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Original article

Biological assay using T cell response for Cry-consensus peptide designed for the peptide-based immunotherapy of Japanese cedar pollinosis

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Abstract

Introduction: Cry-consensus peptide is a linearly linked peptide of T-cell epitopes for the management of Japanese cedar (JC) pollinosis and is expected to become a new drug for immunotherapy. However, the mechanism of T-cell epitopes in allergic diseases is not well understood, and thus, a simple in vitro procedure for evaluation of its biological activity is desired. **Methods:** Peripheral blood mononuclear cells (PBMC) were isolated from 27 JC pollinosis patients and 10 healthy subjects, and cultured in vitro for 4 days in the presence of Cry-consensus peptide and ^{3}H-thymidine. The relationship between growth stimulation (stimulation index; SI) and antigen-specific IgE levels in serum was also investigated in JC pollinosis patients. Moreover, to confirm the importance of the primary sequence in Cry-consensus peptide, heat-treated Cry-consensus peptide and a mixture of the amino acids of which Cry-consensus peptide is composed, and their ^{3}H-thymidine uptake was compared with Cry-consensus peptide. Finally, whether Cry-consensus peptide stimulates PBMCs from healthy subjects was investigated. **Results:** The mean SI of JC patients showed a good correlation with Cry-consensus peptide concentration in the culture medium; however, the SI was independent of the anti-Cry j 1 IgE level. Heat-denatured Cry-consensus peptide retained a PBMC proliferation stimulatory effect comparable to the original Cry-consensus peptide, while the mixture of amino acids constituting Cry-consensus peptide did not stimulate PBMC proliferation. PBMCs from healthy subjects did not respond to Cry-consensus peptide at all. **Discussion:** These data indicate that the PBMC response of patients suffering from JC pollinosis to Cry-consensus peptide is specific for the sequence of T cell epitopes thereof and may be useful for the evaluation of the efficacy of Cry-consensus peptide in vivo.

Keywords: Cry-consensus peptide; Immunotherapy; PBMC; Pollinosis; T-cell epitopes

1. Introduction

The efficacy of immunogenic T cell epitope or specific allergen injection immunotherapy in selected patients with IgE-mediated diseases (Frew, 1993; Malling & Weese, 1993; Müller et al., 1998; Nicodemus, Phillip, Jones, Hirani, & Norman, 1997; Norman et al., 1996) has led to considerable interest in the mechanism underlying these treatments. Immunotherapy research has provided invaluable insights into the immunologic basis of allergic disorders, and in addition, it is hoped that knowledge of these mechanisms may assist the development of more highly targeted forms of allergen and the avoidance of side effects of treatment.

Cry-consensus peptide (Fig. 1) has been developed as a candidate for peptide-based immunotherapy of JC pollinosis, and we reported previously about a determination method using a sandwich enzyme-linked immunosorbent assay (Kozutsumi et al., 2007). JC pollinosis is one of the major allergic diseases in Japan (Murasugi et al., 2005; Sakaguchi et al., 1990; Yasueda, Yui, Shimizu, & Shida, 1983), and T cell determinants of major pollen allergens, Cry j 1 and Cry j 2, have been reported by our group and others (Hashiguchi et al., 1996; Ikagawa, Matshushita, Chen, Ishikawa, & Nishimura, 1996; Ishikawa, Ikagawa, Masuyama, Matshushita, & Nishimura, 1997; Sone et al., 1998, 1999) as a new tool for immunotherapy of JC pollinosis. Recently, Takagi et al. (2005) report on the successful use of orally delivered-analogue peptide vaccine expressed in transgenic rice to reduce allergen-specific Th2 responses. By an overlapping-peptide method, several T-cell determinants of

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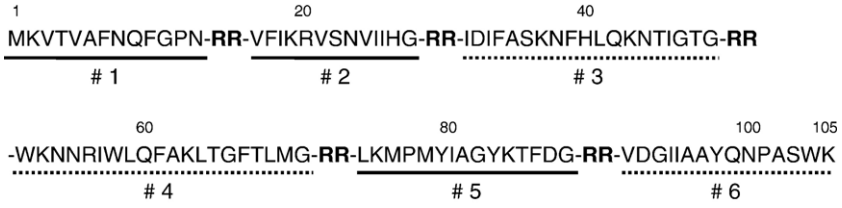

Fig. 1. Amino acid sequence of Cry-consensus peptide. Solid lines (1, 2 and 5) and broken lines (3, 4 and 6) indicate T cell epitopes derived from Cry j 1 and Cry j 2 sequences, respectively. Each epitope is linearly linked with an arginine dimer motif which is a recognition site of lysosomal cathepsin B. Restriction molecules 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).

