



Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

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Induction of Th1 immune responses to Japanese cedar pollen allergen (Cry j 1) in mice immunized with Cry j 1 conjugated with CpG oligodeoxynucleotide

Y. Kaburaki^a, T. Fujimura^b, K. Kurata^c, K. Masuda^d, M. Toda^e, H. Yasueda^f, K. Chida^a, S. Kawarai^g, M. Sakaguchi^{g,*}

^a Department of Animal Resource Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

^b Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

^c Institute of Tokyo Environmental Allergy, 2-2-4 Yushima, Bunkyo-ku, Tokyo 113-0034, Japan

^d Animal Allergy Clinical Laboratories, 5-4-21 Nishihashimoto, Sagamihara, Kanagawa 229-1131, Japan

^e Experimental Allergology, Paul-Ehrlich-Institut, Paul-Ehrlich-Street 59, Langen 63225, Germany

^f Clinical Research Center for Allergy and Rheumatology, National Sagamihara Hospital, 18-1 Sakuradai, Sagamihara, Kanagawa 228-8522, Japan

^g Department of Veterinary Microbiology, School of Veterinary Medicine, Azabu University, 1-17-71 Fuchinobe, Sagamihara, Kanagawa 229-8501, Japan

ARTICLE INFO

Article history:

Received 22 April 2010

Accepted 22 June 2010

Keywords:

Allergens

CpG

Pollen

IgE

ABSTRACT

We determined whether a major Japanese cedar pollen allergen (Cry j 1) conjugated with CpG oligodeoxynucleotide would enhance allergen-specific Th1 responses in mice. Cry j 1 conjugated with CpG (Cry j 1–CpG) induced IL-12 in the spleen cells of naïve mice. Cry j 1–CpG immunization of BALB/c mice suppressed anti-Cry j 1 IgE response and enhanced anti-Cry j 1 IgG_{2a} to subsequent Cry j 1 and alum adjuvant injection. CD4⁺T cells isolated from the spleens in mice immunized with Cry j 1–CpG produced higher IFN- γ levels than did CD4⁺T cells obtained from mice as negative controls. Our results suggested that Cry j 1–CpG immunization can induce Cry j 1-specific Th1 immune responses, thereby inhibiting IgE response to the pollen allergen.

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1. Introduction

Japanese cedar (*Cryptomeria japonica*; CJ) pollinosis is a typical type I allergy induced by CJ pollen and one of the most common allergic diseases in Japan. In areas where there are CJ trees, approximately 10% of the population suffers from CJ pollinosis [1]. Two major allergens, Cry j 1 [2] and Cry j 2 [3] have been isolated from CJ pollen. More than 90% of 145 CJ pollinosis patients had specific IgE antibodies to both Cry j 1 and Cry j 2 allergens: the remainder had specific IgE to either one or the other [4]. The patients with CJ pollinosis had high levels of T-cell reactivity to Cry j 1

and Cry j 2 [5]. Recently, we isolated Cry j 3, which may play crucial roles in the cross-reactivity with oral allergy syndrome [6].

Bacterial DNA and synthetic oligodeoxynucleotides containing CpG are potent adjuvants of Th1 responses characterized by production of IFN- γ and IgG_{2a} [7–9]. Tokunaga and his colleagues had found that DNA extracted from *Mycobacterium bovis* activated NK cells to induce interferon and anti-tumor activities [10,11]. Several short DNA sequences containing CpG motif were determined by the sequencing of mycobacterium DNA.

The administration of allergen conjugated to CpG is an attractive approach to the prevention and treatment of allergic diseases [9]. In a clinical study, ragweed pollen allergen (Amb a 1) conjugated with CpG vaccine showed clinical efficacy in the treatment of ragweed allergic rhinitis

* Corresponding author. Tel.: +81 42 850 2481; fax: +81 42 850 2481.
E-mail address: sakagum@azabu-u.ac.jp (M. Sakaguchi).

