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Immunotherapy with CpG DNA conjugated with T-cell epitope peptide of an allergenic Cry j 2 protein is useful for control of allergic conditions in mice

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Abstract

Immunotherapy using T-cell epitope peptides or CpG DNA conjugated with allergenic protein is useful, although the mechanisms of these therapies differ. However, the combination of CpG DNA and peptide, but not protein, had not been documented. Therefore, we investigated CpG DNA conjugated with peptide to obtain positive synergistic effects. In the first experiment, mice were vaccinated with a conjugate of CpG DNA and Cry j 2 T-cell epitope peptide p246-259 (CpG-peptide); a mixture of CpG DNA and peptide (CpG+peptide); peptide alone, or PBS alone, and immunized with Cry j 2. In the second experiment, mice were immunized with Cry j 2 and injected with CpG-peptide, CpG+peptide, peptide only, or PBS only. In both experiments, Cry j 2-specific IgE, IL-4, and IL-5 were significantly lower in mice given CpG-peptide, versus those given CpG+peptide, peptide alone, or PBS alone. However, IgG2a, IgG2b and IFN-γ did not increase in mice injected with CpG-peptide. In the third experiment, CpG-peptide significantly attenuated nasal symptoms (sneezing and nasal rubbing) compared to CpG+peptide, peptide alone, or PBS alone. Mice were also injected with a conjugate of CpG DNA and Cry j 2 protein (CpG-Cry j 2) or CpG-peptide to compare prime responses. Mice vaccinated with CpG-Cry j 2 generated Cry j 2-specific IgG1, whereas those vaccinated with CpG-peptide did not produce IgG1. This study demonstrated, for the first time, that immunotherapy with CpG DNA conjugated with a T-cell peptide is useful in preventing and treating allergic conditions.

Keywords: CpG DNA; Peptide therapy; Allergy; Immunotherapy; Japanese cedar

1. Introduction

Allergies caused by excessive Th2 immune response, characterized by the production of IgE, IgG1, IL-4, and IL-5, are widespread. It has been reported that

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immunotherapy with T-cell epitope peptide is useful against allergic conditions [1–6]. Recent studies also indicate that conjugates of CpG DNA (oligodeoxynucleotides) that contain CpG motifs and allergenic proteins are potent adjuvants of Th1-like responses, characterized by the production of IL-12, IFN-γ and IgG2a, and the inhibition of IgE synthesis [7–13], and immunotherapy with CpG DNA and an allergenic protein is an attractive approach to treating allergic diseases. However, immunotherapy with a conjugate of CpG DNA and T-cell epitope peptide has not been documented to date. Because the mechanisms of immunotherapy with CpG DNA and immunotherapy with T-cell epitope peptide differ, combinations at low doses may be beneficial and minimize side effects, thereby offering more effective remedies.

Pollinosis caused by pollen from the Japanese cedar (*Cryptomeria japonica*) is the most widespread allergic respiratory disease in Japan, affecting over 15% of the population [14]. More than 90% of patients with Japanese cedar pollinosis have specific IgE antibodies to Cry j 2, a major allergen of the Japanese cedar [15]. Lymph node cells from BALB/c mice immunized with Cry j 2 recognize the Cry j 2 p246-259 peptide [16]. Moreover, it was reported that the administration of Cry j 2 p246-259 reduced allergic responses [6]. Cry j 2 p246-259 peptide, a T-cell epitope, is believed to regulate the allergic response to Japanese cedar pollen.

The present study investigated whether immunotherapy with a conjugate consisting of Cry j 2 T-cell epitope peptide and CpG DNA adjuvant effectively protects against Japanese cedar pollinosis. The results showed that immunotherapy with CpG DNA conjugated with T-cell peptide reduces IgE, IL-4 and IL-5 levels more effectively than therapy with peptide alone or with a mixture of CpG DNA and peptide, and that this strategy is useful for the control of allergic diseases.

2. Materials and methods

We collected pollens of Japanese cedar and purified Cry j 2 according to a method that had been described before [17]. Cry j 2 peptide p246-259 'RAEVSYVHVNGAKF' with purity in excess of 95% and CpG DNA consisting of 20 bases including 2 CpG motifs 'TCCATGACGTTCCTGACGTT' (Rikaken Co., Nagoya, Japan) were used in this study.

