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THERAPEUTIC INTERVENTIONS

A Prophylactic Effect of an Oligodeoxynucleotide Containing a Cytidine–Guanosine Motif against Japanese Cedar Pollen-Induced T-Helper Type 2 Allergic Response

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Background. Over 10% of entire population in Japan suffer from allergic diseases induced by Japanese cedar pollen (JCP) every spring. In terms of preventive medicine, it has become a matter of urgency to establish successful prophylactic and therapeutic strategies for controlling the disorders. The effect of an oligodeoxynucleotide containing a cytidine–guanosine motif (CpG ODN) on the regulation of immune responses induced by JCP was investigated in this study. *Methods.* BALB/c mice were inoculated with CpG ODN intraperitoneally before intranasal sensitization to JCP. Cellular infiltration in the lung of BALB/c mice after treatment with CpG ODN or JCP was performed by hematoxylin and eosin (H&E) staining. Antibody titers and cytokines levels were determined by ELISA. *Results*. Intranasal inoculation of BALB/c mice with JCP induced a T-helper type 2 (Th2-type) dominant immune response, as characterized by the production of interleukin (IL)-4 and IL-5 in the lung and of JCP-specific IgE antibody in serum. Prior intraperitoneal administration of CpG ODN to mice suppressed the subsequent JCP-induced antibody production and infiltration of inflammatory cells in the lung. The inhibitory mechanism of CpG ODN seemed to be attributable to a CpG ODN-induced Th1-type dominant environment, which down-regulated Th2-type response subsequently induced by JCP allergen sensitization. Furthermore, administration with CpG ODN decreased the production of JCP-induced IL-17, which has been found to play a pivotal role in several inflammatory diseases including allergic asthma. The decreased production of IL-17, together with reduced secretion of IL-4 and IL-5, may contribute to diminish the inflammation in the lung of JCP-sensitized mice. *Conclusion*. This work provides evidence that the CpG ODN has a prophylactic effect on the JCP-induced Th2-type allergic responses by establishing or restoring a Th1-type shift of immune environments.

Keywords Japanese cedar pollen (JCP), BALB/c mice, allergic airway inflammation, oligodeoxynucleotide containing a cytidine-guanosine motif (CpG ODN), Th1-/Th2-type responses

INTRODUCTION

The antigen-induced IgE production (1), airway inflammation, and airway hyperreactivity (2–4) have been well documented in patients with allergic asthma and also in its animal model (5,6). Increasing evidences suggest that T-helper type 2 (Th2-type) cytokines, interleukin (IL)-4, IL-5 and IL-13, which are produced by activated $CD4^+$T cells, play a central role in the pathogenesis of allergic asthma (6,7). Thus, intervention by enhancing Th1-type cytokine production that suppresses the action of Th2-type cytokines is inferred to be useful in the treatment of allergic asthma.

The CpG ODN is a synthetic DNA sequence containing an unmethylated cytidine–phosphate–guanosine dinucleotide that mimics immune stimulatory properties of bacterial DNA (8). CpG ODN acts on the prevention or reversal of existing Th2 polarization *in vivo* (9–11) by creating a Th1 cytokine milieu or by restoring a normal Th1/Th2 balance, as demonstrated in several conventional ovalbumin (OVA)-specific allergic model experiments (12–14).

Recently, Japanese cedar (*Cryptomeria japonica*) pollen (JCP) has induced allergic rhinitis and conjunctivitis among more than 10% of population in Japan in every spring. Prophylactic and therapeutic strategies are urgently needed for controlling the illness. The mouse model system of JCP-induced allergic responses has been established in this laboratory (15). It becomes clear that JCP is a strong inducer that elicits Th2-type responses not only in upper airway but also in lower respiratory tract. In this study, we examined allergy-related mediators in the lung of JCP-sensitized mice to gain more insights into the possible therapeutic efficacy of CpG ODN against JCP-induced allergic respiratory disorders.

