

Cry-Consensus Peptide, a Novel Peptide for Immunotherapy of Japanese Cedar Pollinosis, Induces Th1-Predominant Response in Cry j 1-Sensitized B10.S Mice

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Cry-consensus peptide (CCP) is a newly designed peptide for peptide-based immunotherapy of Japanese cedar pollinosis but its mechanism of efficacy is unknown. We investigated the effect of CCP on Cry j 1-specific Th1/Th2 response in a mice model. Subcutaneous injection of CCP decreased Cry j 1-specific IgE and IgG1 in blood slightly, but the IgG2a level was increased significantly in a dose dependent manner. Splenocytes from these mice were stimulated with Cry j 1 *in vitro*. This inhibited IL-4, IL-5 and IL-10 secretion significantly, but IFN- γ secretion was increased. *In vitro* CCP stimulation of splenocytes from Cry j 1-sensitized mice induced more marked Th1-predominancy of cytokine production than native allergen stimulation. Taken together, these data suggest that one of the mechanisms of CCP is dependent on the modulation of the antigen-specific Th1/Th2 response.

Key words immunotherapy; pollinosis; T cell epitope; Th1/Th2; cytokine

Allergy is a significant health problem, currently affecting more than 15% of children and adults worldwide and rising in incidence.¹⁾ Japanese cedar (JC) pollinosis is one of the major allergic diseases in Japan. The prevalence of atopic diseases such as pollinosis has increased during the past few decades,^{2–4)} but the reasons for this increase are still poorly understood. Anti-inflammatory drugs such as histamine release-antagonists and corticosteroids show notable clinical efficacy against allergic diseases; however, the frequent return of inflammation after the termination of drug treatments indicates the need for therapy that will interrupt additional key pathways of the allergic response. The majority of anti-allergic pharmaceuticals are efficacious in the effector phase of allergic response, mainly through the inhibition of multi-specific substances such as chemical mediators, transcription regulatory molecules and enzymes^{5,6)}; however their efficacy is often transient and these pharmaceuticals are generally ineffective in the antigen recognition and T cell proliferation/differentiation stages, and in particular are ineffective against specific IgE regulation in the immune systems.^{7,8)}

Peptide-based immunotherapy (PIT) using T-cell epitopes is a new strategy for allergic diseases or tumors. Regarding allergic diseases, PIT is expected to provide a promising allergen-specific therapy for intractable atopic diseases such as asthma^{9,10)} and pollinosis^{11,12)} through Th1/Th2 balance shift or anergy induction in antigen-specific T helper cells. Various T-cell epitope peptides have been identified for PIT, and especially in tumor immunotherapy, and their efficacy has been already reported.^{13–16)} In contrast, although analyses of T-cell epitopes in major allergens are also advancing in allergic immunotherapy, only a few cases of clinical trials have been reported.^{17–20)}

Previously, our group and others reported on T-cell epitopes of Cry j 1 and Cry j 2, which are major allergens of JC pollinosis.^{21–25)} By using the overlapping-peptide method, several T-cell epitopes have already been characterized, and their MHC class II restriction molecules and presentation to

T-cell clones have been studied.^{21,22)} Based on the above information, CCP has been newly designed as a linked peptide of T-cell epitopes.²⁶⁾ CCP contains six T-cell epitopes chosen from Cry j 1 and Cry j 2 sequences based on the incidence of T cell response in patients suffering from JC pollinosis.

The mechanism of PIT is assumed to be that of classical hyposensitization, but its details with regard to how it shifts the balance and/or induces anergy of T helper cells are still unclear and are now under investigation.^{20,27–31)} In the present study, we investigated the mechanism of CCP efficacy in B10.S mice, which respond to the N-terminal of CCP the same as humans. Cry j 1-specific IgE, IgG1 and IgG2a antibodies in serum and cytokine secretion from cultured splenocytes from Cry j 1-sensitized mice into which CCP was injected subcutaneously were studied. Moreover, the *in vitro* effects of CCP on secreted and intracellular cytokines were also examined to clarify the shift of the balance of T helper cells. Based on these findings, the mechanism of CCP is discussed in comparison with the mechanisms of other PIT.

