

ORIGINAL RESEARCH

Intranasal CpG DNA therapy during allergen exposure in allergic rhinitis

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OBJECTIVE: 1) To estimate the effectiveness of intranasal administration of CpG DNA alone on allergic rhinitis compared with intradermal administration; and 2) to find out how CpG DNA therapy is useful in treatment of allergic rhinitis.

STUDY DESIGN: Mice were intraperitoneally sensitized and intranasally challenged with Japanese cedar. Therapy with CpG DNA alone was also performed during challenge, either intranasally or intradermally. Immunologic variables and nasal symptom were studied.

RESULTS: Intranasal administration of CpG DNA alone significantly reduced the levels of IgE, IL-5 productions from nasal lymphocytes and splenocytes, nasal eosinophilia, and nasal symptoms, although intradermal administration of CpG DNA alone showed no significant reduction.

CONCLUSION: This study demonstrated that CpG DNA has effects not only on splenocytes but also on nasal lymphocytes to attenuate allergic rhinitis, and that intranasal administration, but not intradermal administration, of CpG DNA alone during allergen exposure is useful for control of allergic rhinitis.

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Many people worldwide have allergic rhinitis, induced by Th2 responses characterized by the production of IgE, IgG1, interleukin 4 (IL-4), and interleukin 5 (IL-5). CpG DNA, containing a central unmethylated C-G dinucleotide, is known to induce antigen-presenting cells to promote Th1 responses characterized by the production of interferon-gamma (IFN- γ) and IgG2a, which induce naive

T cells to differentiate into Th1 cells.^{1–4} Consequently, IgE, IL-4, and IL-5 levels decrease.^{1–4} It has been reported that co-administration of CpG DNA and allergens could attenuate allergic rhinitis.^{5,6}

Many patients with allergies are sensitive to multiple allergens.⁷ In recent years, the incidence of patients with multiple allergic sensitizations appears to be increasing. It is often difficult for physicians to identify the specific causative allergen in many allergic patients. It has been reported that the antigen-independent immunity induced by intranasal delivery of CpG alone can offer protection from lung eosinophilia, although an antigen-specific Th1 response elicited by epidermal powder immunization with ovalbumin (OVA) and CpG neither prevented nor treated airway allergy.⁸ Considering these observations, we feel it is important to investigate allergen-independent immunotherapy with CpG DNA alone. However, studies investigating the effects of CpG DNA alone on allergic rhinitis are very rare.

Rhee et al⁹ have reported that therapy with CpG DNA alone, administered intranasally (locally) or intradermally (systemically) before OVA allergen nasal challenge, may be an effective alternative to corticosteroids in the management of allergic rhinitis in BALB/c mice. However, many patients come to the outpatient clinic after they have experienced exposure to allergen and already have symptoms. Therefore, it is necessary to investigate the effect of therapy with CpG DNA alone during allergen exposure. Furthermore, another controversy is whether intradermal or intra-

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nasal administration of CpG DNA is more effective in inhibiting the allergic response.

It is important to find out in an animal model how the CpG DNA therapy is useful. The effects of CpG DNA on mononuclear cells from spleen and bronchial lymph nodes (BLN) have been documented.^{2,3} However, there have been no reports on the effect of CpG DNA on nasal lymphocytes. It is crucial to investigate nasal lymphocytes to develop novel therapy for allergic rhinitis.

Pollinosis caused by pollen from the Japanese cedar (*Cryptomeria japonica*) is the most widespread allergic respiratory disease in Japan and affects over 10% of the population.¹⁰ It was reported that therapy with co-administration of CpG DNA and Japanese cedar was useful for control of Japanese cedar pollinosis.² However, it has not been reported whether therapy with CpG DNA alone is useful for control of Japanese cedar pollinosis.

