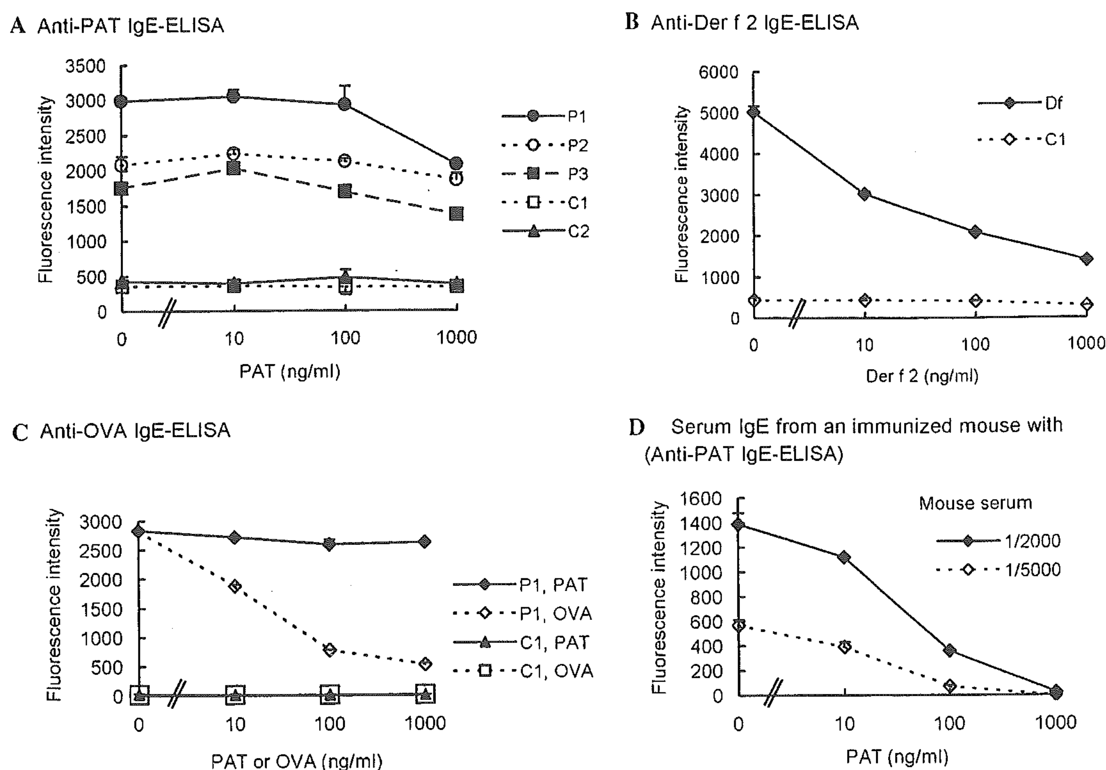

Fig. 2. ELISA-inhibition assays. (A) Three patient sera (P1, P2, and P3) and two control sera (C1 and C2) (at a final dilution of 1:20) were pre-incubated with indicated concentrations of PAT, and the sera were applied to PAT-coated ELISA wells. (B) A mite (*Dermatophagoides Farinae*)-specific patient serum (Df) and a control serum (C1) (at a final dilution of 1:20) were pre-incubated with indicated concentrations of Der f 2 and applied to Der f 2-coated ELISA wells. (C) Serum P1 (anti-OVA-IgE positive) and control serum C1 were pre-incubated with indicated concentrations of PAT or OVA and applied to OVA-coated ELISA wells. (D) Diluted anti-PAT murine serum samples were pre-incubated with indicated concentrations of PAT and applied to PAT-coated ELISA wells. The bound IgE was measured as described in Section 2. Values are the mean of triplicate wells. Similar results were observed in another experiments.

tivity, we used this improved method including NaCl-washing for the following studies.

3.5. Comparison among the four groups using the anti-PAT IgE-ELISA

Serum samples from the 29 patients of Group A, 32 patients of Group B, 23 patients Group C, and 9 subjects of Group D (healthy controls), were examined by the anti-PAT IgE-ELISA with 1 M NaCl-washing. The result is shown in Fig. 4. No statistically significant differences were seen among the four groups (one-way ANOVA). All the absorbance values were within the range of the average $\pm$ 5SD of Group D.

