

Effect of Oral Administration of CpG ODN-OVA on WBB6F1-W/W^v Mice

Reiko Teshima¹, Haruyo Okunuki¹, Yuji Sato², Hiroshi Akiyama², Tamio Maitani² and Jun-ichi Sawada¹

ABSTRACT

Background: We have already reported that antigen-specific IgG1 antibody production in WBB6F1-W/W^v (W/W^v) mice after oral administration of ovalbumin (OVA) was extremely high. Active systemic anaphylaxis (ASA) was induced in these mice after intraperitoneal (i.p.) administration of OVA, and Th2-dominant helper T-cell activation occurred. In this study, we examined the effect of CpG oligodeoxynucleotide (ODN) conjugation of OVA on oral immunization of W/W^v mice.

Methods: W/W^v mice were sensitized by administration of 0.1 mg OVA or CpG ODN-OVA by gavage every day for 4 weeks, and the serum titers of OVA-specific IgG1, IgE, and IgG2a antibody were determined. ASA was induced by i.p. injection of OVA, and the changes in body temperature were monitored. *In vitro* production of Th1- and Th2- type cytokines by splenocytes re-stimulated with antigen was also measured.

Results: The antigen-specific IgG1 antibody titer in the CpG ODN-OVA-sensitized W/W^v mice was lower than in the OVA-sensitized group, but the IgG2a titer was higher. ASA was not induced by i.p. OVA challenge. There were significant increases in the production of Th1-type cytokine (IFN- γ) by splenocytes in the CpG ODN-OVA-sensitized mice, but the Th2-type cytokine (IL-4) level in the splenocyte culture medium was lower.

Conclusions: These results indicated that oral administration of CpG ODN-OVA conjugate significantly induced antigen-specific Th1 responses and reduced Th2 responses (allergic reactions) on re-stimulation. These findings suggest that CpG ODN-antigen conjugate may be useful as an oral vaccine.

KEY WORDS

allergy, CpG motif, oral-sensitization, ovalbumin, WBB6F1-W/W^v mice

INTRODUCTION

Mucosal immunization is characterized by oral and nasal immunization and is known to effectively induce both antigen-specific mucosal and systemic immune responses. Therefore, oral or nasal vaccines may be useful in preventing and curing allergy and infection. Oral immunization seems to be superior to immunization by injection in terms of convenience and safety.

Oligodeoxynucleotides (ODN) containing unmethylated CpG motifs (5'-Pu-Pu-CpG-Pyr-Pyr-3') have recently been reported to activate host defense mechanisms inducing innate and acquired immune responses.¹ CpG ODN directly stimulates cells that express Toll-like receptor (TLR) 9, including macrophages and other antigen-presenting cells (APCs).

Consequently the cells secrete type 1 cytokines, such as IFNs and IL-12, which cause naïve T cells to differentiate into Th1 cells.² Recognition of CpG motifs requires TLR9,³ which triggers the induction of cell-signaling pathways, including mitogen-activated protein kinases (MAPKs) and NF κ B.⁴

Several study groups have conducted research on the application of CpG ODNs in the suppression of allergy,⁵ and many reports ascribe the etiology of allergy to disruption of the balance between Th1- and Th2-T cells and a Th2-dominant state.⁶ Thus, allergic symptoms may be controllable, if the Th-balance is shifted to Th1 dominance.⁷ Shirota *et al.* found that a conjugate between CpG ODN and antigen (Ag) inhibited airway eosinophilia 100-fold more efficiently than a mixture of unconjugated CpG and the Ag.⁸ Administration of CpG ODN alone may cause nonspecific

¹Division of Biochemistry and Immunochemistry and ²Division of Food, National Institute of Health Sciences, Tokyo, Japan.
Correspondence: Dr. Reiko Teshima, Division of Biochemistry and Immunochemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan.

Email: rteshima@nihs.go.jp

Received 31 January 2005. Accepted for publication 5 July 2005.