Previous studies have demonstrated notable differences in the susceptibility to immunization and therapy in different strains of mice. Liu et al¹¹ demonstrated that the expression levels of Toll-like receptor 9 (TLR-9) mRNA in dendritic cells isolated from the spleens of native BALB/c mice differ from those from C57BL/6 mice, even though CpG DNA induces antigen-presenting cells to promote Th1 responses through TLR-9.¹² Therefore, the effect of CpG DNA may differ by mouse strain. In fact, it was reported that vaccination with CpG DNA induces protection against cutaneous leishmaniasis in C57BL/6 mice, but does not prevent progressive disease in BALB/c mice.¹³

Consequently, in this study, we investigated the effect of intradermal versus intranasal administration of CpG DNA alone, given during antigen challenge, both in BALB/c and C57BL/6 mice sensitized with Japanese cedar.

MATERIALS AND METHODS

Reagents

Japanese cedar pollen was provided by Torii Pharmaceutical Co, Ltd. Pollen was dissolved in phosphate buffer saline solution. CpG DNA containing 2 CpG motifs (TCC ATG ACG TTC CTG ACG TT) was synthesized by Rikaken Co. (Nagoya, Japan).

Animals

Specific pathogen-free female BALB/c and C57BL/6 mice (6 weeks of age) were obtained from Japan SLC Inc (Hamamatsu, Japan). All experiments were performed in accordance with the Guidelines for Animal Experimentation at Nagoya City University. All mice were maintained under SPF conditions at the university animal facility.

Immunization Protocol

The protocol used in this study is depicted schematically in Figure 1. BALB/c and C57BL/6 mice were sensitized on days 0 and 7 by intraperitoneal injection with Japanese

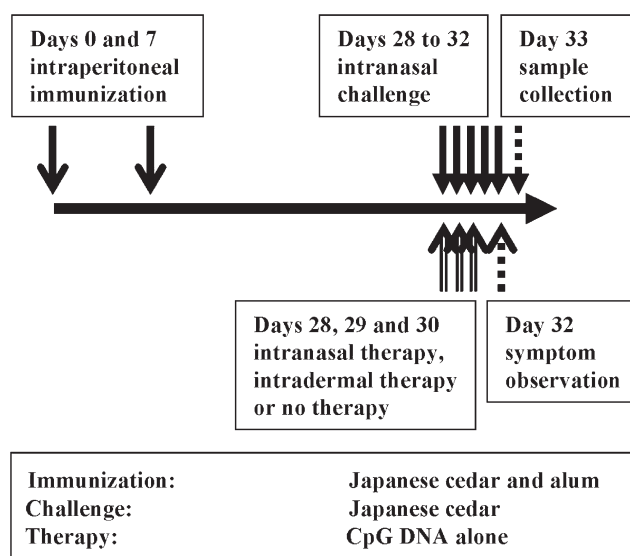


Figure 1 Schematic protocol of immunization and challenge.

cedar pollen (0.5 mg) absorbed to alum (1 mg). Three weeks after the last sensitization, all mice were challenged intranasally on days 28 to 32 inclusively, with Japanese cedar pollen (10 µg). On days 28, 29, and 30, CpG DNA (10 µg) was given intranasally or intradermally 1 hour after nasal challenge. Immediately after the last nasal challenge, the number of nasal rubbing movements was counted for 15 minutes. Mice were sacrificed 24 hours after the last challenge.

Measurement of Japanese Cedar-specific Antibodies

The titers of cedar-specific IgE in sera were measured by ELISA. Briefly, ELISA plates were coated with anti-mouse IgE monoclonal antibodies (Biosource International, Camarillo, CA). After blocking, sera were added to the plates. After washing and adding biotinylated cedar, the plates were incubated for 2 hours, followed by washing and incubation with avidin-peroxidase for 1 hour. After additional washing, a TMB microwell peroxidase substrate system was applied, in accordance with the manufacturer's instructions, and optical density (OD) was measured at 450 nm using a micro-plate reader (Bio-Rad Japan, Tokyo, Japan).