MATERIALS AND METHODS

Culture Media and Reagents The cell culture medium used throughout was RPMI-1640 medium supplemented with 10% FCS (Filtron, Brooklyn, Australia), 1% (100 $\times$) Penicillin–Streptomycin–Glutamine, 1% MEM (100 $\times$) Non-Essential Amino Acid Solution, 1% MEM (100 $\times$) Sodium Pyruvate Solution 100 mM (the above 3 reagents from Invitrogen, Grand Island, NY, U.S.A.), and 50 mM 2-mercaptoethanol (Sigma, St. Louis, MO, U.S.A.). Cry j 1 was purified from JC pollen by our group. *E. coli*-expressed CCP was purified and provided by the bioengineering division in our company.

Sensitization of Mice and Administration of CCP B10.S mice (SLC Japan, Shizuoka, Japan) were housed in a room with a 12/12 h light/dark cycle at constant temperature (22 $\pm$ 3°C), and acclimatized to the colony room for 1 week

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with free access to standard feed and water before the experiments were performed. Cry j 1 (10 µg/0.1 ml saline/head) was subcutaneously injected 4 times together with 0.1 ml of Imject Alum (Pierce, Rockford, IL, U.S.A.) at intervals of 1 week to sensitize the B10.S mice. To determine *in vivo* efficacy, 1 week after the last sensitization, CCP (0.25 or 1 mg/0.2 ml 5% glucose/head) or vehicle (5% glucose) was challenged subcutaneously 5 times at intervals of 1 week. Six weeks after the last sensitization, Cry j 1 (10 µg/head) was injected subcutaneously to augment the antigen-specific immune response, and the sera and splenocytes were prepared.

Measurement of Cry j 1-Specific IgE, IgG1 and IgG2a Levels in the Serum Cry j 1-specific serum IgE, IgG2a were measured by the method previously described.²⁶⁾ Cry j 1-specific serum IgG1 was also assayed according to the IgG2a measurement method using peroxidase-conjugated anti-mouse IgG1 monoclonal antibody (Roche, Indianapolis, IN, U.S.A.) instead of peroxidase-conjugated anti-mouse IgG2a monoclonal antibody.

Splenocyte Culture Splenocytes were prepared under ice-chilled conditions by the following method. Spleens obtained from euthanized mice were gently homogenized through a stainless steel mesh and red blood cells were lysed in ammonium chloride hemolytic buffer. The recovered cells were washed with ice-cold medium and resuspended in culture medium. The cells were cultured in 24-well plate for 3 d at 37 °C, 5% CO₂ in the presence of 1 µM Cry j 1 or 1 µM CCP. Control cultures were incubated with no stimulation. Harvested culture supernatants were stored at -40 °C until analysis by enzyme-linked immunosorbent assay.

Cytokine ELISA Mouse IL-4, IL-5, IL-10 and IFN-γ were determined by using commercial sandwich ELISA sets (BD Pharmingen, San Diego, CA, U.S.A.) by the strategy previously described.²⁶⁾

Statistical Evaluation For comparison between the two groups, the data were firstly analyzed for homogeneity of variance using the *F* test. If the variances were homogeneous, results were evaluated using Student's *t* test. If the variances were not homogeneous, the Aspin-Welch test was performed to analyze statistical differences.

For comparison among multiple groups, the data were firstly analyzed for homogeneity of variance using Bartlett's test. If the variances were homogeneous, the parametric Scheffe multiple comparison test was performed. If the variances were found to be not homogeneous by the Bartlett's test, the non-parametric Scheffe-type multiple comparison test was performed in order to examine the significance of differences among the groups.