©2006 Japanese Society of Allergology

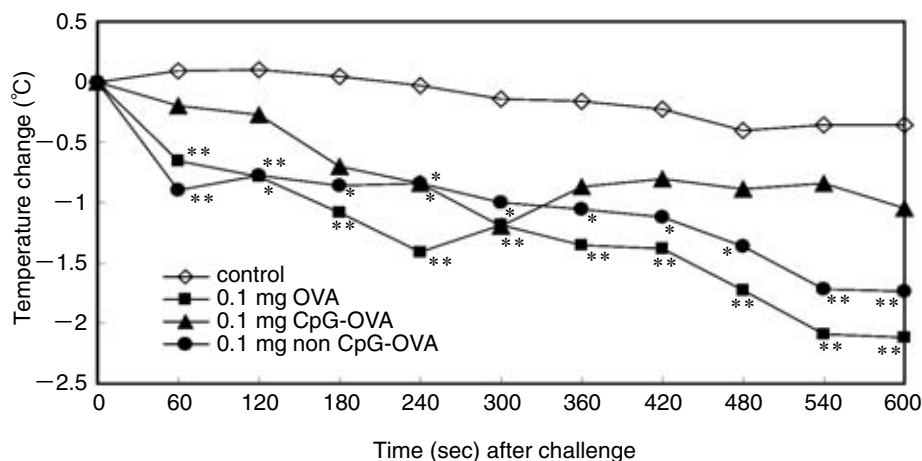


Fig. 1 Changes in body temperature of antigen-sensitized WBB6F1-W/W^v mice after systemic immunization. Body temperature was monitored at 1-minute intervals after OVA challenge. Each value represents the mean for 7 mice. *, ** Significant difference from the control by Student's *t*-test at $p < 0.05$ and $p < 0.01$, respectively.

Th1-like activation and a toxic effect.

We succeeded in inducing antigen-specific antibody production without any adjuvant by administering 0.1 mg or 1.0 mg OVA orally for 9 weeks daily, and we induced ASA by intraperitoneal (i.p.) injection of antigen.⁹ W/W^v mice proved to be a good model for studying induction of food allergy, because their antigen-specific antibody titer has been shown to be much higher than those in other mice (BALB/c, B10A etc).^{10,11} W/W^v mice have mutations in the *c-kit* gene and exhibit defects or deficits in mast cells, erythrocytes, and interstitial cells of cajal, which express *c-kit* protein naturally.^{12,13} The results also suggest that the high susceptibility of the W/W^v mice is attributable to a decrease in *c-kit*-positive cells and/or TCR $\gamma\delta$ -T cells (submitted).

We, therefore, assessed the possibility of using CpG ODN-OVA as an oral vaccine in W/W^v mice. The CpG ODN conjugated to OVA was administered orally to the W/W^v mice, and they were examined for induction of Th1 responses and suppression of allergic reactions.

METHODS

ANIMALS

Female WBB6F1-W/W^v (6 weeks) mice were purchased from Japan SLC (Shizuoka, Japan) and maintained under pathogen-free conditions in our animal facility for 1 week before use. The mice were handled in accordance with NIH Animal Care and Use Guidelines.

CPG AND NON-CPG ODN

The CpG ODN used throughout this study consisted of 20 bases containing two CpG motifs (5'-TCCAT GACGTTCTGACGTT-3').¹⁴ The control ODN was

identical except that the CpG motifs were rearranged (5'-TCCATGAGCTTCCTGAGCTT-3'). Phosphorothioate ODNs were synthesized by TaKaRa Biomedicals (Shiga, Japan). ODN was thiolated for conjugation. The LPS content of the ODN was <200 pg LPS/mg of DNA, as measured by the Limulus HS-J Single Test (Wako Pure Chemicals, Osaka, Japan).

DIRECT CONJUGATION OF ODN TO OVA

ODN was conjugated to OVA by mixing thiolated ODN with maleimide-activated OVA (PIERCE, IL, USA) at 4°C overnight. The molar ratio of CpG to OVA in the conjugate was calculated to be 6 : 1. The molar ratio of non-CpG ODN : OVA was calculated to be 4.6 : 1.

IMMUNIZATION AND INDUCTION OF ACTIVE SYSTEMIC ANAPHYLAXIS (ASA)

Mice were sensitized by daily administration of 0.1 mg OVA (grade V, Sigma Chemical Co, St Louis, MO, USA), CpG ODN-OVA or non-CpG ODN-OVA by gavage for 4 weeks, and ASA challenge was performed by i.p. injection of 1.0 mg of OVA 1 day later. Body temperature changes associated with ASA were monitored with a rectal thermometer for mice (Shibaura Electronics Co. Ltd., Japan) without general anesthesia.