Cytokine Assays

Nasal lymphocytes were prepared by enzyme extraction with collagenase, as previously described.¹⁴ Lymphoid cells (4×10^5 cells per 200 µL) from nose and spleen were cultured with the antigen (100 µg/mL) for 72 hours at 37°C in RPMI-1640 medium (Sigma, St Louis, MO) containing 10% FBS, penicillin G/streptomycin (Sigma, St Louis, MO). After this incubation, the cell-free culture supernatants were collected. To measure the quantities of IL-4, IL-5, and IFN-γ in the culture supernatants, we used commercially available ELISA kits (PharMingen, San Diego, CA) and followed the manufacturer's instructions. The cy-

tokine concentrations were determined by means of standard curves generated with recombinant cytokines.

Pathology

Mice were anesthetized with ketamine/xylazine and then euthanized by cardiac exsanguination. Blood was flushed from the circulation via an intra-cardiac injection of formalin. Each mouse's head was separated from its body and its scalp was removed. After whole heads were fixed in 10% buffered formalin, the heads were sectioned. Five micrometer thick cryosections of nasal tissue were stained with hematoxylin and eosin. The number of eosinophils was evaluated microscopically on both sides of the nasal septum.

Statistics

Means and standard deviations were calculated for each group of mice, and data were statistically analyzed with ANOVA. A $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of Levels of Japanese Cedar-specific IgE

To investigate the effect of CpG DNA on IgE production during challenge with Japanese cedar, we measured Japanese cedar-specific IgE in the sera of 1) mice that had received intranasal therapy with CpG DNA alone (intranasal CpG therapy); 2) mice that had received intradermal therapy with CpG DNA alone (intradermal CpG therapy); and 3) mice that had received no therapy (controls). BALB/c mice that had received intranasal CpG therapy exhibited significantly lower levels of Japanese cedar-specific IgE than controls (Fig 2A, $P < 0.01$). The level of Japanese cedar-specific IgE in C57BL/6 mice that had received intranasal CpG therapy was also significantly lower than in controls (Fig 2b, $P < 0.01$). On the other hand,

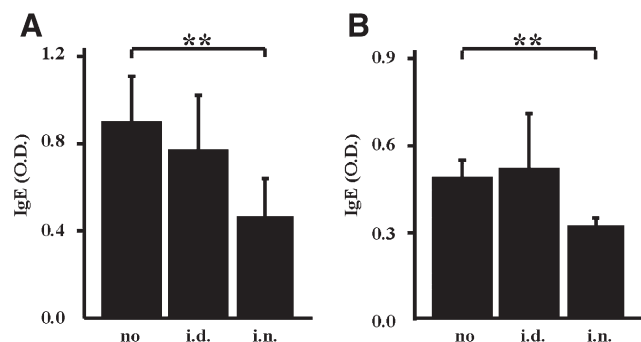


Figure 2 Effect of CpG DNA on levels of Japanese cedar-specific IgE. BALB/c (A) and C57BL/6 (B) mice received no CpG DNA (no); CpG DNA by intradermal injection (i.d.); or CpG DNA intranasally (i.n.). Columns and vertical bars show means $\pm$ SD. ** $P < 0.01$ versus counterpart group.

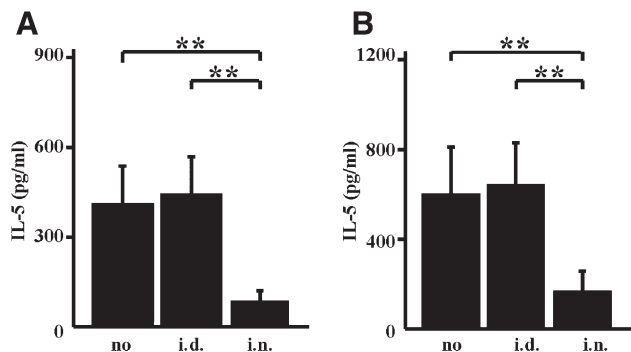


Figure 3 Effects of CpG DNA on IL-5 productions of nasal lymphocytes. BALB/c (A) and C57BL/6 (B) mice received no CpG DNA (no); CpG DNA by intradermal injection (i.d.); or CpG DNA intranasally (i.n.). Columns and vertical bars show means $\pm$ SD. ** $P < 0.01$ versus counterpart group.

among both strains of mice, there was no significant difference in cedar-specific IgE between those that received intradermal CpG therapy and those that did not receive any therapy (Fig 2A and B).