[12]. Moreover, allergens conjugated with CpG reduced the binding activity of specific IgE to native allergens and also reduced allergenesis of the allergens [9,13]. Recently, it was reported that Cry j 2 T-cell epitope peptides conjugated to CpG vaccine have no allergenicity [14].

In this study, we investigated whether Cry j 1–CpG immunization suppressed the development of Cry j 1-specific IgE production in mice. Furthermore, we investigated the effect of Cry j 1–CpG on Th1 and Th2 cytokine production in Cry j 1-specific CD4⁺T cells.

2. Materials and methods

2.1. Mice

Female BALB/c mice (7 weeks old) were obtained from Japan SLC Inc (Shizuoka, Japan). They were housed under specific pathogen-free conditions.

2.2. Antigens

Cry j 1 is a basic glycoprotein isolated from CJ pollen by affinity chromatography using monoclonal antibodies (mAb) specific to Cry j 1 [15,16].

2.3. Conjugation of CpG oligodeoxynucleotides to Cry j 1

The phosphorothioated CpG, consisting of 22 bases containing two CpG motifs 5'-TGACTGTGAACGTTTCGAGATGA-3' (immunostimulatory sequence: ISS 1018) [17,18] was produced by Invitrogen Corp. (Carlsbad, CA, USA). As a negative control, phosphorothioated oligodeoxynucleotide consisting of 22 bases containing the mutated 5'-TGACTGTGAAGTTAGAGATGA-3' (mCpG) was used in this study. Each of these CpGs had a molecular mass of approximately 7000. The 5'-phosphorothioate CpG was coupled to amino groups on Cry j 1. Cry j 1 was modified to incorporate thiol moieties by reacting to it with excess of succinimidyl-S-acetyl thiol acetate (Pierce Biotechnology, Rockford, IL, USA), as described previously [19]. Endotoxin levels were measured by Bio-Whittaker, QCL-1000 (Cambrex Bio Science Walkersville, Walkersville, MD, USA). The endotoxin levels of Cry j 1–CpG and Cry j 1–mCpG were <0.03 endotoxin unit/ μ g.

2.4. In vitro assay for IL-12 p40 assays in the naive spleen cells

Mice were sacrificed by means of anesthesia and their spleens were removed. Spleen cells (2×10^5) were added to wells in a total volume of 100 μ L and stimulated with Cry j 1–CpG, CpG, and mCpG. Cultures were incubated for 0, 6, 11, 24, 35, and 48 h at 37 °C in 5% CO₂. Supernatants were harvested after each incubation period and frozen at –30 °C before being analyzed. The levels of IL-12 p40 in the culture supernatant were determined by sandwich ELISA (BD Biosciences, Franklin Lakes, NJ, USA).

2.5. Immunization

Ten BALB/c mice in each group were subcutaneously immunized with Cry j 1 (5 μ g)–CpG, Cry j 1 (5 μ g)–mCpG, or Cry j 1 (5 μ g) in PBS three times weekly. All mice were intraperitoneally immunized with 5 μ g of Cry j 1 and 2 mg of aluminum hydroxide (alum)(Pierce, Rockford, IL, USA) mixture at 3 and 6 weeks. Blood samples were collected by tail bleeding at weeks 1, 2, 4, 5, and 7 weeks, and serum samples were stored at –30 °C. Spleens for assays of IFN- γ , IL-4, and IL-5 were collected at 7 weeks.

2.6. Measurement of mouse anti-Cry j 1 IgG1, IgG_{2a}, IgE

IgG₁ and IgG_{2a} antibody responses to Cry j 1 were assayed by ELISA as described previously [20]. The IgE antibody response to Cry j 1 was analyzed by fluorometric ELISA as described previously [20].

2.7. ELISA inhibition with human anti-Cry j 1 IgE

The ability of Cry j 1–CpG to bind human Cry j 1-specific IgE was measured by using fluorometric ELISA inhibition as described previously [21].