ANTIBODY (IGE, IGG1, AND IGG2A) TITER DETERMINATION

The serum titers (reciprocal of serum dilution with fluorescence intensity at 50% of the maximum level) of OVA-specific IgE, IgG1 and IgG2a were determined by a previously reported method.⁹ A 50 μ L volume of OVA (40 μ g/mL) in 50 mM sodium car-

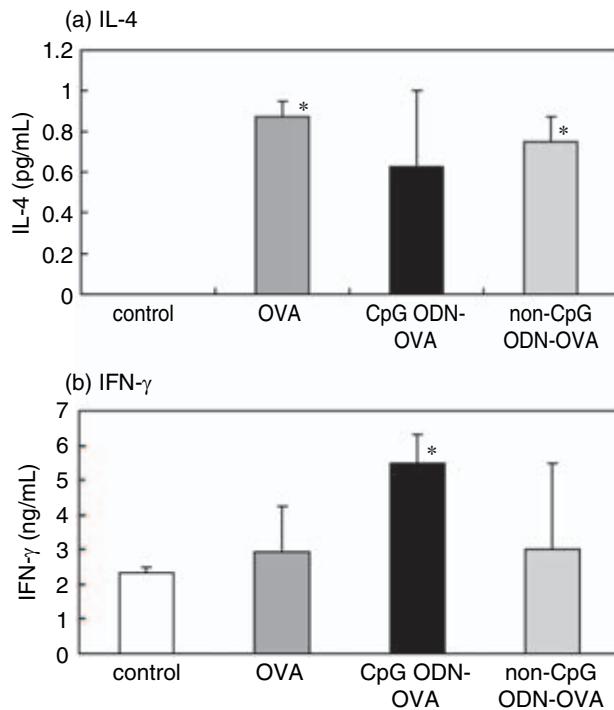


Fig. 2 Cytokine production by splenocytes from antigen-sensitized WBB6F1-W/W^v mice after systemic immunization. Splenocytes were isolated from WBB6F1-W/W^v mice orally sensitized with antigen after i.p. sensitization with 1.0 mg of OVA. (a) IL-4 and (b) IFN-γ cytokine production by splenocytes was measured as described in the Methods section. Each value represents the mean ± S.D. for 7 mice. * Significant difference from the control by Student's *t*-test at *p* < 0.05.

bonate buffer, pH 9.6, was added to each well of a 96-well microtiter plate, and the plate was incubated overnight at 4°C. The solutions were discarded, and each well was washed 4 times with 200 μL PBS containing 0.05% Tween 20 (PBS/Tween). To minimize the nonspecific binding of serum proteins to unoccupied solid-phase sites, 200 μL of 0.1% casein in PBS was added and the plates were incubated for 1 hour at room temperature. The casein solution was removed, and each well was washed in the same manner as above. Fifty μL of the diluted serum containing OVA-specific antibodies were added to each well, and the plates were incubated for 20 hours at 4°C. The solutions were removed, and each well was washed. Fifty μL of rabbit anti-mouse IgE, IgG1, and IgG2a (10⁻³ dilution in PBS containing 0.1% casein (Nordic Immunology, Tilburg, the Netherlands)) were added to each well, and the plates were incubated for 1 hour at room temperature. The solution in each well was removed and washed. Fifty μL of β-galactosidase-linked goat anti-rabbit Ig conjugate (10⁻³ dilution in PBS containing 0.1% casein, (Amersham Pharmacia

Biotech, Uppsala, Sweden)) were added to each well, and the plates were incubated for 1 hour at room temperature. The antibody-enzyme conjugate solution in each well was removed and washed. The wells were incubated for 1 hour at 37°C with 100 μL PBS containing 0.1 mM 4-methylumbelliferyl-β-galactoside (Sigma). Finally, 25 μL of 1 M sodium carbonate was added to each well. The fluorescence intensity of the liberated 4-methylumbelliferone was monitored at 317 and 374 nm for excitation and emission, respectively, by a Titertek Fluoroscan reader (Flow Laboratories Inc., Costa Mesa, CA, USA).