RESULTS

Th1/Th2 Balance of Antigen-Specific Antibodies in Serum CCP is a linked peptide of T cell epitopes chosen from Cry j 1 and Cry j 2 primary sequences (Fig. 1). The effect of subcutaneous injection of CCP on the serum level of Cry j 1-specific antibodies was evaluated by using B10.S mice. As shown in Figs. 2a and b, CCP challenge induced the inclination for decrease level of Cry j 1-specific IgE and IgG1 antibodies. On the contrary, Cry j 1-specific IgG2a antibody was clearly up-regulated by CCP challenge (Fig. 2c). Especially, at a dosage of 1 mg/head, CCP challenge resulted

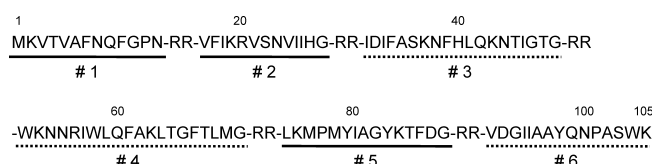


Fig. 1. Amino Acid Sequence of CCP

Solid lines (#1, #2, #5) and broken lines (#3, #4, #6) respectively indicate amino acids from the Cry j 1 and Cry j 2 sequences, with GenBank accession numbers of D26544 and D29772, respectively. Restriction molecules of these epitopes identified in pollinosis patients are the following: #1 and #4: DPB1*0501, #2: DRB5*0101, #3: DPB1*0201 / DRB4*0101 and #6: DRB5*0101 (#5: unknown). B10.S mice sharing #1 peptide with humans were used in all experiments.

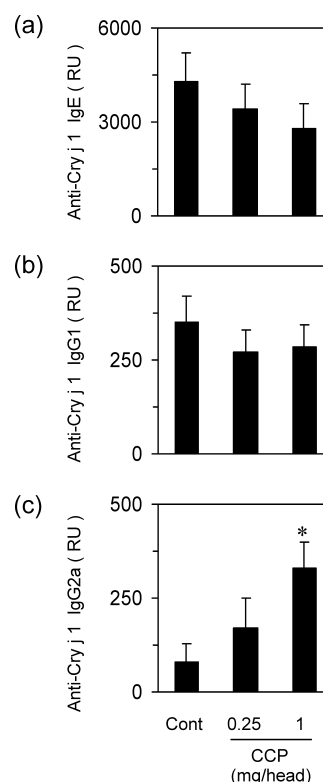


Fig. 2. Effect of CCP Challenge on Cry j 1-Specific IgE, IgG1 and IgG2a Serum Levels

Mice were subcutaneously (s.c.) sensitized with Cry j 1, then injected s.c. with CCP or vehicle challenge as described in Materials and Methods. The sera were obtained 1 week after Cry j 1 boosting, and for each mouse the Cry j 1-specific IgE, IgG1 and IgG2a levels were determined by enzyme-linked immunosorbent assay. The results shown are (a) Cry j 1-specific IgE, (b) Cry j 1-specific IgG1 and (c) Cry j 1-specific IgG2a. Columns and error bars represent the means and standard errors of 15 individual determinations, respectively. An asterisk indicates a significant difference between CCP- and vehicle-challenged mice.

in a significant increase of Cry j 1-specific IgG2a antibody in serum. Since it has been suggested that the antigen-specific IgG2a/IgG1 antibody ratio has correlation with the Th1/Th2 balance, these results suggest that the subcutaneous injection of CCP changes the antigen-specific immunoglobulin secretion from a type 2 to a type 1 response in Cry j 1-sensitized mice.

Th1/Th2 Balance of Secreted Cytokines To investigate CCP effect on antigen-specific cellular response, cytokine secretion was examined using splenocytes from the above Cry j 1-sensitized mice. Type 2 cytokines such as IL-4, IL-5 and IL-10 decreased significantly in the Cry j 1-stimulated splenocytes from CCP-challenged mice (Figs. 3a–c). On the contrary, IFN-γ was significantly greater in CCP-challenged