2.1. Preparation of conjugates

Cry j 2 peptide p246-259 was conjugated with CpG DNA using a protein–protein cross-linking kit (Molecular Probes, Eugene, OR) in accordance with the manufacturer's instructions. Briefly, maleimido groups were introduced into exposed lysine moieties of Cry j 2 peptide p246-259 by incubation with a 5 mg/ml excess of succinimidyl *trans*-4-(maleimidylmethyl)cyclohexane-1-carboxylate (SMCC) for 1.5 h, followed by purification of the spin column that had been provided with the kit. The maleimido-modified Cry j 2 peptide p246-259 (150 μg) and thiol-activated CpG DNA (50 μg) were incubated together for 3 h at room temperature. After purification with HPLC, a conjugate of CpG DNA and peptide (CpG-peptide) was confirmed by staining agarose gel with ethidium bromide. The number of CpG DNA conjugated with a peptide was estimated as the ratio of the molar CpG DNA content divided by the molar peptide content. The CpG DNA content was determined by using A260. Purified Cry j 2 (10 μg) was also conjugated with CpG-DNA (50 μg), in the same manner.

2.2. Immunization

The four protocols used in this study are shown schematically in Fig. 1. In the first experiment, 6-week-old BALB/c mice were injected subcutaneously with CpG-peptide (a conjugate containing 50 μg CpG and 150 μg peptide), CpG+ peptide (a mixture of 50 μg CpG DNA and 150 peptide), peptide alone (150 μg), or PBS alone. Starting 2 weeks later, the same mice were injected intraperitoneally with Cry j 2 (5 μg) in 100 μL of saline containing 4 mg $Al(OH)_3$ as an adjuvant; twice every 2 weeks. Two weeks after the last booster injection, the blood and spleen cells were removed.

In the second experiment, 6-week-old mice were injected intraperitoneally with Cry j 2 (5 μg) and $Al(OH)_3$ (4 mg) twice at 2-week intervals. Four weeks later, the same mice were injected subcutaneously with CpG-peptide (containing 50 μg CpG and 150 μg peptide), CpG+peptide (containing 50 μg CpG and 150 μg peptide) peptide (150 μg), or PBS twice within a 1-week interval. One week later, these mice were challenged subcutaneously with Cry j 2 (5 μg), twice within a 1-week interval. One week after the last challenge, the blood and spleen cells were removed.

In the third experiment, 6-week-old mice were injected intraperitoneally with Cry j 2 (5 μg) and $Al(OH)_3$ (4 mg) twice at 2-week intervals. Four weeks later, the same mice were injected subcutaneously with CpG-peptide (containing 50 μg CpG and 150 μg peptide), CpG+peptide (containing 50 μg CpG and 150 μg peptide), peptide alone (150 μg), or PBS alone twice within a 1-week interval. One week later, these mice were challenged intranasally on days 42 through 48 with Cry j 2 (2 μg). Immediately after the last nasal challenge, the number of sneezing and nasal rubbing movements were counted for 20 min according to the method previously described [18], and blood was removed on day 49.

In the fourth experiment, 6-week-old BALB/c mice were injected subcutaneously with CpG-peptide (containing 50 μg CpG and 10 μg peptide), CpG-Cry j 2 (containing 50 μg CpG and 10 μg Cry j 2), or PBS. Serum from the mice was collected 4 weeks later.

All mice were maintained under SPF conditions in the animal facility at Nagoya City University. The protocols were approved by the Guidelines for Care and Use of Animals at Nagoya City University. Every effort was made to minimize any discomfort of the animals.

