated with 10 μ M peptide and allowed to grow overnight to prepare peptide-loaded antigen-presenting cells (APCs). After the 7-day culture, one-third of the cultured PBMCs in each well were co-cultured for 8 days with peptide-loaded APCs (1×10^5 cells/well) in the presence of 100 U/mL of recombinant human IL-2 and 50 ng/mL of recombinant human IL-4 (PeproTech). Before use in co-culturing, the APCs were washed 4 times with RPMI-1640 (to remove soluble peptides) and then irradiated (45 Gy). The remaining two-thirds of the cultured PBMCs was used for the T cell proliferation assay, in which the PBMCs in each well were stimulated with 2.5 μ M peptide to confirm the presence of peptide-specific T cells. After the 8-day culturing with peptide-loaded APCs, the content of each peptide-specific T cell-positive ($SI \geq 2$) well was collected as a peptide-specific T cell line (TCL). TCLs were maintained by once-weekly stimulation with peptide-loaded APCs in the presence of 100 U/mL of recombinant human IL-2 and 50 ng/mL of recombinant human IL-4.

TCCs were generated from TCLs by limiting dilution (at 3 or 30 cells/well) and expanded by using repeated peptide-loaded APC stimulation in the presence of 100 U/mL of recombinant human IL-2, 50 ng/mL of recombinant human IL-4, and irradiated autologous PBMCs. At the limiting dilution, the frequency of wells yielding T cells was 0.83% (3 cells/well; clone 4G) and 1.67% (30 cells/well; clones 8B and 8E). TCLs and TCCs were confirmed $CD4^+$ $TCR\alpha/\beta^+$ phenotype.

Detection of T cell proliferation

Cultured PBMCs (two-thirds volume of culturing well), TCLs (2×10^4 cells/well), or TCCs (4×10^4 cells/well) were seeded in a 96-well flat-bottomed plate with irradiated (45 Gy) autologous PBMCs (1×10^5 cells/well) in the presence of antigen stimulations and cultured for 3 overnights. T cells also were treated with D-PBS(-) for negative controls. [^3H]-thymidine (37 kBq/mL) was added to each well after 2 overnights from seeding. Proliferation of T cells was assessed by monitoring [^3H]-thymidine incorporation. Statistical significance versus the D-PBS(-) treated condition was evaluated using a two-tailed non-paired Student's t-test (JMP ver. 9.0.2, SAS Institute, Cary, NC, USA).

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Supplementary Table 1. Overlapping peptides spanning the mature Cha o 3 protein, extending from Leu29 to the C-terminal Leu556.