METHODS

Animals

Specific pathogen-free male BALB/c mice (6–7 weeks of age) were purchased from Japan SLC (Hamamatsu, Japan). Mice were given freshwater and autoclaved food

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and were kept at 23°C under bioclean condition throughout all experiments. To avoid laboratory contamination, all allergen-inoculated mice were housed in negatively pressurized isolators equipped with a ventilation system through a high-efficiency particulate air filter (AH model; Nihon-Ika, Osaka, Japan). This work was approved by the Institutional Animal Care and Use, Committee of Fukui University School of Medicine, Fukui, Japan.

Experimental Design

JCP crude extract was purchased from Cosmo Bio Co. (Tokyo, Japan). Nuclease-resistant phosphorothioate-modified ODN 1826 and control non-CpG ODN (Hokkaido System Science, Sapporo, Japan) were dissolved in endotoxin-/pyrogen-free phosphate-buffered saline (PBS). The sequence of ODN 1826 was TCCATGACGTTCCTGACGTT in which CpG motifs were underlined. The sequence of non-CpG ODN was TGGATCCAGCATGTCAGA. Mice, under light anesthesia (pentobarbital sodium, 0.025 mg/g body weight, intraperitoneal administration), were inoculated intranasally with 20 μl of PBS containing 20 μg of JCP per mouse at different time points, according to the schedule shown in Figure 1. To induce strong Th1 immune responses (16), CpG ODN was administered intraperitoneally into mice at an inoculum dose of 100 μg per mouse before JCP sensitization (concentrations of ODNs were based on the work of Kovarik et al. (16)). Samples were then collected at indicated time points.

Preparation of Single-Cell Suspensions from the Lung

Mice were anesthetized and the lung was flushed *in situ* with 20 ml sterile PBS via cannulation of the heart to remove the intravascular blood pool. Minced lung tissues were incubated at 37°C for 60 min on a rocker with 200 μg collagenase D/ml and 40 μg DNase/ml (Roche Molecular Biochemicals, Mannheim, Germany). The enzyme-digested lung tissue was passed through a stainless steel mesh. Single-cell suspensions were collected by density-gradient centrifugation with lymphocyte separation solution (Antibody Institute, Gumma, Japan).

Characterization of Lung Parenchyma Cells

Single-cell suspensions from the lung parenchyma were stained with hematoxylin and eosin (H&E) and the percentage distribution for the different cell types was determined.

Assay of Cytokine Production

Single-cell suspensions (4×10^5 cells in 200 μl per well) were prepared from the lung parenchyma of immunized mice and cultured for 48 h in the presence of CpG ODN (1μg/ml) or JCP (100 μg/ml). The supernatant was then harvested and assayed for IFN-γ, IL-12, IL-4, IL-5, and IL-17 titers using a mouse cytokine detection ELISA kit (BioSource International, Camarillo, CA, USA) in accordance with the manufacturer's instructions.

Quantification of Antibody Level

JCP-specific immunoglobulins (Igs) were measured using an ELISA Ig Quantitative kit (Bethyl Laboratories, Montgomery, TX, USA). Briefly, microtiter plates were coated with 10 μg purified JCP proteins overnight at 4°C. After blocking with 1% BSA for 30 min, serum or bronchoalveolar lavage (BAL) fluids were added to the well and incubated for 1 h. Bound antibodies were reacted with goat horseradish peroxidase-labeled anti-mouse IgE, IgG1, or IgG2a. Plates were read at 450 nm after the addition of 3,3′,5,5′-tetramethylbenzidine. Antibody titers were calculated using a standard curve that was determined from the reference serum using calculation software SPECTRA MAX 250 (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis

The two-tailed Mann–Whitney *U* test and Student's *t*-test were used to determine whether there was a significant difference ($p < .05$) between the experimental and control groups. Statistical analysis was done using StatView J4.11 software (SAS Institute, Cary, NC, USA).