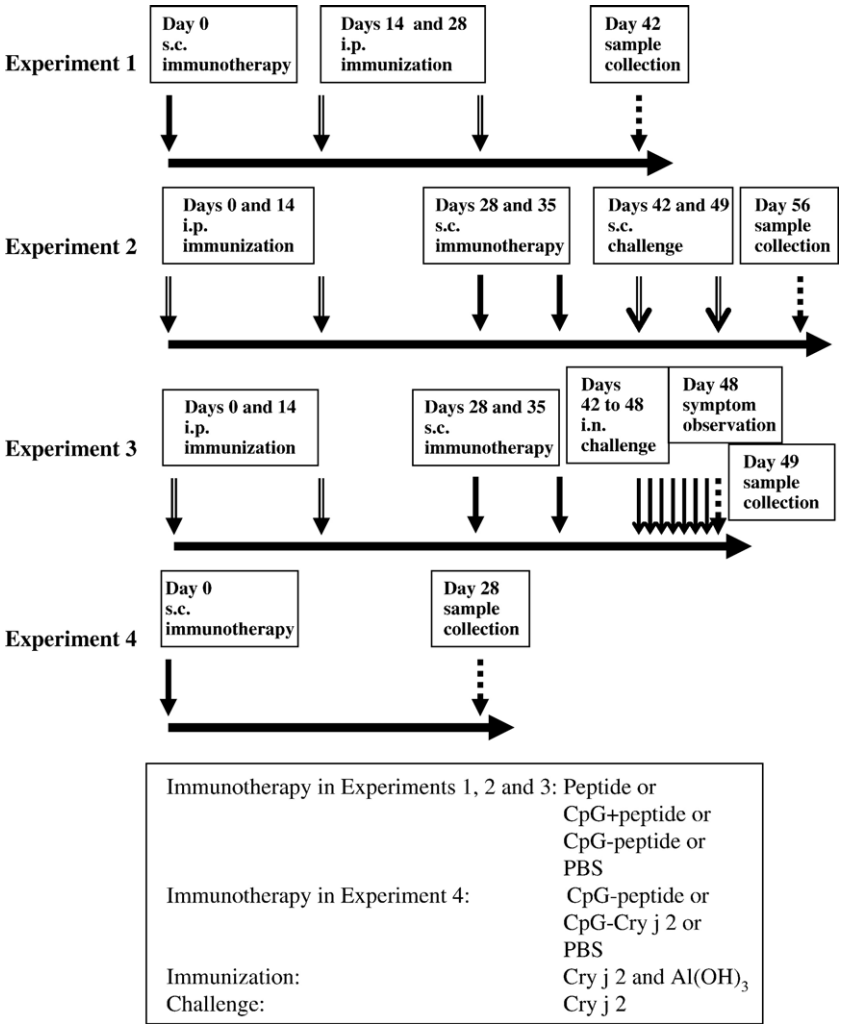

Fig. 1. Schematic protocols of Experiment 1, 2, 3 and 4. CpG-peptide and CpG-Cry j 2 stand for a conjugate of CpG DNA and Cry j 2 peptide p246-259 and a conjugate of CpG DNA and Cry j 2 protein, respectively, and CpG+peptide represents a mixture or CpG DNA and Cry j 2 peptide p246-259. All injections were given subcutaneously (s.c.), intraperitoneally (i.p.) or intranasally (i.n.).

2.3. Measurement of Cry j 2-specific IgE, IgG1, IgG2a and IgG2b

Mouse serum titers of Cry j 2-specific IgE were measured by ELISA. Briefly, ELISA plates were coated with anti-mouse IgE monoclonal antibody (1 μg/ml, Biosource International, Camarillo, CA) overnight at 4 °C. Non-specific binding was blocked, and 5-fold diluted sera were added to the plates, followed by incubation for 1 h at 37 °C. After washing and adding biotinylated Cry j 2, the plates were incubated further for 1 h at 37 °C, followed by washing and incubation with avidin-peroxidase for 1 h at 37 °C. After additional washing, the TMB microwell peroxidase substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was applied according to the manufacturer's instructions, and optical density (O.D.) was measured at 450 nm. Cry j 2 specific IgG1, IgG2a and IgG2b were measured in serum samples by ELISA using peroxidase-labeled second antibodies against each IgG subclass. ELISA plates were coated with Cry j 2 (10 μg/ml) overnight at 4 °C. Non-specific binding was blocked, and serum samples (10-fold diluted) were added to the plates and incubated for 1 h at 37 °C. The plates were washed, and peroxidase-labeled anti-mouse IgG1 (200-fold diluted; The Binding Site, San Diego, CA), IgG2a (100-fold diluted, The Binding Site, San Diego, CA) or IgG2b (100-fold diluted, Southern Biotechnology, Birmingham, AL) was added. The plates were incubated further for 1 h at 37 °C. After washing, the same substrate was applied, as described above.

2.4. Measurement of IFN-γ and IL-4 release by in vitro splenocytes

Spleen cell suspensions (2×10^6 cells/ml) were cultured for 72 h in RPMI-1640 medium containing 10% FCS, penicillin G,

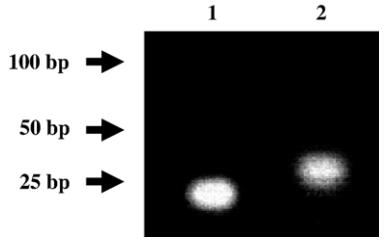

Fig. 2. Detection of a conjugate of CpG DNA and Cry j 2 peptide (CpG-peptide). CpG DNA alone and CpG-peptide were examined by electrophoresis in agarose gels and visualized by staining with ethidium bromide. Lane 1, CpG DNA; lane 2, CpG-peptide.

streptomycin, and 1 μg/ml Cry j 2. Quantities of IFN-γ, IL-4, and IL-5 in the culture supernatants were determined, using a sandwich ELISA with monoclonal antibodies specific for each cytokine. Plates were coated with anti-mouse IFN-γ, anti-mouse IL-4 (Becton Dickinson Co., Hodrege, NE), or anti-mouse IL-5 (Pharmingen, San Jose, CA). Then, the culture supernatant was added, and plates were incubated with the second antibody that is biotinylated anti-mouse IFN-γ antibody, biotinylated anti-mouse IL-4 antibody (Endogen, Woburn, MA), or biotinylated anti-mouse IL-5 antibody (Pharmingen, San Jose, CA). Standard curves were generated using recombinant cytokines.