CYTOKINE ASSAYS OF SPLENOCYTES

Spleen cells were collected from the OVA-immunized mice (5 mice per group), and the cells (5 × 10⁶ cells/ml) were re-stimulated with OVA *in vitro* at a final concentration of 100 μg/mL in a 24-well culture plate at 37°C for 3 days.¹⁵ The levels of IL-4, IL-5, IL-12 (p70), and IFN-γ in the culture medium (RPMI 1640) after 3 days of co-culture with OVA were measured with an OptEIA mouse cytokine ELISA set (PharMingen). Absorbance was measured at 450 nm with a microplate reader (EL 340, Bio-Tek Instruments, Winooski, VT, USA).

ISOLATION OF MOUSE IELS

IELs were isolated as previously described by Nagafuchi.¹⁶ In brief, the small intestine (duodenum, jejunum, and ileum) was removed from the mice, and the small intestine was turned inside-out using polyethylene tubing. Each reversed intestine was cut into four segments, and the segments were placed in a conical 50-mL polypropylene tube containing 45 mL of Hanks' balanced salt solution (HBSS) (GIBCO) containing 5% FBS (Sigma chemical Co). The tube was shaken at 37°C for 45 minutes (horizontal position on an orbital shaker at 135 rpm), and the cell suspension was passed through a glass-wool column to remove adherent cells. The cells were then suspended in 30% (wt/vol) Percoll (Amersham Pharmacia Biotech) and centrifuged for 20 minutes at 1800 rpm. The cell pellet was collected; IELs were purified by density gradient centrifugation using Percoll as the separation medium (1800 rpm, 20 min); and IELs were recovered at the 44% and 70% Percoll interphase. More than 90% of the IELs were recovered.

FLOW CYTOMETRY (FCM)

Two-color or three-color analysis of spleen and IEL subsets was performed. The antibodies used for FCM were phycoerythrin (PE)-labeled anti-mouse CD8α (53-6.7; PharMingen), fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4 (H129.19; PharMingen), peridinin chlorophyll protein (PerCP)-labeled anti-mouse CD3ε (145-2C11; PharMingen), FITC-labeled anti-mouse Integrin β7 chain (M293; PharMingen), PE-labeled anti-mouse TCRγδ (UC7-13D5;

Table 1 Serum OVA-specific antibody of WBB6F1- (+ / +) mice orally sensitized with CpG ODN-OVA

OVA-specific antibody	ELISA titer			
	control	OVA	CpG ODN-OVA	Non CpG ODN-OVA
IgG1	<50	1160 ± 683 *	383 ± 176	275 ± 318
IgG2a	<50	<50	1126 ± 1259	<50
IgE	<50	58.5 ± 18.5	<50	<50

Values are means ± S.D. of data from seven mice per group.

*: Significant difference from the control at $p < 0.05$

Table 2 Flow cytometric analysis of the IELs of W/W^v mice

	control	OVA	CpG ODN-OVA	non-CpG ODN-OVA
slg ⁺ (B cell)	2.879 ± 1.560	2.867 ± 1.891	2.216 ± 1.487	3.494 ± 2.025
CD3 ⁺ β7 ⁺ (T cell)	86.28 ± 1.747	88.97 ± 4.128	91.96 ± 4.411 *	87.95 ± 3.681
CD4 ⁺ CD8 ⁺ (TCRαβCD4CD8αα)	22.85 ± 3.470	20.89 ± 3.597	26.56 ± 7.175	20.58 ± 5.161
CD4 ⁺ CD8α ⁻ (TCRαβCD4)	10.29 ± 4.090	12.22 ± 5.134	14.97 ± 4.422	9.952 ± 3.426
CD8α ⁺ CD8β ⁻ (CD8αα)	32.79 ± 4.202	31.95 ± 6.158	38.57 ± 5.442	36.65 ± 5.054
CD8α ⁺ CD8β ⁺ (TCRαβCD8αβ)	48.92 ± 3.662	51.07 ± 6.515	46.98 ± 5.882	47.69 ± 6.445
TCRαβ ⁻ γδ ⁺ (TCRγδ)	2.933 ± 0.992	3.002 ± 0.906	2.587 ± 1.039	3.478 ± 1.531
TCRαβ ⁺ γδ ⁻ (TCRαβ)	85.72 ± 7.520	90.16 ± 3.115	91.93 ± 3.859	87.77 ± 3.156

Values are means ± S.D. of (% (gated)) data from seven mice per group.

*: Significant difference from control at $p < 0.05$.