Comparison of Cytokine Production from Nasal Lymphocytes and Splenocytes

To investigate the effects of CpG DNA on cytokine production, we measured the production of IL-4, IL-5, and IFN- γ by nasal lymphocytes and splenocytes stimulated with Japanese cedar in vitro. Nasal lymphocytes in BALB/c mice that had received intranasal CpG therapy released significantly lower levels of IL-5 versus those BALB/c mice that had received no therapy and mice that received intradermal CpG therapy (Fig 3A, $P < 0.01$). Nasal lymphocytes in C57BL/6 mice that received intranasal CpG therapy also produced significantly lower levels of IL-5 than control C57BL/6 mice and mice that had received intradermal CpG therapy (Fig 3B, $P < 0.01$). Intranasal CpG therapy significantly decreased IL-5 production by splenocytes in BALB/c mice, when compared with intradermal CpG therapy or no therapy (Fig 4A, $P < 0.01$). Levels of IL-5 by splenocytes in C57BL/6 mice with intranasal CpG therapy were significantly lower than that in C57BL/6 receiving intradermal CpG therapy or no therapy (Fig 4B, $P < 0.05$ and $P < 0.01$, respectively). Levels of IL-4 produced by splenocytes in BALB/c mice receiving either intranasal or intradermal CpG therapy were below detectable levels, although splenocytes in controls produced IL-4 (data not shown, $P < 0.01$). We could not detect IL-4 produced by nasal lymphocytes from either mouse strain or IL-4 produced by splenocytes in C57BL/6 mice. Intradermal or intranasal CpG therapy significantly increased levels of IFN- γ produced by nasal lymphocytes versus no therapy in C57BL/6 mice (data not shown, $P < 0.01$). However, levels of IFN- γ released by nasal lymphocytes from BALB/c mice and levels of IFN- γ produced by splenocytes from both mouse strains were below detectable levels. We also could not detect any cytokines from the supernatants of nasal

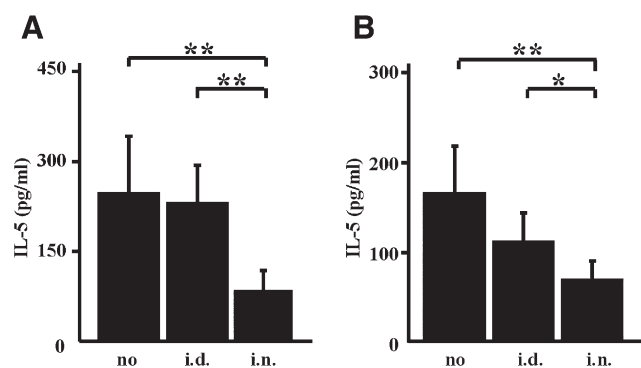


Figure 4 Effects of CpG DNA on IL-5 productions of splenocytes. BALB/c (A) and C57BL/6 (B) mice received no CpG DNA (no); CpG DNA by intradermal injection (i.d.); or CpG DNA intranasally (i.n.). Columns and vertical bars show means $\pm$ SD. * P < 0.05, ** P < 0.01 versus counterpart group.

lymphocytes and splenocytes cultured with no allergen (data not shown).

Comparison of the Degree of Local Eosinophilia

To investigate the effect of CpG DNA on nasal eosinophilia, we counted the number of eosinophils in the nasal septum. Intranasal CpG therapy decreased the number of eosinophils detected in both mice strains compared with no therapy or intradermal CpG therapy (Fig 5A and B, P < 0.01).