2.5. Statistical evaluation

The mean titer and S.D. were calculated for each group, and inter-group differences were determined using Student's *t*-tests. A probability value below 0.05 was considered statistically significant.

3. Results

3.1. Characterization of a conjugate of CpG DNA and peptide (CpG-peptide)

After synthesis and purification of CpG-peptide, we detected CpG-peptide by electrophoresis on agarose gel stained with ethidium bromide (Fig. 2). The number of CpG DNA molecules conjugated to a single peptide molecule estimated from the ratio of the molar CpG DNA content to the molar peptide content revealed an average of CpG DNA per peptide molecule of 0.5.

3.2. Effect of CpG-peptide on IgE, IgG1, IgG2a and IgG2b production before sensitization (Experiment 1)

To determine the ability of CpG-peptide in preventing antibody production in response to Cry j 2 immunization, mice that had been primed with CpG-peptide, a mixture of CpG DNA and peptide (CpG+peptide), peptide alone, or PBS alone,

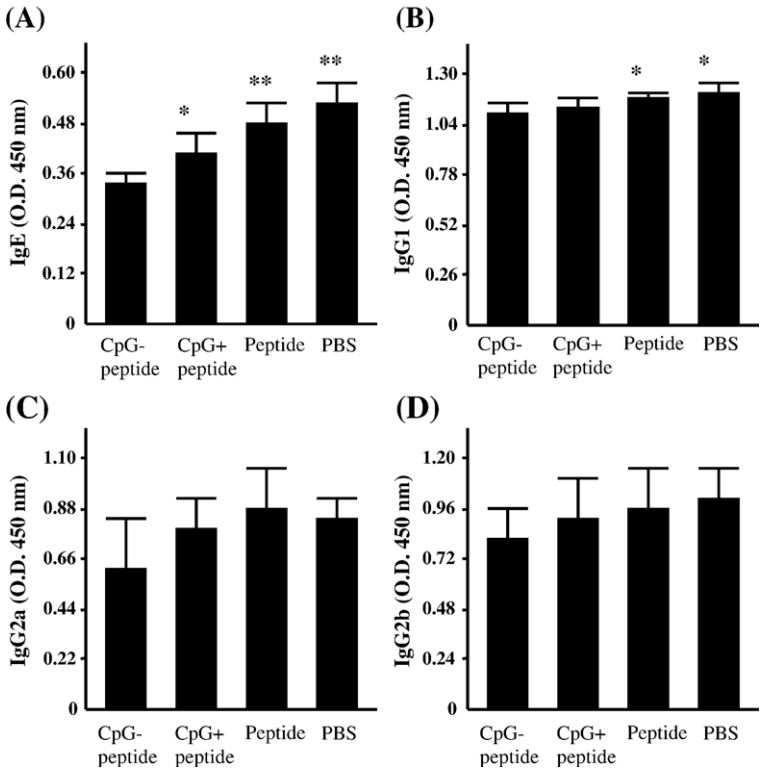

Fig. 3. Cry j 2-specific IgE (A), IgG1 (B), IgG2a (C) and IgG2b (D) levels in blood from mice immunized with Cry j 2 after vaccination. Before immunization with Cry j 2, mice ($n=5$ per group) were injected with CpG-peptide, CpG+peptide, Cry j 2 peptide alone (Peptide), or PBS alone. Levels of antibodies were measured by ELISA. Columns and vertical bars show means±S.D. *$p<0.05$ and **$p<0.01$ versus CpG-peptide group.