3.6. Serum reactivity to non-GM or GM soybean extracts by Western blotting

Two of the Japanese patients' sera (Grpop B), P5 and P6, which were positive for soybean specific IgE test (Immuno CAP scores, 6 and 3, respectively), were used. Their serum IgE detected some proteins in GM (CP4-EPSPS introduced) and non-GM soybean extracts, and, their stained patterns were so similar between GM and non-GM extract-blotted membranes (Fig. 5). No band was detected by a healthy control serum (data not shown).

3.7. A pilot study for the three recombinant proteins using the improved ELISA method

We also used the improved ELISA method described above for CP4-EPSPS or Cry 9C in addition to PAT to detect the antigen-specific IgE in the 4 groups. As shown in Table 1, we screened 151 serum samples for PAT, 140 for Cry9C, and 132 for CP4-EPSPS. To date, no samples positive for IgE to PAT, CP4-EPSPS, and Cry 9C have been found.

4. Discussion

Authorized GM crops for food use in Japan include 61 varieties of 6 plants (as of April 2005). In most of these GM crops, CP4-EPSPS, PAT, or *Bacillus thuringiensis* insecticidal toxins were introduced. Their donor bacterial species are not known to be allergenic, and no significant homology has been found between the three proteins and known allergens. Therefore, it is likely that these proteins are non-allergenic. However, it is important to monitor the unintended occurrence of allergenic reactions to these proteins. The monitoring of serum IgE from Japanese allergic patients for specific binding to CP4-EPSPS, PAT, and Cry9C has not yet been reported. Therefore, serum samples

