

Preclinical evaluation of an immunotherapeutic peptide comprising 7 T-cell determinants of Cry j 1 and Cry j 2, the major Japanese cedar pollen allergens

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Background: Peptide immunotherapy is a new approach to treating allergic diseases, but a therapeutic peptide for Japanese cedar pollinosis has not yet been developed.

Objective: The aim of this study is to prepare and preclinically evaluate a hybrid peptide comprising 7 T-cell determinants of Cry j 1 and Cry j 2, the major Japanese cedar pollen allergens.

Methods: The recombinant hybrid peptide was prepared after immunodominance of 7 T-cell determinants was confirmed by means of PBMC proliferation assay in 113 volunteers with pollinosis. The hybrid peptide was compared with a mixture of the 7 T-cell determinants in a dose-dependent PBMC proliferation assay in 6 volunteers with pollinosis. PBMC proliferation and binding activity of serum IgE antibody against the hybrid peptide, Cry j 1, and Cry j 2 were investigated in 48 volunteers with pollinosis.

Results: The hybrid peptide induced T-cell proliferation with an average 100-fold lower concentration than a mixture of the 7 peptides. PBMCs from 44 (92%) of 48 volunteers proliferated against the hybrid peptide, with significant correlation ($r = 0.87$) in T-cell proliferation against Cry j 1 and Cry j 2. No serum IgE antibodies specific to Cry j 1 or Cry j 2 bound to the hybrid peptide.

Conclusion: A hybrid peptide comprising 7 T-cell determinants has the potential for inducing T-cell proliferative responses that is superior to the potential of a mixture of the T-cell determinants and comparable with that of Cry j 1 and Cry j 2. The hybrid peptide will be of use in specific immunotherapy against Japanese cedar pollinosis. (*J Allergy Clin Immunol* 2001;108:94-100.)

Key words: Japanese cedar pollen, allergen, Cry j 1, Cry j 2, peptide, T-cell determinant, epitope, immunotherapy, IgE

Specific immunotherapy has been widely applied in the treatment of allergic diseases, and its clinical efficacy on seasonal allergic rhinitis has been reported in a World Health Organization position paper.¹ Downregulation of allergen-specific T-cell responses is thought to be an essential mechanism in this therapy.¹⁻³ Peptide-based allergen immunotherapy is a new approach to treating allergen-specific T cells, in which peptides corresponding to T-cell determinants of allergens are used.^{4,5} Clinical application of peptide immunotherapy has been in progress in several allergic diseases.⁶⁻⁸

It is common that multiple T-cell determinants exist in an allergen, and sometimes multiple allergens are involved in an allergic disease. A therapeutic peptide must contain many T-cell determinants of the involved allergens to achieve sufficient efficacy in a large population of allergic patients. Thus far, a mixture of 2 or 3 T-cell determinants has been developed,⁶⁻⁸ but the clinical efficacy of using only a few peptides has been controversial. Instead of developing mixtures of T-cell determinants, it would be more useful to develop a hybrid peptide as an immunotherapeutic peptide in which multiple T-cell determinants are linked together to make one polypeptide. Basic immunologic studies of T-cell determinant-linked peptides have been reported.⁹⁻¹¹ However, a hybrid peptide has never been applied clinically.

Japanese cedar pollinosis is one of the most common seasonal allergic diseases in Japan, with more than 10% of the population being affected. Two major allergens in Japanese cedar (*Cryptomeria japonica*) pollen were isolated and identified as Cry j 1¹² and Cry j 2.¹³ Most patients with *C. japonica* pollinosis have both high IgE titers¹⁴ and T-cell reactivity^{11,15} against Cry j 1 and Cry j 2, indicating that both allergens are important as disease-associated allergens. In a previous study, we identified 3 dominant T-cell determinants in Cry j 1 and 4 in Cry j 2.¹⁶ Thus we sought to develop a hybrid peptide in which these 7 identified T-cell determinants were linked together as a potential therapeutic agent for *C. japonica* pollinosis.