2.8. Cytokine assays in CD4⁺T cells from spleens of immunized mice

CD4⁺T cells were purified from the spleens of mice by MACS [22]. Purified CD4⁺T cells (2×10^5) were cultured with irradiated syngenic splenocytes (3×10^5) as antigen-presenting cells in 96-well plates for 3 days in the presence or absence of Cry j 1 (10 μ g/ml). The culture supernatant was collected 3 days after cultivation and kept at –30 °C until use. IFN- γ , IL-4, and IL-5 levels were determined by ELISA using anti-IFN- γ mAb, anti-IL-4 mAb, and anti-IL-5 mAb (BD Biosciences) [20].

2.9. Statistics

The Kruskal–Wallis test was applied to compare data among the four groups. If the difference was significant, post hoc analysis using the Mann–Whitney U-test with Bonferroni correction in antibody levels and the Turkey–Kramer test in cytokine levels were performed to analyze the difference between the ODN–Cry j 1 group and the other group. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. IL-12 p40 from naive spleen cells

We investigated whether Cry j 1–CpG induced IL-12 p40 in the spleens of naive mice. After spleen cells were cultivated for 36 h, significant IL-12 p40 production was observed in the cells stimulated with CpG–Cry j 1 and CpG as compared with mCpG as a negative control (Fig. 1).

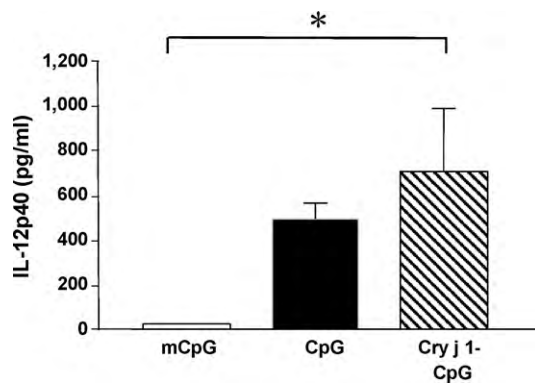


Fig. 1. IL-12 p40 production by Cry j 1-CpG or CpG stimulation in the spleens of naive mice. mCpG (1 μ g/ml; 142 pmol), CpG (1 μ g/ml; 142 pmol), Cry j 1-CpG (1 μ g/ml; 142 pmol). * p < 0.05 for Cry j 1-CpG compared with mCpG.

3.2. Human anti-Cry j 1 IgE binding to Cry j 1-CpG

To determine whether the conjugation of CpG to Cry j 1 diminished the ability of Cry j 1 to react with anti-Cry j 1 IgE in pooled serum from patients with CJ pollinosis, the ability of Cry j 1-CpG to bind anti-Cry j 1 IgE was tested by the ELISA inhibition. Cry j 1-CpG reacted less well with anti-Cry j 1 IgE than did Cry j 1. An approximate 50-fold higher concentration of Cry j 1-CpG was necessary to achieve 50% inhibition compared with Cry j 1 (Fig. 2).

3.3. Anti-Cry j 1 IgE in mice immunized with Cry j 1-CpG

We investigated whether initial immunization of Cry j 1-CpG suppressed anti-Cry j 1 IgE development after immunization of Cry j 1 and alum. In groups immunized with Cry j 1 or Cry j 1-mCpG, anti-Cry j 1 IgE production was induced by boosting with Cry j 1 and alum at 4 and 6 weeks. Anti-Cry j 1 IgE production was significantly inhibited in mice immunized with Cry j 1-CpG up to 7 weeks (Fig. 3). Cry j 1-CpG immunization suppressed anti-Cry j 1

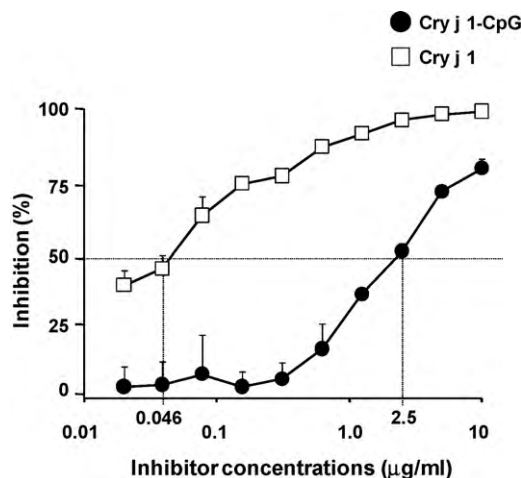


Fig. 2. Inhibition of human anti-Cry j 1 IgE from CJ allergic patients by Cry j 1 or Cry j 1-CpG.