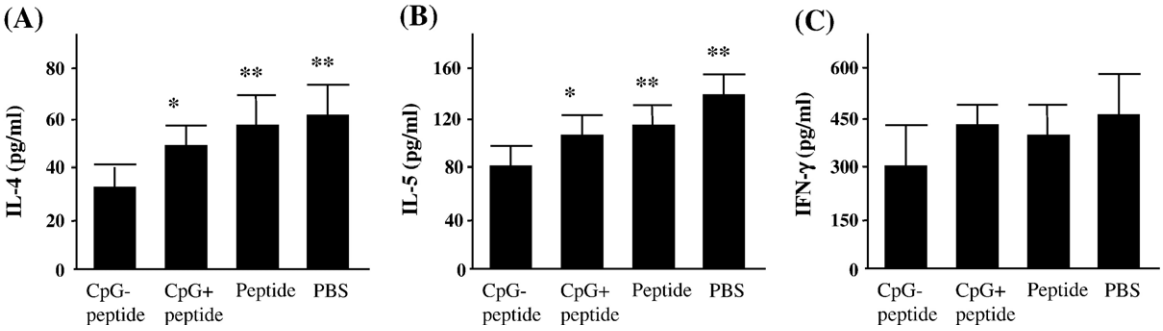

Fig. 4. IL-4, IL-5 and IFN-γ responses of splenocytes from mice immunized with Cry j 2 after vaccination. IL-4 (A), IL-5 (B) and IFN-γ (C) responses of splenocytes, stimulated with Cry j 2 in vitro, were measured by ELISA. Splenocytes were collected from mice injected with CpG-peptide, CpG+peptide, Cry j 2 peptide (Peptide), or PBS before immunization with Cry j 2. Data are means±S.D. *$p<0.05$ and **$p<0.01$ versus CpG-peptide group.

was immunized with Cry j 2 and alum. Mouse serum levels of Cry j 2-specific IgE, IgG1, IgG2a and IgG2b were measured by ELISA. Mice immunized with Cry j 2 after receiving CpG-peptide produced significantly less Cry j 2-specific IgE than mice given CpG+peptide, peptide alone, or PBS alone (Fig. 3A). Mice immunized with Cry j 2 after CpG-peptide generated significantly less Cry j 2-specific IgG1 than mice given peptide or PBS alone (Fig. 3B). However, the production of IgG2a and IgG2b did not significantly differ between mice primed with CpG-peptide, CpG+peptide, peptide alone, or PBS (Fig. 3C and D).

3.3. Effect of CpG-peptide on IL-4, IL-5 and IFN-γ before sensitization (Experiment 1)

To investigate the preventative effects of CpG-peptide on cytokine release in response to Cry j 2 immunization, we measured Cry j 2-stimulated splenocyte release of IL-4, IL-5,

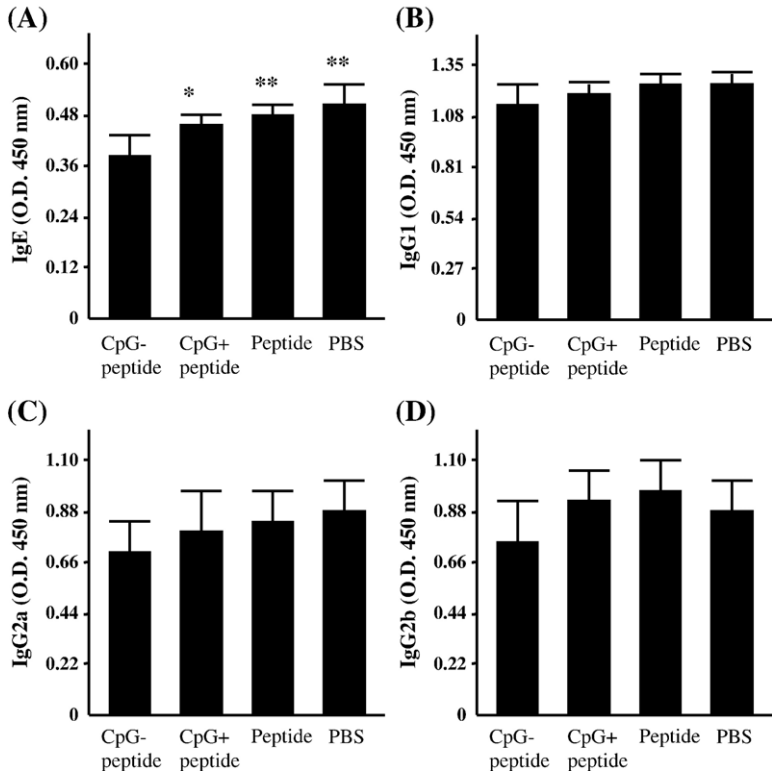

Fig. 5. Cry j 2-specific IgE (A), IgG1 (B), IgG2a (C) and IgG2b (D) levels in blood from mice that received immunotherapy after immunization with Cry j 2. After immunization with Cry j 2, mice ($n=5$ per group) were injected with CpG-peptide, CpG+peptide, Cry j 2 peptide (Peptide), or PBS. Levels of antibodies were measured by ELISA. Columns and vertical bars show means±S.D. *$p<0.05$ and **$p<0.01$ versus CpG-peptide group.