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Abbreviation used
SI: Stimulation index

METHODS

Blood donors

One hundred thirteen volunteers (67 male subjects with an average age of 42 years and 46 female subjects with an average age of 32 years) who exhibited clinical symptoms of typical seasonal *C japonica* pollinosis were recruited from our institute. The institutional ethical review board approved this study, and the volunteers participated with informed consent. Blood samples were used for this study from volunteers who were regarded as patients with *C japonica* pollinosis on the basis of confirmation both of allergen-specific IgE levels and of significant T-cell proliferation against Cry j 1, Cry j 2, or both. One hundred thirteen volunteers participated in the study in the pollen season from March to April in 1998, and 48 volunteers randomly selected from this group participated in the study in the pollen season in April 1999.

Allergens and their T-cell determinants

Cry j 1 (353 amino acids; 45 kd) and Cry j 2 (388 amino acids; 38 kd) were purified with mAbs, as described previously.¹³ Peptides corresponding to 7 T-cell determinants in Cry j 1 and Cry j 2 were synthesized by using a solid-phase peptide synthesizer, Symphony (Protein Technologies, Tucson, Ariz), which uses standard Fmoc chemistry. The peptides were purified by means of HPLC and used after verifying that the purity was greater than 95% and after validating the amino acid sequences.

Preparation of a hybrid peptide

A hybrid peptide (96 amino acids; molecular weight, 10,612) was prepared by using an *Escherichia coli* expression system. Briefly, 8 pairs of complementary DNA fragments (F1-F8c) that encode parts of the hybrid peptide (Table I) were synthesized by using a DNA synthesizer (model 394; Applied Biosystems, Foster City, Calif). These fragments were ligated with a DNA ligation kit (Takara Biomedicals, Tokyo, Japan). PCR was performed to amplify the ligated DNA fragments with primers PRv (5'-ggatccgcgt ggtatcatcg ca-3') and PFW (5'-aggctgactt acagatctgc ac-3'). These primers were designed to give rise to the *Bam*HI site at the 5' end of the fragment and the *Bgl*II and *Sal*I sites at the 3' end. The amplified DNA fragments were digested with *Bam*HI and *Sal*I and cloned into the *Bam*HI and *Sal*I sites of pUC18 to produce pUC(h7-3), which was digested with *Bam*HI and *Sal*I. The resultant fragment encoding the hybrid peptide was cloned into the *Bgl*II and *Sal*I site of pUC(h7-3) to construct pUC(h7-3)₂. The plasmid pUC(h7-3)₂ encodes a fusion peptide that has a repeat structure of the hybrid peptide with linker amino acids between and at each end of the hybrid peptides. pUC(h7-3)₂ was transformed into bacterial competent YA21 cells. After cultivation, the transformed YA21 cells were pelleted by means of centrifugation and then lysed in TRIS-HCl buffer containing 0.4% Triton X-100. The resulting crude fusion peptide was digested with trypsin. The hybrid peptide was purified by means of HPLC and was used after verifying that the purity was greater than 95% and after validating the amino acid sequence and molecular weight.

PBMC proliferation assay

PBMCs were isolated from heparinized blood by means of density centrifugation with Ficoll-Paque (Amersham Pharmacia Biotech, Tokyo, Japan). A single cell suspension was prepared in

TABLE I. Oligonucleotides used to construct the hybrid peptide

Product	Nucleotide sequence
F1	5'-gatccgcgtg gtatcatcgc agcataccag aaccggcgt cttgg-3'
F1c	5'-agatttccaa gatgccgggt tctggtatgc tgcgatgata ccacgcg-3'
F2	5'-aaatctatga aagttaccgt tgccttcaac cagttcggtc cg-3'
F2c	5'-gatgtccgga ccgaactggt tgaagcaac ggtaacttgc at-3'
F3	5'-gacatcttcg catctaaaaa ctccatctg ca-3'
F3c	5'-gttttctcgc agatggaagt ttttagatgc gaa-3'
F4	5'-gaaaaacaaa ctgacctctg gtaaatcgc atcttgc-3'
F4c	5'-gttcaggcaa gatgcgattt taccagaggt cagttt-3'
F5	5'-ctgaactacg gtctggttca tgttgcaaac acaactacg a-3'
F5c	5'-gacgggtcgt agttgttgtt tgcaacatga accagaccgt a-3'
F6	5'-ccgctcgtgt aaatcgaag gtgtaacat ctacacaaa a-3'
F6c	5'-cttctttttt ggttagatg ttaccacctt cgtattacc a-3'
F7	5'-aagaagcatt caacgttga cagttgccta aactg-3'
F7c	5'-accggtcagt ttacggaact gtccaacgt gaattg-3'
F8	5'-accggttcca ccctgatggg tctgtcagat ctgtaag-3'
F8c	5'-tcgacttaca gatctgcacg acccatcagg gtgaa-3'