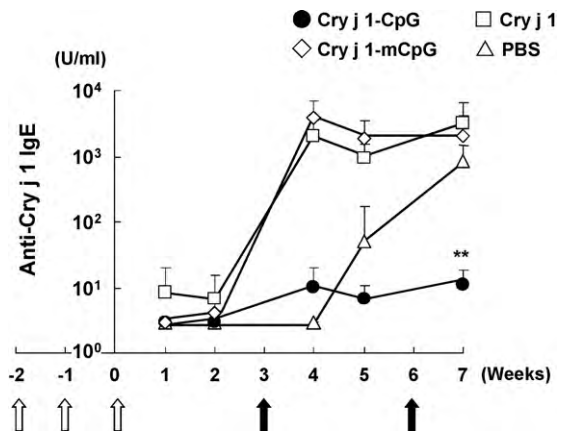


Fig. 3. Inhibition of anti-Cry j 1 IgE production by Cry j 1-CpG inoculation. BALB/c mice were immunized with 5 μ g each of Cry j 1, Cry j 1-CpG, Cry j 1-mCpG, or PBS. ** p < 0.01 for the group immunized with Cry j 1-CpG compared with other groups.

IgE production to a secondary immunization of Cry j 1 and alum.

3.4. Anti-Cry j 1 IgG₁ and IgG_{2a} in mice immunized with Cry j 1-CpG

The effects of initial immunization of Cry j 1-CpG on the production of anti-Cry j 1 IgG₁ production were examined. After first immunization of Cry j 1 and alum at 3 weeks, anti-Cry j 1 IgG₁ production in groups immunized with Cry j 1 and Cry j 1-mCpG was enhanced (Fig. 4A). After secondary immunization of Cry j 1 and alum at 7 weeks, anti-Cry j 1 IgG₁ production showed no significant difference among any of the groups.

The effects of initial immunization of Cry j 1-CpG on the production of anti-Cry j 1 IgG_{2a} production were examined. At 1 and 2 weeks, anti-Cry j 1 IgG_{2a} levels showed no differences among any of the groups. After immunization of Cry j 1 and alum at 7 weeks, Cry j 1-CpG immunization developed significantly higher levels of anti-Cry j 1 IgG_{2a} compared with groups immunized with PBS, Cry j 1, or Cry j 1-mCpG (p < 0.01) (Fig. 4B).

3.5. Cytokine production in Cry j 1-specific CD4⁺T cells cultures

The levels of IFN- γ , IL-4, and IL-5 in response to Cry j 1 were measured in CD4⁺T cells obtained 2 weeks after the second boosting of Cry j 1 in alum. CD4⁺T cells from mice immunized with Cry j 1-CpG secreted significantly higher levels of IFN- γ than did CD4⁺T cells obtained from mice immunized with PBS, Cry j 1, or Cry j 1-mCpG (p < 0.05) (Fig. 5A).

Cry j 1-specific IL-5 levels in mice immunized with Cry j 1-CpG showed significantly lower than those in mice immunized with Cry j 1-mCpG, but showed no significant differences among other groups (Fig. 5B). Cry j 1-specific IL-4 levels in mice immunized with Cry j 1-CpG showed no significant differences among other groups (Fig. 5C).