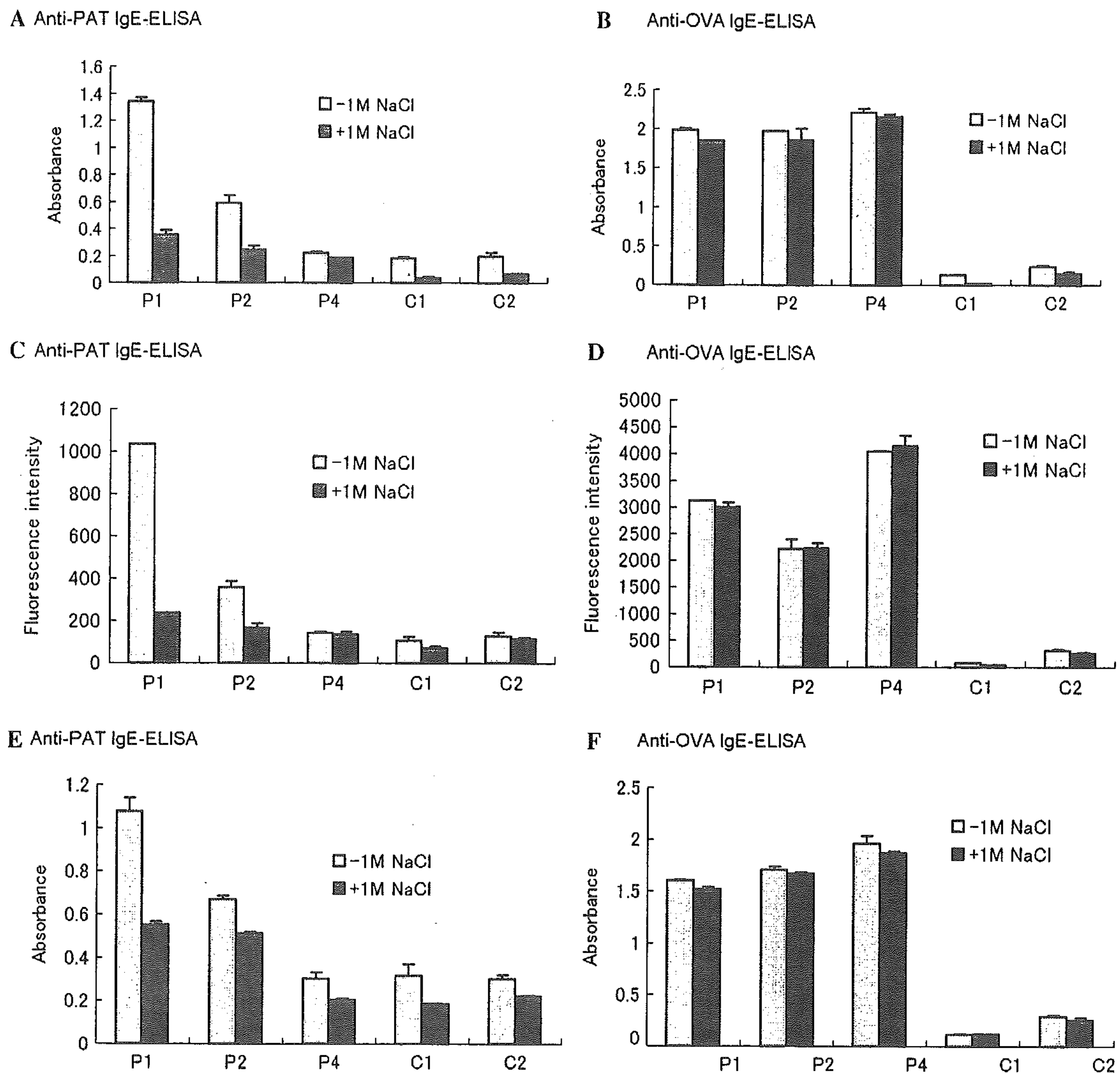

Fig. 3. Effect of 1 M NaCl-washing on the binding of patient serum IgE to the coated PAT and OVA, and comparison of detection methods and blocking reagents. Two patient sera (P1 and P2), an OVA-specific IgE positive serum (P4), and two control sera (C1 and C2) were applied to PAT-(A, C, and E) or OVA coated-(B, D, and F) wells. After incubation, the wells were washed with PBS-T or PBS-T containing 1 M NaCl, and the bound IgE was detected by colorimetric method (A, B, E, and F), or fluorometric method (C and D). And two kinds of blocking reagents were compared, 0.1% casein (A–D) and 10% FBS (E and F) in PBS, pH 7.0. Values are the mean of triplicate analyses.

selected randomly from an allergic patient population were tested for IgE specific for these proteins.

As indicated in the Appendix of the Codex guideline (2003), one clinical issue for testing the allergenicity of novel protein introduced into foods is monitoring the allergy to the novel proteins, where the availability of sufficient numbers of human sera was critical. In addition, the quality of the sera and assay procedure need to be standardized to obtain valid conclusions. Nonetheless, such post-marketing studies on GM foods are very important for public acceptance of the novel foods. For these purposes, convenient assay methods are mandatory to test a large number of human sera for novel protein-specific IgE.

However, the presence of human serum IgE specific for novel proteins is rather difficult to precisely conclude because no definitive positive and negative control sera to be used for ELISA and Western blotting are currently available. Furthermore, non-specific IgE binding to test proteins has been observed (Bernstein et al., 2003; Goodman and Leach, 2004). Assessment of the allergenicity of a GM food requires both a standard assay protocol and a case-by-case approach, and the specificity of any binding with human serum IgE must be individually confirmed.

Also in this study, we found that, in several serum samples, IgE non-specifically bound to PAT. The non-specific binding was confirmed by the inhibition ELISA and the 1 M NaCl-washing. Since the washing did not significantly affect the IgE binding to Cry9C and CP4-EPSPS, it is likely that PAT can weakly bind to IgE. The solubility of PAT is poor in water and soluble in a basic solution. Because of the low hydrophilicity, the IgE in high concentration serum (1:20) might bind non-specifically to the coated PAT.