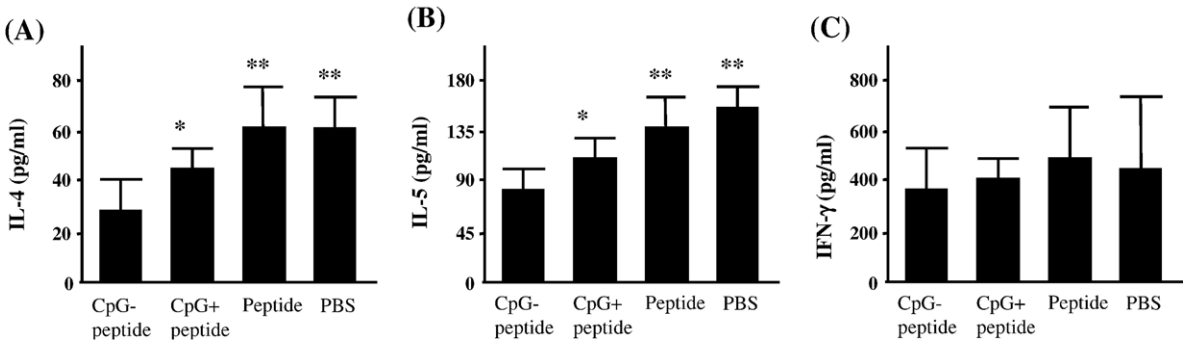

Fig. 6. IL-4, IL-5 and IFN-γ responses of splenocytes from mice received immunotherapy after immunization with Cry j 2. After immunization with Cry j 2, mice were injected with CpG-peptide, CpG+peptide, Cry j 2 peptide (Peptide), or PBS. IL-4 (A), IL-5 (B) and IFN-γ (C) responses of splenocytes, stimulated with Cry j 2 in vitro, were measured by ELISA. Data are means±S.D. *$p<0.05$ and **$p<0.01$ versus CpG-peptide group.

and IFN-γ, in vitro, from mice immunized with Cry j 2 after vaccination with CpG-peptide, CpG+peptide, peptide, or PBS. Splenocytes from mice injected with CpG-peptide produced significantly lower IL-4 and IL-5 responses than those from mice given CpG+peptide, peptide, or PBS (Fig. 4A and B). The IFN-γ responses, however, did not differ between the groups (Fig. 4C).

3.4. Effect of CpG-peptide on IgE, IgG1, IgG2a and IgG2b production after sensitization (Experiment 2)

Mice were injected with CpG-peptide, CpG+peptide, peptide or PBS after immunization with Cry j 2, so as to examine the therapeutic effects of CpG DNA on antibody production in mice with Japanese cedar pollinosis. Serum levels of Cry j 2-specific IgE, IgG1, IgG2a, and IgG2b were measured by ELISA. Mice injected with CpG-peptide produced significantly less Cry j 2-specific IgE than those given CpG+peptide, peptide or PBS (Fig. 5A). Levels of IgG1, IgG2a, and IgG2b production did not significantly differ between the groups (Fig. 5B–D).

3.5. Effect of CpG-peptide on IL-4, IL-5 and IFN-γ production after sensitization (Experiment 2)

Fig. 6 shows the levels of Cry j 2 peptide-stimulated splenocyte release of IL-4, IL-5 and IFN-γ, in vitro, from mice injected with CpG-peptide, CpG+peptide, peptide, or FBS after Cry j 2 immunization. Splenocytes from mice injected with CpG-peptide released significantly less IL-4 and IL-5 than splenocytes from mice that had received CpG+peptide, peptide or PBS (Fig. 6A and B). The amount of IFN-γ release did not significantly differ between the four groups (Fig. 6C).

3.6. Effect of CpG-peptide on nasal symptoms (Experiment 3)

To investigate the effect of CpG-peptide on nasal symptoms, we studied the numbers of sneezing and nasal rubbing in mice that were intranasally challenged with Cry j 2 after immunotherapy with CpG-peptide, CpG+peptide, peptide alone, or PBS alone. The numbers of sneezing and nasal rubbing in mice with CpG-peptide were significantly fewer than in mice with CpG+peptide, peptide or PBS (Fig. 7A and B). Mice with CpG-peptide