RESULTS

Diminished Cellular Infiltration in the Lung by Prior Treatment with CpG ODN

Although there was no significant difference observed in the value of airway resistance between JCP-sensitized mice and control mice (not shown), intranasal inoculation with JCP caused a severe infiltration of inflammatory cells, such as neutrophils and eosinophils, in the lung

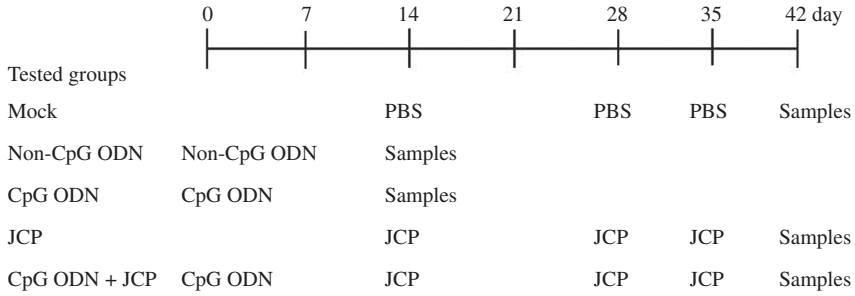

FIGURE 1.—Time schedule of experimental protocol. Mock group—mice were inoculated intranasally with PBS; non-CpG ODN group—mice were administered intraperitoneally with non-CpG ODN at a dose of 100 μg per mouse; CpG ODN group—mice were administered intraperitoneally with CpG ODN at a dose of 100 μg per mouse; JCP group—mice were inoculated with JCP at a dose of 20 μg per mouse; CpG ODN + JCP group—CpG ODN was administered intraperitoneally to mice prior to JCP sensitization. Five mice were used in each group.

TABLE 1.—Cellular infiltration in the lung of BALB/c mice after treatment with CpG ODN or JCP.

Tested group	No. of cells ($\times 10^5$) per lung			
	Total cells	Neutrophil	Eosinophil	Others
Mock	9.1 ± 3.2	0.2 ± 0.01	0.04 ± 0.02	8.9 ± 1.2
Non-CpG ODN	8.7 ± 4.5	0.2 ± 0.03	0.08 ± 0.04	9.5 ± 1.2
CpG ODN	9.2 ± 3.8	0.3 ± 0.02	0.06 ± 0.03	8.8 ± 0.9
JCP	23.9 ± 4.8*	9.3 ± 0.6*	1.70 ± 0.8*	12.9 ± 6.7
CpG ODN+JCP	18.0 ± 7.2**	5.4 ± 0.8**	0.90 ± 0.2**	11.7 ± 1.1

Notes: The lung parenchyma cells of BALB/c mice in each tested group were obtained at the time point indicated in Figure 1. Data are presented as means ± SD of results for each group of five mice tested.

*Significant difference ($p < .01$ by Mann–Whitney U test) compared with corresponding mock group. **Significant difference ($p < .05$ by Mann–Whitney U test) compared with corresponding JCP group.

parenchyma of mice (Table 1). Prior intraperitoneal administration of mice with CpG ODN, before sensitization to JCP, reduced the subsequent JCP-induced inflammatory reaction. The total number of inflammatory cells was reduced from $23.9 \pm 4.8 \times 10^5$ per lung in JCP-sensitized mice to $18.0 \pm 7.2 \times 10^5$ per lung in CpG ODN-pretreated and then JCP-sensitized mice. The number of neutrophils and eosinophils was also decreased by prior administration with CpG ODN (Table 1).

Suppression of Th2-Type Cytokine Production by Prior Treatment with CpG ODN

To elucidate the role of CpG ODN on the regulation of JCP-induced allergic reaction, IFN-γ, IL-12, IL-4, IL-5, and IL-17 in the culture fluids of lung parenchyma cells were assayed. The lung parenchyma cells from mock group of mice responded well *in vitro* to the stimulation with CpG ODN and produced a large amount of Th1-type cytokines such as IFN-γ and IL-12, whereas the lung cells did not react to the JCP stimulation (Table 2). The CpG ODN-sensitized mice showed a significant increase in the level of Th1-type cytokines after the boosting of lung cells with the homologous stimulator of CpG ODN. On the contrary, the reverse pattern of cytokine production was observed in the JCP group. Upon stimulation *in vitro* with the homologous allergen of JCP, an appreciable amount of Th2-type cytokines was released. The titers of IL-4, IL-5, and IL-17 were increased from each undetectable level to 25.7 ± 3.8, 37.2 ± 6.2, and 20.2 ± 4.5 pg/ml, respectively. In the same JCP group of mice, the production of Th1-type cytokines by a heterogenous stimulator of CpG ODN was slightly suppressed. Pre-sensitization with both CpG ODN and JCP (the CpG ODN + JCP group) reduced the production of Th2-type cytokines following subsequent stimulation with JCP *in vitro*. These results demonstrated that CpG ODN was a strong inducer on the production of Th1-type, but not Th2-type, cytokines. Intraperitoneal administration of CpG ODN before JCP sensitization increased the secretion of Th1-type cytokines and suppressed the Th2-type cytokine production.