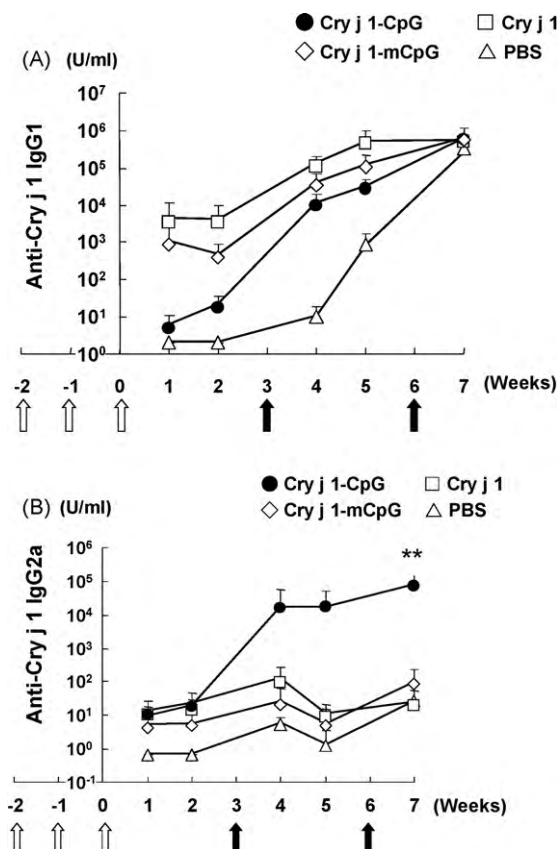


Fig. 4. Anti-Cry j 1 IgG₁ and IgG_{2a} production by Cry j 1-CpG inoculation. BALB/c mice immunized with 5 µg each of either Cry j 1, Cry j 1-mCpG, or PBS. (A) IgG₁ and (B) IgG_{2a}. ***p* < 0.01 for the group immunized with Cry j 1-CpG compared with other groups.

4. Discussion

It has been reported that IFN-γ induction is an IgM to IgG_{2a} switch factor, which accounts for the association of IgG_{2a} with a Th1 response [23]. Cry j 1-CpG immunization significantly induced the production of IFN-γ from Cry j 1-specific CD4⁺T cells. These observations are similar to those from the study of Iho et al. [24]. Those investigators showed that CpG oligodeoxynucleotide containing AACGTT induced the production of IFN-γ and the expansion of antigen-specific human peripheral blood mononuclear cells. In our study, we used CpG oligodeoxynucleotide containing AACGTT. This CpG might be a Th1 cell differentiation inducer and promote IFN-γ production.

In this study, inoculation with Cry j 1-CpG reduced anti-Cry j 1 IgE antibody and enhanced IgG_{2a} responses after immunization with alum-precipitated Cry j 1. Cry j 1-CpG induced the allergen-specific Th1-type immune response, resulting in the inhibition of the IgE response to the allergen. Tight et al. reported that in mice primed for Th2 response, injection with Amb a 1 (a major ragweed allergen)-conjugated CpG suppressed specific Amb a 1 IgE [9]. Also, Suzuki et al. reported that anti-Cry j 2 IgE levels were lower in mice immunized with Cry j 2 T-cell peptide conjugated with CpG [14]. These findings indicated the