RPMI-1640 medium (Life Technologies, Rockville, Md) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (Life Technologies), and 5% human AB serum (Sigma Chemical, St Louis, Mo). For assay, 4 × 10⁵ PBMCs were stimulated with peptides or allergens in 200 µL of medium in either duplicate or triplicate wells in 96-well flat-bottom plates (Corning, Corning, NY) for 3 days. Each well then received 0.5 µCi of tritiated thymidine (Amersham Pharmacia Biotech), and the cells were incubated for another 18 hours. The cells were harvested, and the incorporated radioactivity was measured by means of a liquid scintillation counter. The conditions of culture were optimized at 4 days in the pollen season. The results for some experiments are expressed as stimulation indices (SIs), which are calculated as follows: mean counts per minute in the presence of antigen divided by mean counts per minute in the absence of antigen. An SI value greater than twice the background level was considered positive.

ELISA for specific IgE

Specific IgE antibodies to the hybrid peptide and *C japonica* allergens were measured by means of ELISA, as previously described,¹⁷ with slight modifications. Briefly, microplates were coated with or without 1 µg/mL antigens. After incubation of human plasma (diluted 1:20) with the immobilized antigens or negative control wells, anti-human IgE antibody conjugated with peroxidase (International Reagents, Kobe, Japan) was added to each well. As the enzyme reaction substrate, *o*-phenylenediamine (International Reagents) was added, and the plates were incubated at 37°C for 1 hour. The resulting color development was stopped, and OD was measured at 490 nm. The results are indicated as the mean OD in duplicate wells. Positive-negative criteria for IgE-binding activity were defined as follows: mean + (3 × SD of OD in the negative control wells) (n = 48).

Statistics

For evaluating T-cell responses in the population, the geometric mean of individual SI values was calculated because converting SI values to logarithmic values normalizes the data. The Pearson correlation coefficient was calculated by using computer software (Kaleidagraph, version 3.09; Synergy software, Reading, Pa), with log (SI) values also used for this calculation.

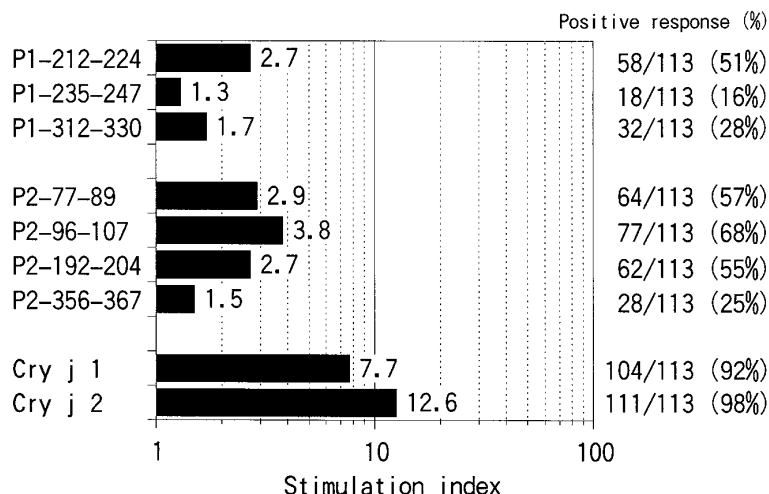


FIG 1. Proliferative responses of PBMCs against Cry j 1, Cry j 2, and the 7 T-cell determinants in the 2 allergens. Bars and adjacent numbers in the graph show geometric mean values of SIs in 113 volunteers with pollinosis. Percentage of positive (SI > 2) response is presented on the right side of the graph.