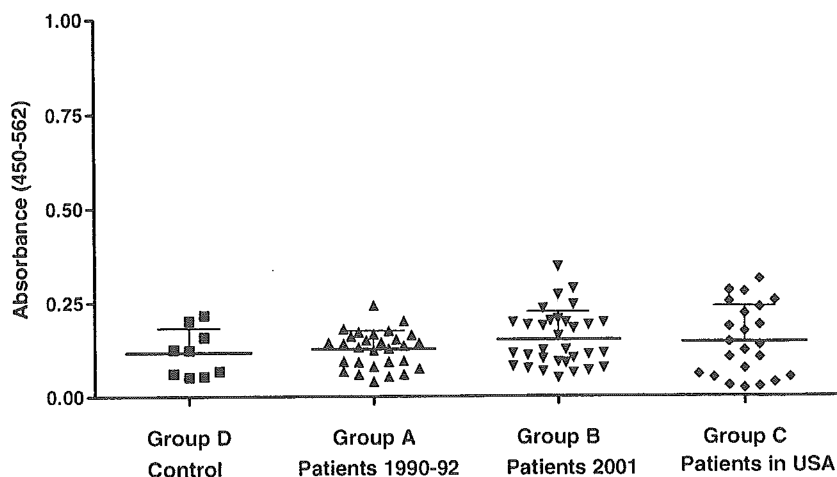

Fig. 4. Data obtained from 93 serum samples by improved IgE-ELISA method. Each serum sample of the four groups (A–D, described in Section 2.1) was diluted (1:20) and assayed for anti-PAT IgE-ELISA. A; Twenty-nine patients (1990–1992), B; 32 patients (2001), C; 23 commercially available sera, D; 9 healthy volunteers. The sera were assayed for anti-PAT IgE by the improved ELISA method. Colorimetric detection was used. The average (horizontal bold line) and standard deviation (upwards error bar) of each group are indicated.

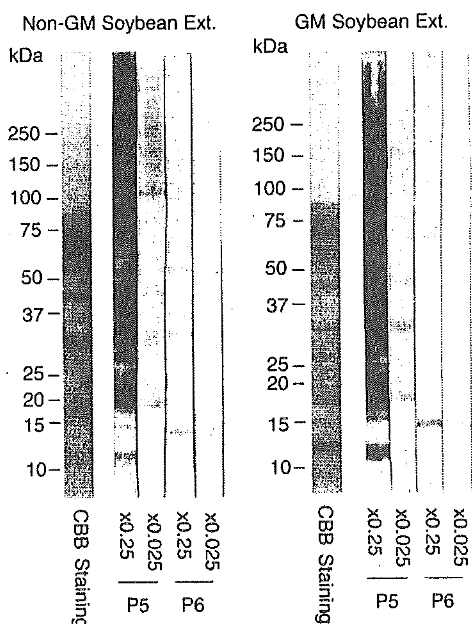

Fig. 5. IgE-Western blotting of sera from patients to GM or non-GM soybean extracts. Whole extracts from GM (CP4-EPSPS introduced) or non-GM soybeans were applied to SDS–PAGE and blotted to nitrocellulose membranes. The membranes pieces were reacted with 4- or 40-times diluted sera (0.25× or 0.025×, respectively) from two of patients allergic to soybean (P5 and P6).

And we have tested two of the subjects allergic to soybean about the reactivity to whole extract from GM or non-GM soybean. Their reactivity was almost equal to both extracts by Western blotting.

Batista et al. (2005) performed a post-marketing study for CP4-EPSPS and Cry1Ab, and they reported that no allergenic response to the two proteins was observed by skin prick testing and Western blotting. Our data also show that specific IgE-positive sera for PAT, CP4-EPSPS, and

Table 1
Detection of novel protein-specific IgE in patients' sera

Sampling year	PAT		Cry9C		CP4-EPSPS	
	No. of samples	Positive samples	No. of samples	Positive samples	No. of samples	Positive samples
1990–1992	29	0	0	0	0	0
1999	0	0	3	0	3	0
2000	0	0	30	0	30	0
2001	32	0	32	0	32	0
2002	31	0	31	0	31	0
2003	20	0	20	0	20	0
2004	16	0	16	0	16	0
US samples[a]	23	0	8	0	0	0
Total	151	0	140	0	132	0

[a] Commercially available patient sera collected in the United States.

Cry9C have not been found by the ELISA or Western blotting method. These studies by us and Batista et al. could be considered as pilot post-marketing studies for GM-food, and a large number of additional serum samples should be tested. In such studies, our improved ELISA method might be a convenient screening method prior to the confirmatory methods such as Western blotting or ELISA-inhibition.

If specific serum IgE to a protein was detected, that would not necessarily cause IgE receptors cross-linking on mast cells or basophils, and degranulation of the cells by the stimulation with the protein. When specific IgE was detected, further study is needed like basophil degranulation test, skin test, or oral challenge. Serum IgE test is located in the first step of allergenicity evaluation.