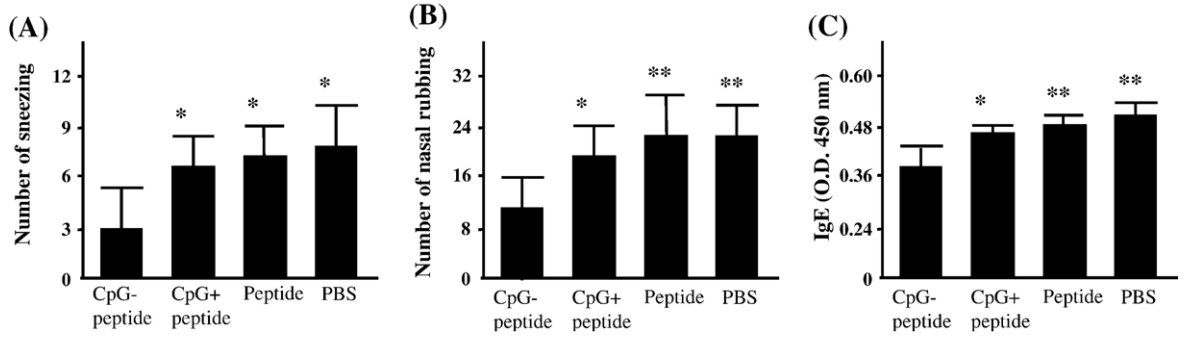

Fig. 7. Numbers of sneezing (A), number of nasal rubbing (B), and level of Cry j 2-specific IgE (C) in mice that received nasal challenge with Cry j 2 after therapy. After immunization with Cry j 2, mice ($n=5$ per group) were injected with CpG-peptide, CpG+peptide, Cry j 2 peptide (Peptide), or PBS, and mice were challenged intranasally with Cry j 2. The numbers of sneezing and nasal rubbing were counted for 20 min immediately after the last nasal challenge. Level of IgE in blood was also measured by ELISA. Columns and vertical bars show means±S.D. *$p<0.05$ and **$p<0.01$ versus CpG-peptide group.

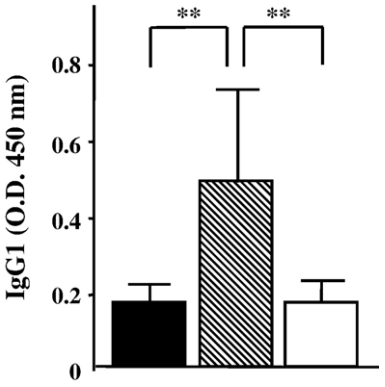

Fig. 8. Cry j 2-specific IgG1 level in prime responses to CpG-peptide or CpG-Cry j 2. Mice were injected with CpG-peptide (black bar), CpG-Cry j 2 (shaded bar), or PBS alone (white bar) to investigate prime responses. Titer of IgG1 antibody was measured by ELISA ($n=5$ per group). Data are means ± S.D. $^{**}p<0.01$ versus counterpart group.

also generated significantly lower levels of IgE than mice with CpG+peptide, peptide, or PBS after nasal challenge (Fig. 7C).

3.7. Effect of CpG-peptide or a conjugate of CpG DNA and Cry j 2 protein (CpG-Cry j 2) on IgE and IgG1 production in primary responses (Experiment 4)

To study the primary response of CpG DNA, we investigated IgE and IgG1 production in mice that had been vaccinated with CpG-peptide or CpG-Cry j 2. Vaccination with CpG-Cry j 2, but not with CpG-peptide, produced Cry j 2-specific IgG1 antibody (Fig. 8). Mice injected with CpG-peptide and CpG-Cry j 2 did not produce IgE (data not shown).

4. Discussion

Specific immunotherapy with increasing doses of injected allergen induces hyporesponsiveness. This phenomenon has been applied widely in the treatment of allergic diseases; however, anaphylactic reactions can be severe and life-threatening. The long duration of treatment and frequent injections also result in low rates of patient compliance. Therefore, new strategies, such as peptide therapy and immunotherapy with CpG DNA, have been studied. CpG DNA induces antigen-presenting cells to promote Th1-like responses characterized by the production of IL-12, INF-γ and IgG2a, which induce naive T cells to differentiate into Th1 cells [7–13]. This strategy also inhibits IgE, IgG1, and PCA reactions, and decreases IL-4 levels [8–13]. The mechanism by which these effects occur has been explained by many investigators. CpG DNA undergoes endocytosis and interacts with toll-like receptor 9 (TLR-9), which further interacts with the adaptor protein, MyD88 [13,19–21]. Through MAP kinase, p38, JUN1/2, IKK, AP-1, and NF-κB translocate to the nucleus, where they promote the transcriptional activation of target proinflammatory genes like TNF-α, IL-12, and CD 40 [13,22]. On the other hand, the subcutaneous administration of peptide can modulate IgE, IgG1 and IL-4 responses, as well as airway hyperresponsiveness [1,2]. The tolerogenic effects of peptides on T cells and antibody responses against whole protein allergens have been demonstrated using antigenic epitopes [3–6], suggesting that one key explanation for the underlying mechanism of peptide immunotherapy is the induction of tolerance within a specific T-cell population. Consequently, the mechanisms by which CpG DNA and peptide therapy work are thought to differ.