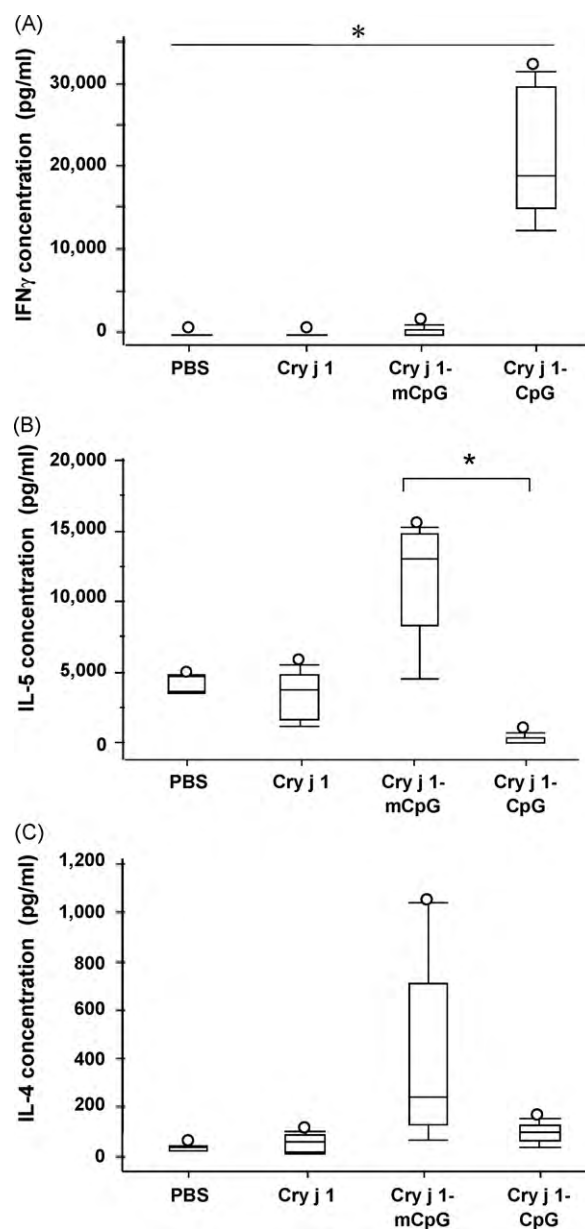


Fig. 5. The effect of Cry j 1-CpG on cytokine production in Cry j 1-specific CD4⁺T-cell cultures. CD4⁺T cells from mice immunized with Cry j 1, Cry j 1-CpG, Cry j 1-mCpG, or PBS were stimulated with 10 µg/ml of Cry j 1 for 3 days. (A) IFN-γ, (B) IL-5 and (C) IL-4. **p* < 0.05 for the group immunized with Cry j 1-CpG compared with other groups.

potential for a CpG-conjugated allergen or T-cell peptide vaccine to serve as a tool for the prevention and treatment of CJ pollinosis.

In the present study, IL-4 levels in mice immunized with Cry j 1-CpG were no different from those in any other immunized mice. IL-5 levels in mice immunized with Cry j 1-CpG showed significantly lower than those in mice immunized with Cry j 1-mCpG, but showed no significant differences among other groups. Similarly, our previous study showed that the inoculation of DNA vaccine encoding the CD4⁺T-cell epitope in Cry j 2 (a major CJ allergen)

inhibited the allergen-specific IgE response to subsequent allergen and alum injection, but did not inhibit allergen-specific IL-4 and IL-5 production [22]. The alum can induce strong antigen-specific Th2 response and Th2-associated IL-4 production [25,26]. The subsequent allergen and alum injection in the present study might induce too strong Th2 responses to suppress Th2-cytokines in mice.

CpG oligodeoxynucleotide is a potent adjuvant of Th1 response, so it is hoped that it will be useful in the development of immunotherapies for allergic diseases and cancer. Marshall et al. demonstrated that ISS 1018-conjugated Amb a 1 allergen would induce IFN- γ production in peripheral blood mononuclear cells from human patients with ragweed allergy [18]. We used the same single strand synthetic ISS 1018-conjugated Cry j 1. Our data suggested that CpG played a role in switching on Th1 immunity to a protein antigen through the upregulation of IgG2a and IFN- γ . Furthermore, Cry j 1–CpG induced Th1 cell differentiation and suppressed anti-Cry j 1 IgE. This immunization may be useful as an immunotherapy for CJ pollinosis.

Acknowledgments

This work was supported by a Grant-in Aid for Scientific Research from the Ministry of Health, Labor, and Welfare of Japan, by the Promotion and Mutual Aid Corporation for Private Schools of Japan, by a Grant-in-Aid for Matching Fund Subsidy for Private University, and by a project grant awarded by the Azabu University Research Services Division.

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