RESULTS

T-cell responses to 7 T-cell determinants in 113 patients with *C japonica* pollinosis

We first investigated T-cell responses to the 7 peptides corresponding to the 3 T-cell determinants in Cry j 1 and the 4 T-cell determinants in Cry j 2 in a large population of patients with *C japonica* pollinosis. PBMCs from 113 volunteers with *C japonica* pollinosis were stimulated in vitro with 1 μ g/mL each of the 7 T-cell determinants of Cry j 1 and Cry j 2. The results are expressed as geometric mean values of SIs for each stimulant, and the percentage of positive response (SI > 2) is also indicated (Fig 1). Among the 7 peptides, P2-96-107 from Cry j 2 showed the strongest activity for inducing T-cell proliferation, with a 68% positive response. Although P1-235-247 from Cry j 1 showed relatively weak activity, some volunteers showed strong T-cell response to it without T-cell response to the other 2 T-cell determinants of Cry j 1 (data not shown). On the basis of these results, we decided to use all of the 7 T-cell determinants for constructing a hybrid peptide.

T-cell response of hybrid peptide versus a mixture of T-cell determinants

We designed and prepared a hybrid peptide (Fig 2). We compared the hybrid peptide with a mixture of the 7 T-cell determinants in a dose-dependent T-cell proliferation assay. PBMCs from 6 volunteers with *C japonica* pollinosis were stimulated in vitro with serial concentrations of the hybrid peptide and the mixture of the 7 T-cell determinants (1 nmol/L of the mixture of the 7 T-cell determinants containing 1 nmol/L of each peptide). PBMCs from all 6 volunteers responded to the hybrid peptide with an average 100-fold lower concentration than to the mixture of the 7 T-cell determinants when the

concentration reached a sufficient level (eg, 5000 cpm for patients 1, 3, and 6; Fig 3). Thus the hybrid peptide had stronger activity for inducing T-cell proliferation than a mixture of T-cell determinants.

T-cell response and IgE-binding activity to hybrid peptide in 48 patients with *C japonica* pollinosis

We next investigated T-cell proliferation and IgE binding to the hybrid peptide in 48 volunteers with *C japonica* pollinosis and compared those values with values to Cry j 1 and Cry j 2. PBMCs from the volunteers were stimulated in vitro with 10 nmol/L of the hybrid peptide Cry j 1 and Cry j 2. Forty-four (92%) of 48 volunteers showed positive T-cell responses to the hybrid peptide (Fig 4, A), with significant correlation ($r = 0.87$) between the hybrid peptide and *C japonica* allergens. Accordingly, the geometric mean value of SI and the frequency of positive response for the hybrid peptide were comparable with those for Cry j 1 and Cry j 2 (Fig 4, B). Therefore the hybrid peptide had comparable activity with that of *C japonica* allergens for inducing T-cell proliferation.

On the other hand, IgE antibodies specific to *C japonica* allergens were evident in most of the volunteers, whereas no IgE antibody to the hybrid peptide was detected (Fig 5, A). The median values of OD and frequency of positive response (OD > 0.033) for the hybrid peptide, Cry j 1, and Cry j 2 are shown in Fig 5, B. The negative response to the hybrid peptide was not caused by lack of the hybrid peptide coated on the microplate because anti-hybrid peptide antibodies detected the peptide on the plate (data not shown). Thus the hybrid peptide did not show any binding activity to anti-*C japonica* allergen IgE antibodies.