Location in mature Cha o 3	Peptide number	N-terminal							Amino acid residue								C-terminal						
(1-20)	#1	L	P	L	L	T	R	G	R	W	I	V	D	E	A	T	G	L	R	V	K		
(11-30)	#2	V	D	E	A	T	G	L	R	V	K	L	A	C	V	N	W	V	G	H	L		
(21-40)	#3	L	A	C	V	N	W	V	G	H	L	E	P	G	L	P	E	G	L	N	R		
(31-50)	#4	E	P	G	L	P	E	G	L	N	R	L	P	V	A	T	V	A	H	T	I		
(41-60)	#5	L	P	V	A	T	V	A	H	T	I	S	S	L	G	F	N	C	V	R	L		
(51-70)	#6	S	S	L	G	F	N	C	V	R	L	T	Y	S	I	H	M	L	T	R	T		
(61-80)	#7	T	Y	S	I	H	M	L	T	R	T	S	Y	T	N	A	T	V	A	Q	T		
(71-90)	#8	S	Y	T	N	A	T	V	A	Q	T	F	A	R	L	N	L	T	E	A	A		
(81-100)	#9	F	A	R	L	N	L	T	E	A	A	S	G	I	E	H	N	N	P	E	L		
(91-110)	#10	S	G	I	E	H	N	N	P	E	L	L	D	L	G	H	V	A	A	Y	H		
(101-120)	#11	L	D	L	G	H	V	A	A	Y	H	H	V	V	A	A	L	S	E	A	G		
(111-130)	#12	H	V	V	A	A	L	S	E	A	G	V	M	V	I	L	D	N	H	V	S		
(121-140)	#13	V	M	V	I	L	D	N	H	V	S	K	P	K	W	C	C	A	V	D	D		
(131-150)	#14	K	P	K	W	C	C	A	V	D	D	G	N	G	F	F	G	D	R	Y	F		
(141-160)	#15	G	N	G	F	F	G	D	R	Y	F	N	P	N	T	W	V	E	G	L	G		
(151-170)	#16	N	P	N	T	W	V	E	G	L	G	L	M	A	T	Y	F	N	N	T	P		
(161-180)	#17	L	M	A	T	Y	F	N	N	T	P	N	V	V	A	M	S	L	R	N	E		
(171-190)	#18	N	V	V	A	M	S	L	R	N	E	L	R	G	N	R	S	T	P	I	S		
(181-200)	#19	L	R	G	N	R	S	T	P	I	S	W	S	R	H	M	Q	W	G	A	A		
(191-210)	#20	W	S	R	H	M	Q	W	G	A	A	T	V	H	K	A	N	P	K	V	L		
(201-220)	#21	T	V	H	K	A	N	P	K	V	L	V	I	L	S	G	L	Q	F	D	T		
(211-230)	#22	V	I	L	S	G	L	Q	F	D	T	D	L	S	F	L	P	V	L	P	V		
(221-240)	#23	D	L	S	F	L	P	V	L	P	V	T	L	P	F	K	E	K	I	V	Y		
(231-250)	#24	T	L	P	F	K	E	K	I	V	Y	E	G	H	W	Y	S	F	G	V	P		
(241-260)	#25	E	G	H	W	Y	S	F	G	V	P	W	R	T	G	L	P	N	D	V	C		
(251-270)	#26	W	R	T	G	L	P	N	D	V	C	K	N	E	T	G	R	F	K	S	N		
(261-280)	#27	K	N	E	T	G	R	F	K	S	N	V	G	F	V	T	S	S	A	N	A		
(271-290)	#28	V	G	F	V	T	S	S	A	N	A	T	A	A	P	L	F	M	S	E	F		
(281-300)	#29	T	A	A	P	L	F	M	S	E	F	G	I	D	Q	R	Y	V	N	D	N		
(291-310)	#30	G	I	D	Q	R	Y	V	N	D	N	D	N	R	Y	L	N	C	I	L	A		
(301-320)	#31	D	N	R	Y	L	N	C	I	L	A	Y	L	A	E	E	D	L	D	W	A		
(311-330)	#32	Y	L	A	E	E	D	L	D	W	A	L	W	T	M	G	G	S	Y	Y	Y		
(321-340)	#33	L	W	T	M	G	G	S	Y	Y	Y	R	S	D	K	Q	P	V	K	D	F		
(331-350)	#34	R	S	D	K	Q	P	V	K	D	F	E	E	T	Y	G	F	F	N	H	D		
(341-360)	#35	E	E	T	Y	G	F	F	N	H	D	W	S	R	I	R	N	P	D	F	I		
(351-370)	#36	W	S	R	I	R	N	P	D	F	I	S	R	L	K	E	I	Q	Q	P	I		
(361-380)	#37	S	R	L	K	E	I	Q	Q	P	I	Q	D	P	Y	L	A	P	G	P	Y		
(371-390)	#38	Q	D	P	Y	L	A	P	G	P	Y	Y	Q	I	I	Y	H	P	A	S	G		
(381-400)	#39	Y	Q	I	I	Y	H	P	A	S	G	L	C	V	E	S	G	I	G	N	T		
(391-410)	#40	L	C	V	E	S	G	I	G	N	T	V	H	L	G	S	C	Q	S	V	R		