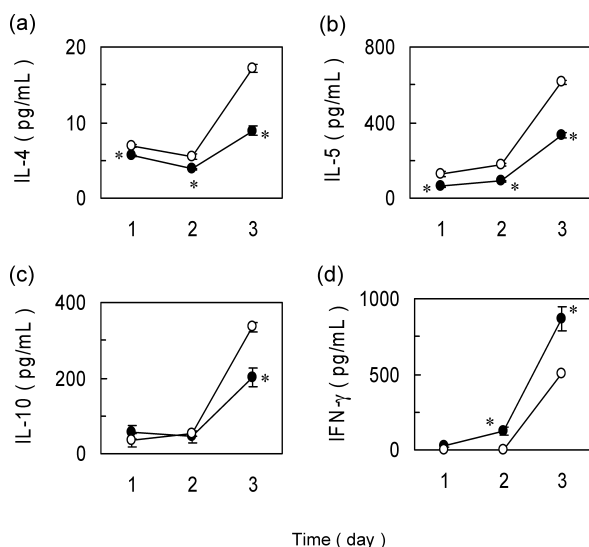


Fig. 3. Cytokine Profiles of Splenocytes from B10.S Mice Sensitized with Cry j 1

The cells were prepared from boosted mice and cultured for the indicated times in the presence of $1 \mu\text{M}$ Cry j 1 as described in Materials and Methods. The results shown are (a) IL-4, (b) IL-5, (c) IL-10 and (d) IFN- γ concentrations in the culture supernatants. Open and closed circles represent the mean value of vehicle- and CCP-challenged mice, respectively, and error bars indicate standard errors of three determinations of CCP and vehicle challenged mice, respectively. Asterisks indicate significant differences between CCP- and vehicle-challenged mice.

mice than control mice (Fig. 3d). These findings indicate that Th2 type cytokine secretion in antigen response shifted to Th1 dominant secretion after CCP challenge, in accordance with the results of the antigen-specific antibody profile.

Effects of CCP on Cytokine Production *in Vitro* The *in vitro* effect of CCP on cytokine production was also investigated with splenocytes from Cry j 1-sensitized mice. The cells were cultured in the presence of CCP or Cry j 1, and the levels of secreted cytokines were measured by ELISA. As shown in Figs. 4a–c, the production of Th2 type cytokines from CCP-stimulated cells was significantly lower than those from Cry j 1-stimulated cells. On the other hand, IFN- γ production was increased much more by CCP stimulation than by Cry j 1 stimulation (Fig. 4d). These cytokine profiles were reproducible in several independent experiments, and were secreted in a dose-dependent manner at a range from 0.01 to $1 \mu\text{M}$ of CCP or Cry j 1 (data not shown). At day 4, the IFN- γ /IL-4 ratio obtained with CCP stimulation was approximately 5-fold that obtained with Cry j 1 stimulation. These results indicate that CCP, as a T cell epitope peptide, and Cry j 1, as a native allergen, induce different T cell response *in vitro* as well as *in vivo*.

DISCUSSION

Previously, our group established various T-cell clones from peripheral blood mononuclear cells of patients suffering from JC pollinosis, and on the basis of the proliferative response of these clones and the frequency of MHC class II alleles in the pollinosis patients, six T cell epitopes were identified.^{21,22} CCP is a *de novo*-designed linear recombinant peptide combining these six T-cell epitopes as shown in Fig. 1. For PIT of allergic diseases, T-cell epitopes are promising candidates because they do not cause the severe side-effects

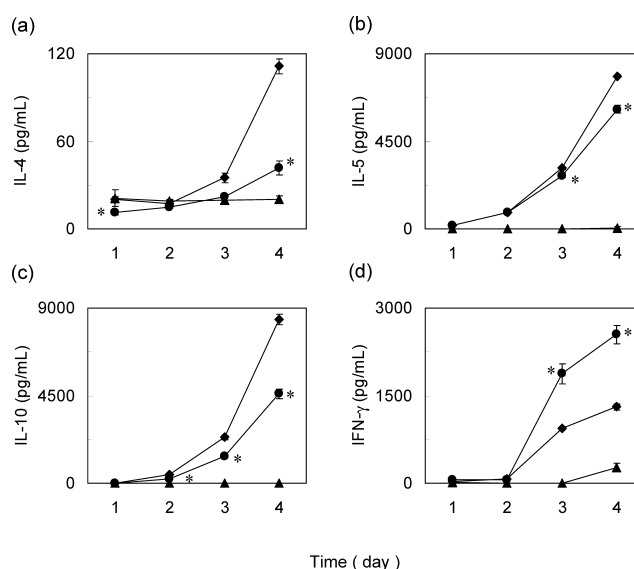


Fig. 4. Induction of Th1 Predominance *in Vitro*

The cells were prepared from boosted mice and cultured for the indicated times in the presence of $1 \mu\text{M}$ CCP or Cry j 1 as described in Materials and Methods. The data shown are the time-courses of (a) IL-4, (b) IL-5, (c) IL-10 and (d) IFN- γ production. Circles, diamonds and triangles represent CCP, Cry j 1 and control (no stimulation), respectively. Symbols and error bars indicate means and standard errors of three determinations. Asterisks indicate significant differences between CCP- and Cry j 1-stimulation.