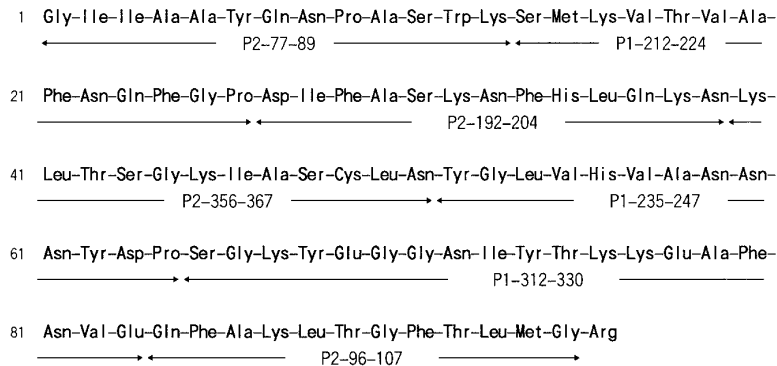


FIG 2. Amino acid sequence of the hybrid peptide. The hybrid peptide comprises 7 T-cell determinants. The position of each T-cell determinant is indicated under the sequence.

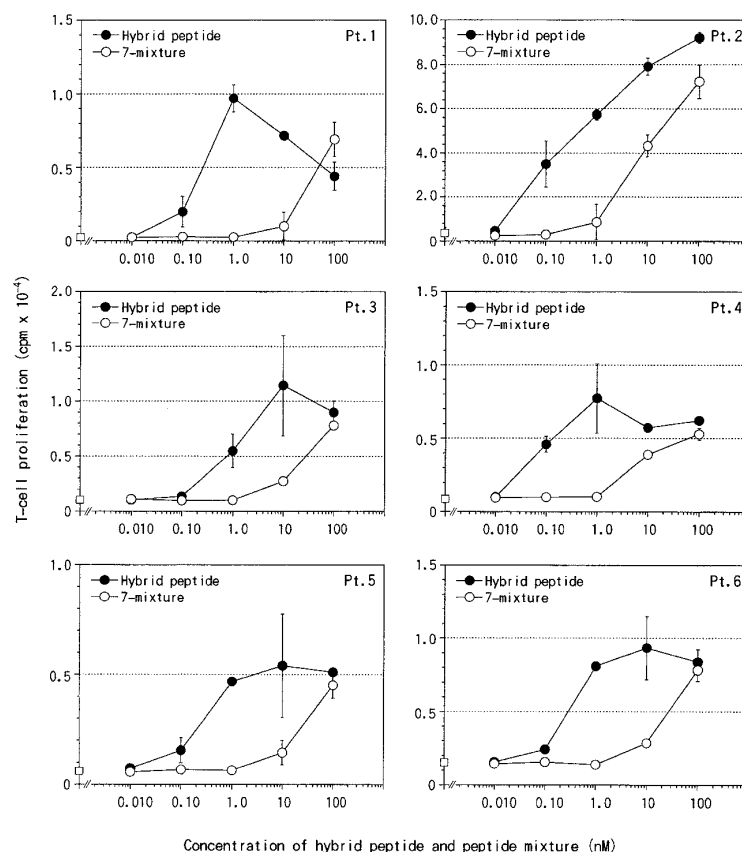


FIG 3. Comparison of the hybrid peptide with a mixture of 7 T-cell determinants to T-cell response. Proliferative responses of PBMCs from 6 volunteers with pollinosis are indicated. The results are expressed as means $\pm$ SD in counts per minute. The open square on the vertical axis shows background response.

DISCUSSION

The aim of this study was to prepare a hybrid peptide comprising 7 T-cell determinants of Cry j 1 and Cry j 2 and preclinically evaluate its potential efficacy for regulating *C japonica* allergen-specific T-cell responses by means of PBMC proliferation assay.

It is generally considered that a peptide with high activity for inducing T-cell proliferation in vitro correlates with

its ability for inhibiting T-cell responses in vivo. We previously reported that a dominant T-cell determinant of Cry j 2 induced oral tolerance in mice, in which we demonstrated that there was correlation between immunodominance of a peptide and its ability to induce immunologic tolerance.¹⁸ In this report we showed that P2-96-107 from Cry j 2 exhibited strong activity for inducing T-cell proliferation with a 68% positive response, suggesting that P2-96-