Several T-cell epitopes have been identified [16,22]. However, Cry j 2 p246-259 peptide was used in this study because Cry j 2 p246-259 peptide contains a dominant T-cell determinant of Cry j 2 in mice [16], and it was reported that immunotherapy with this peptide reduced IgE and IL-4 in BALB/c mice [6]. Furthermore, the Cry j 2 p245-259 peptide 'SRAEVSYVHVNGAKF' is a T-cell epitope in humans [23]. Thus, this study using this epitope will contribute to the development of new immunotherapy not only in mice with allergy to Japanese cedar, but also in humans with allergy to Japanese cedar.

The present study discovered that immunotherapy with a conjugate of CpG DNA and Cry j 2 peptide (CpG-peptide), but not Cry j 2 protein, reduced IL-4, IL-5, and IgE more effectively than Cry j 2 peptide alone or a mixture of CpG DNA and Cry j 2 peptide (CpG+peptide). This phenomenon was evident in both preventative and therapeutic protocols. This finding suggests that immunotherapy with a conjugate of CpG DNA and peptide is more useful for the control of allergic conditions than therapy with peptide therapy alone or a mixture of CpG DNA and peptide, not only as a vaccine but also as a therapeutic drug. Why a conjugate of CpG DNA and peptide reduces Th2 response more effectively than the peptide alone or a mixture of the two remains obscure. However, one explanation may be that CpG DNA enhances the effect of peptide therapy, because CpG DNA works as an adjuvant to induce the maturation, activation and survival of dendritic cells [24,25]. In addition, conjugation of CpG DNA might enhance the effect of peptide therapy, since CpG DNA enhances antigen uptake by dendritic cells when conjugated together [26,27].

In this study, mice vaccinated with a conjugate of CpG DNA and Cry j 2 protein (CpG-Cry j 2) produced Cry j 2-specific IgG1 antibody. This suggests that vaccination with CpG-Cry j 2 protein induces an allergic response, since systemic anaphylaxis and antigen-dependent airway obstruction can be mediated through IgG1 in the mouse

via a mechanism that is induced by IgE in humans [28]. Increasing IgG1 may also cause other problems, such as autoimmune disease. Mice vaccinated with CpG-peptide did not generate Cry j 2-specific IgG1; therefore, vaccination with a conjugate of CpG DNA and peptide should be notable.

CpG DNA is believed to increase IgG2a, IgG2b and IFN-γ (Th1 response) [7–13]. In fact, immunotherapy with CpG DNA and Cry j 2 protein increased IgG2a and IFN-γ in mice with allergy to Japanese cedar [9]. However, this study demonstrated that immunotherapy with CpG-peptide did not increase IgG2a, IgG2b and IFN-γ in both the preventive and therapeutic protocols. Our findings suggest that this therapy should be hopeful, since an increased Th1 response might cause other problems such as autoimmune disease. Although the underlying mechanisms have not been defined, peptide together with CpG DNA could lead to the absence of IgG2a and IgG2b production, because antibody production in response to a peptide is more difficult than that to a protein. In addition, Th1 cells caused by CpG DNA might undergo tolerance in response to large amounts of peptides, which would prevent Th1 responses.

It was reported that a conjugate of CpG DNA and an allergenic protein is less anaphylactogenic and less allergenic than a native allergen [7]. Considering this, a conjugate of CpG DNA and T-cell epitope peptide may be less allergenic than a native peptide. Therefore, a conjugate of CpG DNA and T-cell epitope peptide should be paid attention to, since it has been reported that high-dose peptide-based immunotherapy could cause anti-peptide antibodies, immediate hypersensitivity reactions and anaphylactic reactions [29,30].

Sneezing and pruritus of the nose are clinically major symptoms of allergic rhinitis. Nasal rubbing is a good parameter of pruritis of the nose. In this study, we demonstrated that CpG.-peptide significantly reduced the numbers of sneezing and nasal rubbing compared with CpG+peptide, peptide alone, or PBS alone. This suggests that a conjugate of CpG DNA and T-cell epitope peptide is effective in controlling the symptoms of allergic rhinitis in mice, and a conjugate of CpG DNA and peptide is useful not only on the induction phase of allergic sensitization but also on the effector phase of allergic reaction.

Immunotherapy with T-cell epitope peptide is a useful approach to treating allergic diseases, but the present study demonstrated that therapy using a conjugate of CpG DNA and peptide was more useful than that with peptide alone. Thus, the immunotherapy with a conjugate of CpG DNA and T-cell peptide should provide a new effective treatment strategy.

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