Comparison of Nasal Symptom

To investigate the effect of CpG DNA on nasal symptom, we counted the number of nasal rubbing. Intranasal CpG therapy decreased the number of nasal rubbing in both mice strains compared with no therapy or intradermal CpG therapy (Fig 6A and B, P < 0.01).

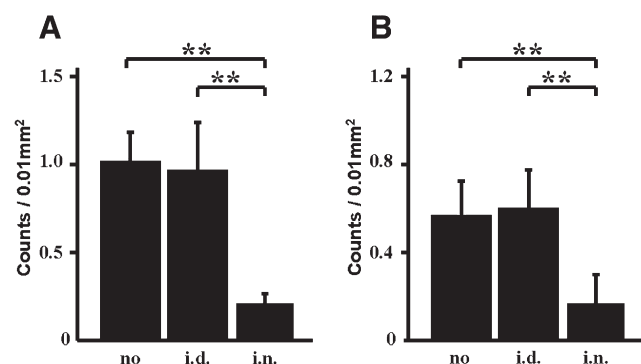


Figure 5 Effect of CpG DNA on nasal eosinophilia. Mice received no CpG DNA (no); CpG DNA by intradermal injection (i.d.); or CpG DNA intranasally (i.n.). The number of eosinophils infiltrated into the nasal mucosa was measured microscopically. Results in BALB/c (A) and C57BL/6 (B) mice demonstrate means $\pm$ SD. ** P < 0.01 versus counterpart group.

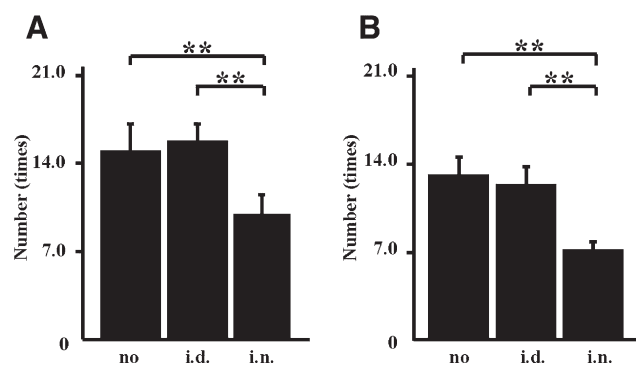


Figure 6 Effect of CpG DNA on nasal rubbing. Mice received no CpG DNA (no); CpG DNA by intradermal injection (i.d.); or CpG DNA intranasally (i.n.). After the last nasal challenge, number of nasal rubbing was counted. Results in BALB/c (A) and C57BL/6 (B) mice demonstrate means $\pm$ SD. ** P < 0.01 versus counterpart group.

Comparison of IgE, IL-5, Eosinophilia and Nasal Symptom Between BALB/c and C57BL/6 Mice

To investigate strain-dependent induction of allergic rhinitis, we compared levels of IgE, IL-5, eosinophilia, and nasal symptom between BALB/c mice receiving no therapy and C57BL/6 mice receiving no therapy. There were significant differences in the levels of IgE and the count of eosinophils in nasal mucosa in the control mice from the two strains (Figs 2 and 5, P < 0.01), whereas no significant differences were observed in the levels of IL-5 produced by nasal lymphocytes or splenocytes or in the count of nasal rubbing (Figs 3, 4, and 6).

DISCUSSION

Intradermal therapy with CpG DNA alone during allergen exposure did not show any significant difference with the control group in level of IgE, levels of IL-5 productions from nasal lymphocytes and splenocytes, the degree of eosinophilia in nasal mucosa, and the severity of nasal symptom in both mouse strain, BALB/c and C57BL/6. However, intranasal therapy with CpG DNA alone during allergen exposure did reduce level of IgE, levels of IL-5 productions from nasal lymphocytes and splenocytes, nasal eosinophilia, and nasal symptom in both mice. Also, intranasal therapy with CpG DNA during allergen exposure significantly reduced IL-5 from nasal lymphocytes and splenocytes, nasal eosinophilia, and nasal symptoms compared with intradermal administration in both mice. This finding suggests that intranasal (local) administration of CpG DNA alone during allergen exposure is more effective than intradermal (systemic) administration of CpG DNA alone for control of allergic rhinitis, especially for the induction of airway allergen tolerance in allergic rhinitis. Rhee et al⁹ reported that intradermal administration of CpG