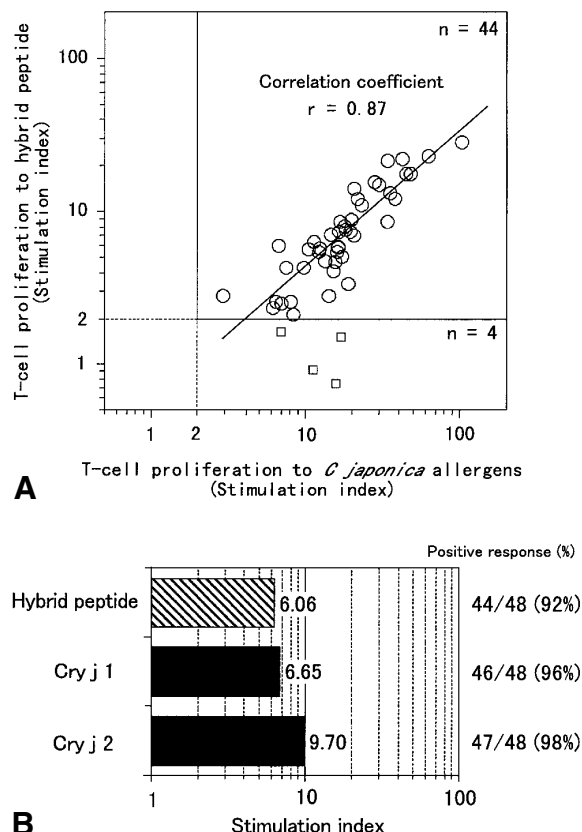


FIG 4. Proliferative responses of PBMCs from 48 volunteers with pollinosis against the hybrid peptide, Cry j 1, and Cry j 2. **A**, Individual SIs are plotted vertically for the hybrid peptide and horizontally for *C. japonica* allergens (combined SI values for Cry j 1 and Cry j 2). Pearson correlation coefficient was analyzed in the 44 positive (SI > 2) responders to the hybrid peptide. **B**, Bars and adjacent numbers in the graph show geometric mean values of SI. Percentage of positive response is presented on the right side of the graph.

107 is a clinically important T-cell determinant in the majority of patients with *C. japonica* pollinosis. We¹⁶ and others^{11,19} have demonstrated the immunodominance of P1-212-224 in Cry j 1, but no other reports^{11,20} have ever regarded P2-96-107 as a dominant T-cell determinant in Cry j 2. Because each volunteer showed different patterns in T-cell proliferation against the 7 T-cell determinants, we concluded that all 7 T-cell determinants were important for constructing the hybrid peptide.

The hybrid peptide comprises the 7 T-cell determinants in random order. In our previous study we synthesized several kinds of hybrid peptides in which 2, 3, or 4 T-cell determinants were linked in various orders with or without linkers between them. After extensive study, we found that neither order of T-cell determinants nor insertion of amino acid linkers between T-cell determinants affects activity of the hybrid peptide or its component T-cell determinants for inducing T-cell proliferation. In addition, we synthesized hybrid peptides for mice, in which 3 mouse T-cell determinants of *C. japonica* allergens were directly linked, and confirmed that each T-cell determinant keeps its immunologic tolerance-inducing

activity (unpublished data). Therefore in this study we designed and prepared a hybrid peptide in which T-cell determinants were linked in random order and without a linker between them.

It is notable that the hybrid peptide elicited T-cell proliferation in much lower doses than a mixture of T-cell determinants because the hybrid peptide may regulate *C. japonica* allergen-specific T-cell response much more efficiently than a mixture of peptides. This notion is supported by a study on mice by Falk et al,²¹ who prepared a polypeptide comprising 16 repeated copies of a T-cell determinant and found that the polypeptide induced T-cell tolerance in vivo much more efficiently than a dose-matching single T-cell determinant in addition to its greater activity for inducing T-cell proliferation in vitro. The mechanism for exceptionally strong activity of the hybrid peptide for inducing T-cell proliferation may be efficient antigen presentation of the peptide to T cells by antigen-presenting cells. Molecular association of the hybrid peptide with HLA molecules might be an important process for efficient antigen presentation, but further study is necessary to elucidate the precise mechanism. Our results suggest that the hybrid peptide has