Acknowledgments

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ORIGINAL ARTICLE

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

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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

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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 NFκB.[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

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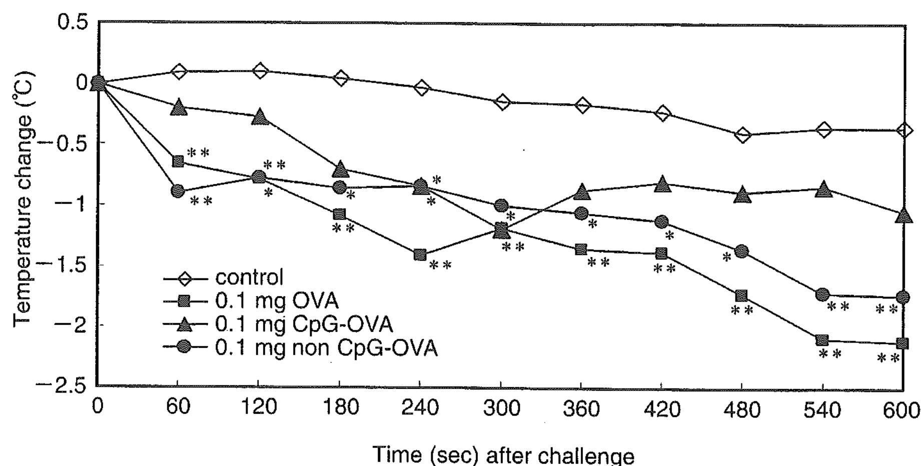

Fig. 1 Changes in body temperature of antigen-sensitized WBB6F1-W/W^v mice after systemic immunization. Body temperature was monitored at 1-minute intervals after OVA challenge. Each value represents the mean for 7 mice. *, ** Significant difference from the control by Student's *t*-test at $p < 0.05$ 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 TCRγδ-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 NIHS 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 GACGTTCCTGACGTT-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℃ 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 µL volume of OVA (40 µg/mL) in 50 mM sodium car-

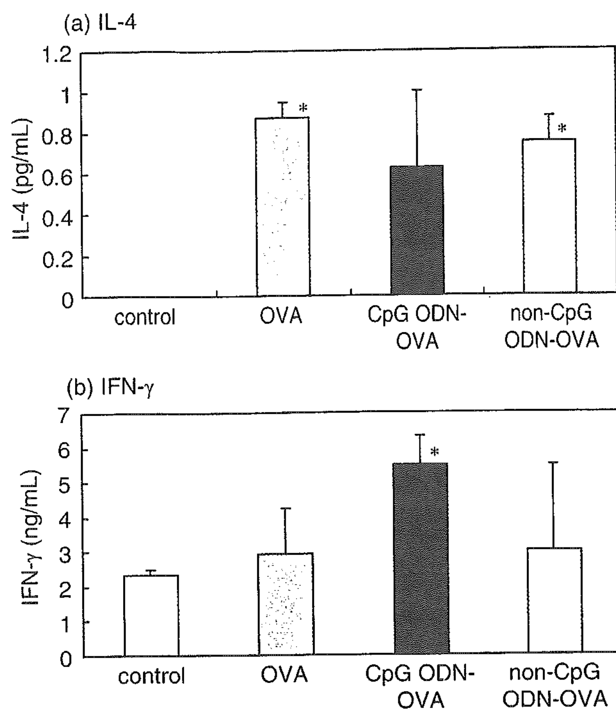

Fig. 2 Cytokine production by splenocytes from antigen-sensitized WBB6F1-W/W^v mice after systemic immunization. Splenocytes were isolated from WBB6F1-W/W^v mice orally sensitized with antigen after i.p. sensitization with 1.0 mg of OVA. (**a**) IL-4 and (**b**) IFN-γ cytokine production by splenocytes 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℃. 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℃. 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℃ 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 (5×10^6 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℃ for 3 days.[15] The levels of IL-4, IL-5, IL-12 (p 70), 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℃ 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
sIg^+ (B cell)	2.879±1.560	2.867±1.891	2.216±1.487	3.494±2.025
$CD3^+$ $\beta7^+$ (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
$CD4^+$ $CD8\alpha^-$ (TCRαβCD4)	10.29±4.090	12.22±5.134	14.97±4.422	9.952±3.426
$CD8\alpha^+$ $CD8\beta^-$ (CD8αα)	32.79±4.202	31.95±6.158	38.57±5.442	36.65±5.054
$CD8\alpha^+$ $CD8\beta^+$ (TCRαβCD8αβ)	48.92±3.662	51.07±6.515	46.98±5.882	47.69±6.445
$TCR\alpha\beta^-$ $\gamma\delta^+$ (TCRγδ)	2.933±0.992	3.002±0.906	2.587±1.039	3.478±1.531
$TCR\alpha\beta^+$ $\gamma\delta^-$ (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℃ 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 between 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.

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