induced by allergen extracts, such as anaphylactic shock. Several hypotheses have been proposed regarding the mechanism of PIT for allergic diseases,^{32,33} but the details are still unknown.

The sequence of CCP is specialized for PIT of JC pollinosis in humans, and thus it is hard to evaluate the efficacy of each of these T cell epitopes in animal models. In this study, we used B10.S mice, which show a unique response to the N-terminal domain of the CCP sequence (#1 in Fig. 1) for evaluation of the pharmacological effect of CCP. When CCP were injected in B10.S mice subcutaneously at the dosage of 1 mg/body, antigen-specific IgE and IgG1 antibodies decreased slightly, whereas antigen-specific IgG2a antibody increased significantly (Fig. 2). This result suggests that a shift toward Th1 predominance of the T helper cells is caused by CCP in B10.S mice sensitized with Cry j 1. The lack of significant difference in IgE and IgG1 levels may indicate that the effect of CCP in B10.S mice is less than the expected effect in humans because B10.S mice respond to only the N-terminal domain of CCP.

Injection of T-cell epitopes subcutaneously 4–6 times at intervals of 1 week or less were also effective for cat allergy.¹⁹ However, there were unexpected side effects in clinical studies, possibly because cat allergen Fel d is so small that its IgE epitopes and its T cell epitopes cannot be separated completely, and T cell peptides of cat allergens induced effects of IgE epitope sequences which they contained. There are no IgE-binding motifs of Cry j 1 and Cry j 2 in the CCP sequence, and therefore, histamine is not secreted *in vitro* by challenging peripheral basophils of patients suffering from JC pollinosis with CCP (data not shown). Moreover, CCP does not activate PBMC from healthy subjects at all (data not shown). Recently, Yoshitomi *et al.* indicated that T-cell epitopes retain their immunogenicity and tolerogenicity in PIT

when linked together in a peptide.³⁴⁾ This evidence also indicates that CCP will be useful as a new tool for PIT of JC pollinosis.

Cytokine secretion profiles from spleen cells showed inhibited Cry j 1-specific Th2 cytokine production in CCP-challenged mice (Fig. 3). In contrast, the production of a Th1 cytokine, IFN- γ , was activated by CCP injection. Similar results were also observed with *in vitro* CCP stimulation of splenocytes from Cry j 1-sensitized mice (Fig. 4). The balance shift of cytokine production is probably a crucial step in common with both the PIT using CCP and conventional immunotherapy using allergen extracts.³⁵⁾ How the Th2/Th1-shift is induced in PIT as opposed to classical hyposensitization using allergen extracts, which causes a decrease of IL-5 in humans suffering from JC pollinosis,³⁶⁾ is of great interest.

The induction of Th2/Th1-shift by CCP is also supported by intracellular cytokine analysis (data not shown). It is well known that IFN- γ and IL-4 are very important cytokines in the pathogenesis of allergic diseases. IL-4 exerts effects on naive T cells *via* the IL-4 receptor and Stat-6 signaling pathway.^{37–42)} Recently, Elser *et al.* reported that the repression of IL-4 production by IFN- γ occurred at the transcriptional level *via* the repression of IL-4 promoter activity by the IFN- γ -inducible factors IRF-1 and IRF-2.⁴³⁾ This suggests that the balance between IL-4 and IFN- γ may be regulated by IFN- γ .

In conclusion, CCP induced Th2/Th1 shift of the T helper cells in the JC pollen-sensitized mice model both *in vivo* and *in vitro*. Induction of the Th1 response by CCP might play an essential role in the efficacy of PIT. Clinical studies of CCP are now making good progress, and we expect that a Th2/Th1 shift will be induced by CCP in humans as well as in mice.

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