Santa Cruz Biotechnology, Delaware, CA, USA), PE-labeled anti-mouse TCRγδ (GL3; Immunotech, Marseille, France), FITC-labeled anti-mouse TCRαβ (H57-597; Immunotech), PerCP-labeled anti-mouse CD4 (H129.19; PharMingen), and FITC-labeled anti-mouse CD8β (53-5.8; PharMingen) antibodies. All incubations were performed in the dark. A single cell suspension of lymphocytes in PharMingen Stain Buffer containing 2% FBS was incubated with 50 μL of properly diluted mAb at 4°C for 30 min. The cells were washed in HBSS by centrifugation, and after staining, a total of at least 10,000 cells was analyzed with a FACS Calibur (Becton Dickinson, Sunnyvale, CA, USA). The data were analyzed with Cellquest[®] software.

STATISTICAL ANALYSIS

All values are expressed as means ± standard deviation of the mean. Statistical comparisons were performed with Student's *t*-test or Mann-Whitney's *U*-test. Each experiment was repeated at least twice.

RESULTS

Antibody production in CpG ODN-OVA- or OVA-sensitized mice was investigated by measuring the serum titer of OVA-specific IgG1, IgE, and IgG2a antibody of each mouse by indirect ELISA (Table 1). There were marked rises in specific IgG1 antibody titer (1160 ± 683) in the OVA-sensitized mice, and the IgE antibody titer also rose slightly. In the CpG ODN-OVA-sensitized W/W^v mice, on the other hand, the

antigen-specific IgG1 antibody titer (383 ± 176) was lower than that in the OVA-sensitized group, while IgG2a titer (1126 ± 1259) was higher than that (<50) in the OVA-sensitized group. OVA-specific IgA antibody titer, which is a marker of mucosal immunity, was slightly increased in the OVA-sensitized mice (unpublished data).

The body temperature of the sensitized mice was measured at 1-minute intervals for 10 minutes after the i.p. challenge (systemic immunization) with 1.0 mg of OVA (Fig. 1). Gradual decreases were observed in the OVA- and non-CpG ODN-OVA-sensitized mice, and the decreases in the body temperature in both groups of mice at 600 seconds after i.p. OVA immunization were significant in comparison with the control mice. The hypothermia confirmed the induction of ASA. However, there were no significant changes in body temperature in the CpG ODN-OVA-sensitized mice at 600 seconds after i.p. OVA immunization.

Figure 2 shows the *in vitro* production of cytokines by splenocytes re-stimulated with 100 μg of OVA. There were significant rises in the production of Th2-type cytokines (e.g., IL-4) by the splenocytes from the OVA- and non-CpG ODN-OVA-sensitized mice (Fig. 2a), whereas the Th1-type cytokine (IFN-γ) levels in the splenocyte culture media increased in the CpG ODN-OVA-sensitized group (Fig. 2b). These results suggested that oral sensitization with the CpG ODN-OVA conjugate resulted in a state of Th1-dominant helper T-cell activation.

The duodenum, jejunum, and ileum were excised from antigen-sensitized mice, and IELs were isolated. The population of IELs from each group was analyzed by FCM (Table 2). Only total T cells increased slightly as a proportion of IELs in the CpG ODN-OVA-sensitized mice, and the populations of other cells were almost the same as in the control mice. The populations of IELs in the OVA-sensitized group and the non-CpG ODN-OVA-sensitized group were almost the same as in the control mice.

DISCUSSION

Several reports have described suppression of the allergic reaction in mice by subcutaneous or i.p. administration of CpG ODN combined with antigen.^{17,18} and Shirota showed that inhalation of CpG ODN-OVA conjugate inhibits airway eosinophilia in OVA-sensitized mice.⁸ Another report claimed that, when the CpG ODN-antigen conjugate is given to Amb a1-sensitized mice intraperitoneally, the Th1 reaction was induced and the antigen-specific IgE antibody titer did not increase after additional administration of Amb a1.¹⁹ However, there have been no reports of oral administration of CpG ODN-antigen conjugate causing suppression of the allergic reaction.