Suppression of JCP-Induced Th2-Type Antibody Production by Prior Treatment with CpG ODN

The production of antigen-specific Th2-type antibodies is prerequisite for the immunological response leading to allergic diseases. The antibody titer in the serum of tested mice was determined by ELISA method. JCP-sensitized mice exhibited Th2-type dominant antibody responses, characterized by increased titers of total serum IgE, JCP-specific IgE as well as JCP-specific IgG1 (Table 3). By intraperitoneal administration of CpG ODN before JCP sensitization, the levels of these allergy-related antibodies diminished significantly. The total serum IgE has decreased to normal level and JCP-specific IgE antibody became undetectable. The level of JCP-specific IgG1 antibody has decreased from 16.5 ± 6.7 to 6.8 ± 2.2 ng/ml. These results demonstrated that CpG ODN

TABLE 2.—Cytokine production of the lung parenchyma cells stimulated *in vitro* with CpG ODN or JCP.

Tested group	Stimulated with	Cytokine titer (pg/ml)				
		IFN-γ	IL-12	IL-4	IL-5	IL-17
Mock	CpG ODN	138.2 ± 67.1	105.6 ± 35.2	<5.0	<5.0	<8.0
	JCP	<1.0	<5.0	<5.0	7.1 ± 0.3	<8.0
Non-CpG ODN	CpG ODN	123.1 ± 58.3	112.6 ± 42.8	<5.0	<5.0	<8.0
	JCP	<1.0	<5.0	<5.0	<5.0	<8.0
CpG ODN	CpG ODN	425.8 ± 28.6*	342.8 ± 63.2*	<5.0	6.4 ± 0.2	<8.0
	JCP	<1.0	6.8 ± 0.7	<5.0	5.2 ± 0.3	<8.0
JCP	CpG ODN	56.9 ± 8.9	61.9 ± 46.2	<5.0	<5.0	<8.0
	JCP	<1.0	<5.0	25.7 ± 3.8**	37.2 ± 6.2**	20.2 ± 4.5**
CpG ODN+JCP	CpG ODN	242.8 ± 38.4	184.8±53.1	<5.0	<5.0	<8.0
	JCP	4.3 ± 1.0	11.8 ± 3.2	<5.0***	9.8 ± 3.7***	11.1 ± 2.6***

Notes: The lung parenchyma cells of BALB/c mice in each tested group were obtained at the time point, as indicated in Figure 1. Lung cell suspension obtained from one mouse was divided into two equal parts. One part was stimulated *in vitro* with CpG ODN (1 μg/ml) and the other part was stimulated with JCP allergen (100 μg/ml) for 48 h. The concentration of cytokines in the culture supernatant was determined by ELISA. Data are presented as mean ± SD of results for each group of five mice tested.

*Significant difference ($p < .01$ by Mann–Whitney U test) compared with corresponding mock group and JCP group. **Significant difference ($p < .01$ by Mann–Whitney U test) compared with corresponding mock group and CpG ODN group. ***Significant difference ($p < .01$ by Mann–Whitney U test) compared with corresponding JCP group.

TABLE 3.—Production of total serum IgE and JCP-specific Ig isotypes in BALB/c mice after treatment with CpG ODN or JCP.

	Antibody titer (ng/ml) in						
	Serum				BAL		
Tested groups	Total IgE	Anti-JCP IgE	Anti-JCP IgG1	Anti-JCP IgG2a	Anti-JCP IgE	Anti-JCP IgG1	Anti-JCP IgG2a
Mock	77.1 ± 26.7	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9
Non-CpG ODN	79.3 ± 15.2	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9
CpG ODN	59.5 ± 14.8	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9
JCP	142.8 ± 76.9*	20.3 ± 8.8*	16.5 ± 6.7*	<3.9	18.3 ± 8.8*	12.7 ± 4.5*	<3.9
CpG ODN+JCP	108.0 ± 26.7**	<3.9***	6.8 ± 2.2***	<3.9	<3.9***	<3.9***	<3.9

Notes: The BAL fluids and sera were collected at the time indicated in Figure 1. Total IgE and JCP-specific IgE, IgG1 and IgG2a were assayed by ELISA kit. Data are presented as mean ± SD of results for each group of five mice tested.