DNA alone can reduce Th2 responses in mice with allergic rhinitis. However, we could not show any effectiveness of intradermal administration of CpG DNA alone in mice with allergic rhinitis. This discrepancy may be caused by two differences between our study and the study by Rhee et al.⁹ The first is that mice received CpG DNA before nasal challenge in the report of Rhee et al,⁹ whereas mice received CpG DNA after nasal challenge in our report. The second is a difference of the total amount of CpG DNA administered; Rhee et al⁹ used 100 μ g and we used 30 μ g.

In this study, we used 30 μ g CpG DNA and could not show any significant effectiveness of intradermal administration of CpG DNA alone. Intradermally delivered CpG may attenuate allergic rhinitis more effectively, if the amount of CpG DNA is increased. However, the side effects of CpG DNA at high doses should be considerable.^{15,16}

All mice were challenged with Japanese cedar intranasally, but not intradermally, during allergen exposure in this study, although CpG DNA was administered intranasally or intradermally. Mice were challenged with cedar intranasally because people are exposed to pollen allergens via their nose. We think that the experiment protocol in this study is very close to a real-life clinical situation.

CpG DNA has been reported to attenuate allergic diseases in mice.^{5,9} However, the effects of CpG DNA on nasal lymphocytes have been not documented to date. In this study, we demonstrated that CpG DNA reduces IL-5 productions and that CpG DNA increases IFN- γ production by nasal lymphocytes in C57BL/6 mice only. This suggests that CpG DNA exhibits an effect on allergic rhinitis through nasal lymphocytes.

We discovered that intradermal CpG therapy does not significantly decrease IgE, eosinophilia, or IL-5 productions by nasal lymphocytes or splenocytes in C57BL/6 mice, but it does increase IFN- γ production by nasal lymphocytes, which suggests an increase of IFN- γ by CpG DNA does not always accompany reduction of IgE, eosinophilia, or IL-5. This finding also suggests a different mechanism between the effects of CpG DNA on Th1 response and Th2 response. It remains obscure why intradermal administration led to an increase of IFN- γ but no effect on the parameters such as IgE, eosinophilia, and IL-5 by nasal lymphocytes and splenocytes. However, one explanation is that CpG DNA effects the inhibition by a Th independent mechanism or by means of other cytokines secreted by Th1 cells that were not measured in this study. Conversely, a previous report¹⁷ stated that CpG DNA can decrease IL-5 in sera of IFN- γ knockout mice. Also, intradermal CpG therapy did not significantly decrease the levels of IL-5 produced by splenocytes in our BALB/c mice, but did decrease splenocyte IL-4 production. Consequently, CpG DNA may prevent the Th2 response via multiple mechanisms.

In this study, we investigated the effects of CpG DNA alone on allergic responses in two different mouse strains, BALB/c and C57BL/6 mice, because it has been reported

that the effects of CpG DNA are different in the two strains.¹¹⁻¹³ However, intranasal therapy with CpG DNA alone similarly reduced level of IgE, eosinophilic infiltration, nasal symptom, and IL-5 from nasal lymphocytes and splenocytes in both mouse strains. This finding certainly proves that intranasal CpG DNA therapy is useful for control of allergic rhinitis.

It was reported that intradermal administration of CpG DNA conjugated with Amb a 1, a major allergen of ragweed, may be useful for control of ragweed pollinosis in human beings.⁶ CpG DNA is expected to treat patients with allergic diseases. We have investigated the effect of therapy with intranasal administration of CpG DNA alone in mice, but not in human beings. However, our findings may give hope that a new therapy, such as CpG DNA nasal spray, soon may emerge clinically to control allergic rhinitis in human beings, although further studies are needed.

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