In this study, we conjugated CpG ODN with the antigen and administered it orally to mice. The CpG-B ODN with nuclease-resistant phosphorothioate (PS) backbones, which were used in this study, has dramatically enhanced B-cell-stimulating properties and stability compared to CpG-A ODN with phosphodiester (PO) backbones. CpG-B ODN stimulates NK cells and induces IFN- γ secretion, although the degree of stimulation is weaker than by use with CpG-A ODN.^{20,21} CpG ODN might give rise to adverse effects, because it causes antigen-non-specific reactions. Therefore, we produced the CpG ODN-antigen conjugate to reduce the adverse effects of the antigen-non-specific reactions of CpG ODN and investigated whether it can induce antigen-specific Th1 activity.

Table 1 shows that the antigen-specific IgG1 (Th2-like) antibody titer in the CpG ODN-OVA-sensitized W/W^v mice was lower than in the OVA-sensitized group, whereas the IgG2a (Th1-like) titer was higher, and there were significant rises in the production of Th1-type cytokine (IFN- γ) by splenocytes in the CpG ODN-OVA-sensitized mice. On the other hand, the Th2-type cytokine (IL-4) level in the splenocyte culture medium decreased (Fig. 2). Th2-type lymphocytes produce cytokines (IL-4, IL-5, etc.) in response to the antigenic peptide presented on the antigen-presenting cells (APCs). These cytokines activate inflammatory cells, such as mast cells and eosinophils, and eventually cause allergy. Th1 cells, on the other hand, have the ability to suppress cytokine production by Th2 cells. Many reports ascribe the development of allergy to the disruption of the balance be-

tween Th1-and Th2-T cells and a Th2-dominant state.⁶ We demonstrated that the CpG ODN-OVA conjugate orally administered to W/W^v mice altered the Th1/Th2 balance by inducing strong Th1 responses. The slight increase of total T cells in a proportion of IELs in the CpG ODN-OVA-sensitized mice was observed (Table 2). This might reflect the stimulatory effect of CpG-ODN-OVA on Th1 cells.

Allergen immunotherapy is associated with a risk of anaphylaxis, and it is important to prevent anaphylaxis for the creation of good vaccines. Our experimental OVA sensitization conditions (dose and period) are generally strong enough to induce ASA and to induce shock on antigen provocation (Fig. 1). However, ASA was slightly inhibited in the CpG ODN-OVA conjugate-administered mice, and almost no hypothermia was observed, the same as in the control. Horner reported finding that the intradermal or intravenous administration of CpG ODN-antigen conjugate to mice reduced the possibility of anaphylactic shock,²² and since our study showed that oral administration of CpG ODN-antigen reduced anaphylaxis reactions, the CpG ODN-antigen conjugate seems to be useful as an oral vaccine. Especially, the oral administration of CpG ODN antigen seems to be applicable to food-derived sensitivity, such as food allergy or celiac disease.

The mechanism of action of the CpG ODN-antigen conjugate has not been examined in detail. Shirota proposed the following hypothesis.⁸ The APCs surrounded by CpG ODN-OVA present the antigenic peptide on their membrane surface and simultaneously secrete IL-12. The anti-OVA Th cells recognize antigen peptides on APCs, approach APCs very closely, and become an effective target for IL-12. The effect of the CpG ODN is then concentrated on T cells specific for the antigen combined with CpG ODN, and an antigen-specific reaction occurs. These IL-12-induced Th1 cells weaken the effect of Th2 cells, and the antigen-specific allergy reactions are inhibited.

As intraperitoneal injection with Amb a1-CpG conjugate to Amb a1-sensitized mice induced a de novo Th-1 response and suppressed IgE antibody formation after additional challenge with Amb a1.¹⁹ Oral administration of OVA-CpG to OVA immunized mice might suppress IgG1 or IgE antibody production after additional challenge with OVA.

In conclusion, we conjugated CpG ODN to OVA and succeeded in inducing antigen-specific Th1 reactions by oral administration to W/W^v mice. The CpG-ODN-OVA conjugate seems to be useful as an oral vaccine.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Ministry of Health, Labor and Welfare, and the Cooperative System for Supporting Priority Research of the Japan

Science and Technology Agency. We thank Dr. Mamoru Totsuka for his helpful suggestions in regard to preparation of the IEL cells.