*Significant difference ($p < .01$ by Mann–Whitney U test) compared with corresponding mock group and CpG ODN group. **Significant difference ($p < .05$ by Mann–Whitney U test) compared with corresponding JCP group. ***Significant difference ($p < .01$ by Mann–Whitney U test) compared with corresponding JCP group.

treatment down-regulated the JCP-induced Th2-type humoral immune responses.

DISCUSSION

Previous studies have reported that administration of CpG ODN during antigen sensitization of naive mice, or before antigen challenge of sensitized mice, prevents the Th2-directed allergic airway inflammation and airway hyperreactivity (12,17,18). However, in this study, we found that intraperitoneal administration of CpG ODN into mice before JCP sensitization reduced the JCP-induced Th2-type cytokine secretion, eosinophilic inflammation, and JCP-specific antibody production of IgE and IgG1.

A Th1-/Th2-type cytokine balance plays an important role in the regulation of a certain allergen-induced allergic reaction. The Th2-type cytokine like IL-4 and IL-13 promotes B cell switching to IgE production and hypersecretion of airway mucus. IL-5 has been shown to be primary determinant of eosinophil priming, activation, recruitment, and survival (7). In fact, mice genetically deficient in IL-4 or IL-5 exhibited a reduced level of allergen-induced airway hyperreactivity and eosinophilia (19,20). Thus, Th2-type cytokine profile is a critical component of allergen-induced allergic diseases. JCP sensitization induced a dominant neutrophilia and eosinophilia, and administration of CpG ODN reduced JCP-induced allergic responses (Table 1). JCP-sensitized mice showed an increase in the production of Th2-type cytokines. However, the prior treatment of CpG ODN caused the reduced production of Th2-type cytokines and augmented the production of Th1-type cytokines like IFN-γ and IL-12 (Table 2). IL-12 is rapidly released by antigen-presenting cells in response to infection and is essential for the development of a type 1 immune response (12). IFN-γ plays an important role in the differentiation of $CD4^+$T cells to T-helper type 1 cells and also down-regulates the Th2-type responses including allergic reactions (21). These results together with these findings suggested that the prophylactic effect of CpG ODN against the Th2-driven allergic airway response is associated with the Th1-type dominant cytokine production. It seems likely that the administration of CpG ODN into BALB/c mice establishes or restores a type 1 shift immune environment which down-regulates type 2 immune responses subsequently elicited by JCP sensitization.

It is of interest to find that CpG ODN treatment inhibited the production of IL-17 in the lung of JCP-sensitized mice (Table 2). The proinflammatory cytokine of IL-17 has been forwarded as a link between the activated T-lymphocytes as well as the recruitment and activation of neutrophils in various types of airway inflammation (22). The concentration of IL-17 is increased in BAL fluid (BALF), sputum, and blood from patients with asthma (23,24). In a mouse model of allergic airway inflammation, systemic blockade of IL-17 inhibits allergen-induced accumulation of neutrophils in the airway (25). These findings suggest that IL-17 is involved in the pathophysiology of asthma. Thus, the reduced production of IL-17 by CpG ODN administration may contribute to diminish the inflammation in the lung of JCP-sensitized mice, particularly to decrease the neutrophil accumulation.

Because natural allergic inflammatory reactions are mediated by a combination of Th2-type cytokines, the single administration of CpG ODN therapy for allergic diseases may offer some advantages over the therapeutic administration of antibodies to IL-4, IL-5, IL-13, or to their receptors. This work supports further researches focusing on the development of a stimulator of Th1 immune responses for clinical use.

CONCLUSION

This work provides evidence that the CpG ODN has a prophylactic effect on the JCP-induced Th2-type allergic responses by establishing or restoring a Th1-type shift of immune environment.

Declaration of Interest

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