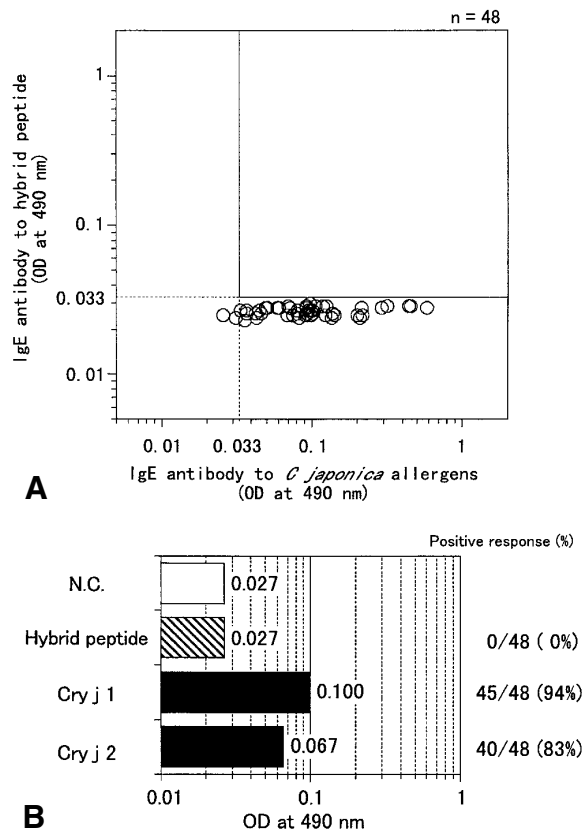


FIG 5. Binding activity of serum IgE from 48 volunteers with pollinosis to the hybrid peptide, Cry j 1, and Cry j 2. **A**, Individual ODs are plotted vertically for the hybrid peptide and horizontally for *C. japonica* allergens (combined OD values for Cry j 1 and Cry j 2). **B**, Bars and adjacent numbers in the graph show median values of OD. Positive (OD > 0.033) response is presented on the right side of the graph. The background of the data in the negative control was 0.027 ± 0.002 .

immunologic effectiveness that is superior to that of a mixture of T-cell determinants.

It was demonstrated that 92% of volunteers showed positive T-cell responses to the hybrid peptide, with significant correlation between the peptide and the *C. japonica* allergens. Because more than 90% of the population tested responded to the hybrid peptide, which is comparable with the response to Cry j 1 and Cry j 2, we believe that the hybrid peptide has sufficient potential to be used in a clinical trial. On the other hand, 4 volunteers who showed positive responses to Cry j 1, Cry j 2, or both did not show positive response to the hybrid peptide. PBMCs from these subjects also did not proliferate against any of the 7 T-cell determinants, indicating that they have T-cell determinants distinct from the selected ones. Therefore the more T-cell determinants the hybrid peptide has, the greater therapeutic efficacy it will have. We also confirmed that *C. japonica* allergen-specific IgE antibodies in the sera from the 48 volunteers did not bind to the hybrid peptide. The hybrid peptide did not inhibit IgE binding to native allergens either (data not shown). These results were consistent with another study indicating that the 7 peptides do not contain IgE-binding residues of *C. japonica*

allergens²² (data not shown). Although clinical safety of the peptide cannot be predicted, even though it does not show any IgE-binding activity in vitro, it is expected that the hybrid peptide has lower risk of causing IgE-mediated side effects than native protein allergens.

In summary, we successfully developed a hybrid peptide comprising 7 T-cell determinants of Cry j 1 and Cry j 2. The hybrid peptide has potential for inducing T-cell proliferative responses that is superior to that of a mixture of the 7 T-cell determinants and comparable with that of Cry j 1 and Cry j 2. Therefore it is expected that the hybrid peptide will downregulate *C. japonica* allergen-specific T cells more efficiently than a mixture of the peptides and will inhibit pathogenic T-cell responses as effectively as Cry j 1 and Cry j 2. We are planning a clinical study with the hybrid peptide in the near future. We believe that the hybrid peptide will be an effective immunotherapeutic peptide for treating patients with *C. japonica* pollinosis.

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