REFERENCES

1. Krieg AM, Yi AK, Matson S *et al*. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995;**6522**: 546-549.
2. Roman M, Martin-Orozco E, Goodman J *et al*. Immunostimulatory DNA sequences function as Th1 inducing adjuvants. *Nat. Med.* 1997;**3**:849-854.
3. Takeshita F, Liefer AC, Gursel I *et al*. Role of toll-like receptor 9 in CpG DNA-induced activation of human cells. *J. Immunol.* 2001;**167**:3555-3558.
4. Krieg AM. CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 2002;**20**:709-760.
5. McCluskie MJ, Davis HL. CpG DNA as mucosal adjuvant. *Vaccine* 1999;**18**:231-237.
6. McHugh SM, Deighton J, Stewart AG, Lachmann PJ, Ewan PW. Bee venom immunotherapy induces a shift in cytokine responses from a TH-2 to a TH-1 dominant pattern: comparison of rush and conventional immunotherapy. *Clin. Exp. Allergy* 1995;**25**:828-838.
7. Krieg AM. Mechanisms and applications of immune stimulatory CpG oligodeoxynucleotides. *Biochim. Biophys. Acta* 1999;**1489**:107-116.
8. Shirota H, Sano K, Kikuchi T, Tamura G, Shirato K. Regulation of murine airway eosinophilia and Th2 cells by antigen-conjugated CpG oligodeoxynucleotides as a novel antigen-specific immunomodulator. *J. Immunol.* 2000;**164**:5575-5582.
9. Okunuki H, Teshima R, Sakushima J *et al*. Induction of active systemic anaphylaxis by oral sensitization with ovalbumin in mast-cell-deficient mice. *Immunol. Lett.* 2000;**74**:233-237.
10. Akiyama H, Teshima R, Sakushima J *et al*. Examination of oral sensitization with ovalbumin in Brown Norway rats and three strains of mice. *Immunol. Lett.* 2001;**78**:1-5.
11. Okunuki H, Teshima R, Harikai N *et al*. Oral sensitization of W/W^v mice with ovalbumin and possible involvement of the decrease in $\gamma\delta$ -T cells. *Biol. Pharm. Bull.* 2003;**26**: 1260-1265.
12. Nocka K, Majumder S, Chabot B *et al*. Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice—evidence for an impaired c-kit kinase in mutant mice. *Genes Dev.* 1989;**3**:816-826.
13. Nocka K, Tan JC, Chiu E *et al*. Molecular bases of dominant negative and loss of function mutations at the murine c-kit/white spotting locus: W37, W^v, W41 and W. *EMBO J.* 1990;**9**:1805-1813.
14. Sano K, Shirota H. CpG oligodeoxynucleotides as a future vaccine for allergic disease. *Allergol. Int.* 2005;**54**:17-23.
15. Akiyama H, Hoshino K, Tokuzumi M *et al*. The effect of feeding carrots on immunoglobulin E production and anaphylactic response in mice. *Biol. Pharm. Bull.* 1999;**22**: 551-555.
16. Nagafuchi S, Totsuka M, Hachimura S *et al*. Dietary nucleotides increase the proportion of a TCR $\gamma\delta$ subset of intraepithelial lymphocytes (IEL) and IL-7 production by intestinal epithelial cells (IEC); implications for modification of cellular and molecular cross-talk between IEL and IEC by dietary nucleotides. *Biosci. Biotechnol. Biochem.* 2000;**64**:1459-1465.
17. Broide DH, Stachnick G, Castaneda D *et al*. Systemic administration of immunostimulatory DNA sequences mediates reversible inhibition of Th2 responses in a mouse model of asthma. *J. Clin. Immunol.* 2001;**21**:175-182.
18. Santeliz JV, Van Nest G, Traquina P, Larsen E, Wills-Karp M. Amb a 1-linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. *J. Allergy Clin. Immunol.* 2002;**109**:455-462.
19. Tighe H, Takabayashi K, Schwartz D *et al*. Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. *J. Allergy Clin. Immunol.* 2000;**106**:124-134.
20. Ballas ZK, Rasmussen WL, Krieg AM. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* 1996;**157**:1840-1845.
21. Yamamoto S, Yamamoto T, Kataoka T *et al*. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN [γ] and augment IFN-mediated [γ] natural killer activity. *J. Immunol.* 1992;**148**:4072-4076.
22. Horner AA, Takabayashi K, Beck L *et al*. Optimized conjugation ratios lead to allergen immunostimulatory oligodeoxynucleotide conjugates with retained immunogenicity and minimal anaphylactogenicity. *J. Allergy Clin. Immunol.* 2002;**110**:413-420.