which Cry-consensus peptide is composed have been already characterized, and their MHC class II restriction molecules have been also identified (Sone et al., 1998, 1999). As shown in Fig. 1, Cry-consensus peptide includes *human* T cell epitopes derived from Cry j 1 and Cry j 2. Each epitope is linearly linked with an arginine dimer motif which is a recognition site of cathepsin B, a lysosomal serine protease that cleaves T cell epitopes from endosomal antigens in antigen presenting cells (Authier, Kouach, & Briand, 2005). In our preliminary study, Western blotting revealed that Cry-consensus peptide was digested by cathepsin B in vitro (data not shown).

In this study, we examined the induction of T cell proliferation by Cry-consensus peptide to evaluate the biological activity of T cell epitopes in peptide-based immunotherapy. PBMCs from JC pollinosis patients and healthy subjects were used throughout all experiments because PBMCs include antigen-specific T cells acting as responders and autologous B cells acting as antigen presenting cells. PBMCs were stimulated by Cry-consensus peptide and dose-dependent PBMC proliferation was examined. The relationship between the anti-Cry j 1 IgE level in serum and the stimulation index (SI) in response to Cry-consensus peptide was also investigated to evaluate the usefulness of IgE levels as a reliable indicator of responsiveness to Cry-consensus peptide. Moreover, whether the potency of Cry-consensus peptide to induce PBMC proliferation was dependent on the primary sequence or the tertiary conformation was studied by assessing the potency of heat-denatured Cry-consensus peptide and an amino acid mixture corresponding to the composition of Cry-consensus peptide. Finally, the response of PBMCs from healthy subjects was examined to assess the specificity of the induction of PBMC proliferation by Cry-consensus peptide in JC pollinosis patients.

2. Methods

2.1. Subjects

A total of 37 subjects (27 JC pollinosis patients and 10 healthy subjects, median ages 37 and 39, ranges 29–55 years and 30–51 years, respectively) participated in this study, although not all of the experiments were conducted on each subject. All patients who were selected had typical case histories and RAST (radioallergosorbent test, Amersham Biosciences, Uppsala, Sweden) class>2 (0.70 UA/ml) in response to JC pollen allergen Cry j 1 and were otherwise healthy and taking no medication. All subjects, including healthy subjects, gave informed consent to participate in this study, which was approved by the Ethics Committee of Meiji Dairies Corporation.

2.2. Materials

Cry-consensus peptide was expressed by using *Escherichia coli* and purified in our institute. Cry j 1 was also prepared in our laboratory from JC pollen. Partial denaturization of Cry-consensus peptide was performed by gentle heating at 70 °C for 24 h. The conditioned medium used throughout this study was RPMI-1640 culture medium supplemented with 10% FCS (Filtron, Brooklyn, Australia), 1% (100×) penicillin–streptomycin–glutamine, 1% MEM (100×) non-essential amino acid solution, 1% MEM (100×) sodium pyruvate solution 100 mM (the above three reagents from Invitrogen, Grand Island, NY, USA), and 50 μM 2-mercaptoethanol (Sigma, St. Louis, MO, USA). Pokeweed mitogen (PWM) for nonspecific stimulation was purchased from Honen Corporation (Tokyo, Japan). Other reagents were of analytical grade.

2.3. Isolation of cells and cultures

Peripheral venous blood (10–60 ml) was collected into a heparinized syringe. Collected blood was centrifuged at 700×*g* for 20 min at 20 °C, and then the plasma fraction was recovered for use in PBMC culture. The cell fraction was diluted with phosphate-buffered saline (PBS, pH 7.2) to twice the initial volume and laid over Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden). Following density gradient centrifugation at 300×*g* for 35 min at 20 °C, the PBMC layer was harvested from the PBS/Ficoll-Paque interface and washed twice in RPMI-1640, and a part of the PBMC was removed to estimate the number of viable cells (cells that excluded 0.1% trypan blue). The remaining cells were then resuspended at a concentration of 5×10^6 cells/ml in the conditioned medium.

2.4. Stimulation

The cells were cultured in flat-bottomed 96-well plates at a final concentration of 5×10^5 cells/well in the presence of Cry-consensus peptide (0.04 to 25 μg/ml), Cry j 1 (10 μg/ml), PWM (0.5 μg/ml), an amino acid mixture containing L-alanine/L-glutamine/L-leucine/ L-serine/L-arginine/L-lysine/L-threonine/L-asparagine/L-glycine/L-methionine/L-tryptophan/L-asparatic