(401-420)	#41	V	H	L	G	S	C	Q	S	V	R	S	R	W	N	Y	D	A	S	V	K
(411-430)	#42	S	R	W	N	Y	D	A	S	V	K	G	P	I	G	L	M	G	S	S	S
(421-440)	#43	G	P	I	G	L	M	G	S	S	S	C	I	S	T	Q	G	N	G	L	P
(431-450)	#44	C	I	S	T	Q	G	N	G	L	P	A	I	M	T	E	N	C	S	A	P
(441-460)	#45	A	I	M	T	E	N	C	S	A	P	N	N	T	L	W	S	T	V	S	S
(451-470)	#46	N	N	T	L	W	S	T	V	S	S	A	Q	L	Q	L	G	T	R	V	L
(461-480)	#47	A	Q	L	Q	L	G	T	R	V	L	G	K	D	G	K	E	K	W	M	C
(471-490)	#48	G	K	D	G	K	E	K	W	M	C	L	D	G	S	K	S	P	L	I	S
(481-500)	#49	L	D	G	S	K	S	P	L	I	S	T	N	E	C	I	C	I	T	D	S
(491-510)	#50	T	N	E	C	I	C	I	T	D	S	H	C	Y	P	K	L	N	P	E	K
(501-520)	#51	H	C	Y	P	K	L	N	P	E	K	Q	W	F	K	V	I	T	T	N	K
(511-528)	#52	Q	W	F	K	V	I	T	T	N	K	Q	L	L	H	Q	L	Q	L		
(1-20)P30T	#1-P30T	L	T	L	L	T	R	G	R	W	I	V	D	E	A	T	G	L	R	V	K
(211-230)V258I	#22-V258I	V	I	L	S	G	L	Q	F	D	T	D	L	S	F	L	P	V	L	P	I
(221-240)V258I	#23-V258I	D	L	S	F	L	P	V	L	P	I	T	L	P	F	K	E	K	I	V	Y
(261-280)V299I	#27-V299I	K	N	E	T	G	R	F	K	S	N	I	G	F	V	T	S	S	A	N	A
(271-290)V299I	#28-V299I	I	G	F	V	T	S	S	A	N	A	T	A	A	P	L	F	M	S	E	F

In the present article, peptides were named based on the distance from Leu29; for example, a peptide corresponding to Cha o 3 residues Leu29 to Lys48 was designated as “location 1-20”. Peptides corresponding to the mature Cha o 3 protein were designed as a series of 20-mer peptides overlapping by 10 amino acids each. P30T, V258I, and V299I indicate amino acid substitutions replacing Pro30 with Thr, Val258 with Ile, and Val299 with Ile, respectively.

Supplementary Table 2. Gene frequency of HLA-DRB1 alleles for the study subjects (17 out of 20 subjects who gave consent for HLA typing) enrolled in the present study.

HLA-DRB1 allele	Subject gene frequency %	Ikeda et al gene frequency %
DRB1*01:01	5.882	5.823
DRB1*04:05	17.647	13.491
DRB1*08:02	5.882	4.207
DRB1*08:03	11.765	8.32
DRB1*09:01	8.824	14.283
DRB1*11:01	2.941	2.518
DRB1*12:01	5.882	3.774
DRB1*12:02	2.941	1.814
DRB1*13:07	2.941	0.005
DRB1*14:05	5.882	2.09
DRB1*15:01	5.882	7.778

The HLA-DRB1 genotype was assessed by DNA typing through a clinical test service conducted by SRL, Inc. (Tokyo, Japan) or ReproCELL Inc. (Kanagawa, Japan). The control HLA-DRB1 gene frequency data from a previous report⁸ (n = 19,183 in the Japanese population) was used as the control.

Supplementary Table 3. Subject background.

Subject No.	Japanese cedar		Japanese cypress		sex	Any allergic symptoms during cedar/cypress season
	score	UA/mL	score	UA/mL		
1	4	20.6	2	1.58	male	yes
2	3	9.57	2	2.31	male	yes
3	4	17.6	2	2.15	male	yes
4	6	100<	4	18.3	male	yes
5	3	13.6	2	1.77	male	yes
6	5	50.1	2	2.76	male	yes
7	4	39.3	2	3.25	male	yes
8	3	16.1	2	1.18	male	yes
9	4	48.6	3	7.94	male	yes
10	5	67.8	3	4.8	male	yes
11	6	100<	4	34.5	male	yes
12	4	19.7	2	1.37	male	yes
13	5	58.8	3	4.49	male	yes
14	5	79.4	3	5.96	male	yes
15	3	8.81	2	1.26	male	yes
16	5	72.8	3	6.91	male	yes
17	4	42.9	3	3.81	male	yes
18	4	44.4	2	2.55	female	yes
19	5	50.0	2	3.45	male	yes
20	3	14.1	2	1.09	female	yes

Japanese cedar– and Japanese cypress–specific IgE levels in subject serum were measured with the ImmunoCAP test. “Yes” subjects had allergic symptoms during the Japanese cedar and Japanese cypress dispersion season.