

The Hyperresponsiveness of W/W^v Mice to Oral Sensitization Is Associated with a Decrease in TCR $\gamma\delta$ -T Cells

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We have already reported that WBB6F1-W/W^v (W/W^v) mice, which have mutations in the c-kit gene, are highly susceptible to oral sensitization, and that the proportion of TCR $\gamma\delta$ -T cells among the intraepithelial lymphocytes (IELs) ($\gamma\delta$ -IELs) of W/W^v is much lower than in congenic wild-type (+/+) mice. In this study we examined an inhibitory role of $\gamma\delta$ -IELs in oral sensitization using two different methods. First, wild-type (+/+) mice were sensitized by oral administration of 1.0 mg ovalbumin (OVA) by gavage every day for 9 weeks after anti-TCR $\gamma\delta$ antibody treatment 4 times. The treatment resulted in an enhanced OVA-specific IgG1 antibody production, active systemic anaphylaxis (ASA), and Th2-dominant cytokine production. Next, W/W^v mice whose bone marrow cells were reconstituted from C57BL/6J mice for 5 months were sensitized by oral administration of OVA. The OVA-specific IgG1 antibody titer in the bone marrow-reconstituted W/W^v mice was neither significantly enhanced, nor ASA was induced. The proportion of $\gamma\delta$ -IELs in the reconstituted mice was much higher than that in the untreated W/W^v mice. The above findings suggest that the decrease or increase in number of $\gamma\delta$ -IELs enhances or decreases oral sensitization respectively. These results show that $\gamma\delta$ -IELs have an important role in the oral tolerance to food antigens.

Key words rodent; food allergy; $\gamma\delta$ -T cell; flow cytometry

The number of patients with food allergies has been increasing for various reasons (*e.g.*, changes in diet, increased stress, *etc.*), but the mechanisms of the development and regulation of food allergy have not yet been fully elucidated. Thus, oral sensitization models in animals are important for better understanding the induction and regulation mechanisms of food allergy.^{1–3)}

When the allergenicity of dietary proteins is evaluated in animal, it is desirable to perform the sensitization orally, preferably without using adjuvants. However, since orally administered food antigens induce tolerance by various mechanisms,^{4–6)} it is important to optimize the dosage of the allergen and the duration of immunization in order to achieve oral sensitization without inducing tolerance.

In previous studies, we compared animal food allergy models using various strains of mice and rat,⁷⁾ and found that significant antigen-specific antibody production was successfully induced without any adjuvant by orally administering OVA for 9 weeks in B10A mice and BALB/c mice. More recently, W/W^v mice have been proven to be a good responder to food allergens, because their antigen-specific antibody titer was much higher than in the other murine strains used.⁸⁾

It is important to clarify the cause of the high susceptibility of the W/W^v mice to oral sensitization in order to elucidate the mechanism of development of food allergy.

W/W^v mice have mutations in the c-kit gene and exhibit defects or a deficiency of mast cells, red blood cells, and interstitial cells of Cajal, which ordinarily express c-kit protein.^{9,10)} The c-kit protein is a member of the type III receptor tyrosine kinase family, and its dimer is a ligand for the stem cell factor (SCF).¹¹⁾ We have reported low susceptibility of the congenic wild-type (+/+) mice to oral sensitization. The lack of high sensitivity in c-kit-positive wild-type (+/+) mice suggested that the susceptibility of W/W^v mice to oral sensitization is related to the mutations in the c-kit gene.¹²⁾

The population of $\gamma\delta$ -IELs is much smaller in W/W^v mice than in wild-type mice.¹²⁾ Moreover, it has been demonstrated that the ratio of the $\gamma\delta$ -IELs to total T cells in mouse intestinal mucosa is much higher than in other peripheral lymphoid tissues,¹³⁾ and that $\gamma\delta$ -IELs have been found to develop thymus independently in cryptopatches (CPs), which are located in the lamina propria. CPs are the small clusters of immature lymphocytes (c-kit+, IL-7R+, Thy-1+, LFA-1+).^{14,15)} Thus, $\gamma\delta$ -IELs are thought to play an important role in immune responses in the mucosa, although their function in oral sensitization is not fully understood.¹⁶⁾

The objective of this study was to identify the cause of the high susceptibility of W/W^v mice to oral sensitization, and we performed two experiments to achieve this objective. In the first experiment, congenic wild-type (+/+) mice were treated with the anti-TCR $\gamma\delta$ antibody before oral sensitization to OVA. In the second experiment, bone marrow cell-reconstituted W/W^v mice with increased $\gamma\delta$ -IELs were orally sensitized with OVA.

MATERIALS AND METHODS

Mice Female C57BL/6J (Jms) (5 weeks), WBB6F1-+/+(6 weeks) and WBB6F1-W/W^v (3 weeks) mice were purchased from Japan SLC (Shizuoka, Japan) and kept under pathogen-free conditions in our animal facility for 1 week before use. Mice were handled in accordance with NIHs Animal Care and Use Guidelines.

Treatment with Anti-mouse TCR $\gamma\delta$ Antibody and Oral Immunization of Wild-Type Mice WBB6F1-+/+(wild-type) mice (7 weeks) were treated with 0.2 mg of purified hamster anti-mouse TCR $\gamma\delta$ antibody (GL-3; CEDAR-LANE) or purified hamster IgG (ICN Pharmaceuticals, Inc, Ohio, U.S.A.) on the day 0, 7, 14 and 35 by intraperitoneal (i.p.) injections.¹⁷⁾ From the day 7 to the day 70, the mice

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were sensitized by administration of 1.0 mg OVA (grade V, Sigma Chemical Co, St Louis, MO, U.S.A.) by gavage daily for 9 weeks.

Reconstitution of Bone Marrow Cells in W/W^v Mice Bone marrow cells were obtained from C57BL/6J (Jms) (6 weeks) mice,^{18,19} and 1×10^7 of them were injected i.v. into 4-week-old W/W^v mice according to the method of Harrison and Astle.²⁰ Five months later the W/W^v mice were sensitized by oral administration of 1.0 mg OVA by gavage every day for 9 weeks.

Hematological Examination Red blood cell (RBC) counts, white blood cell (WBC) counts, platelet (PLT) counts, hemoglobin (HGB) volumes, and hematocrits (HCT) were determined with an automatic hematology analyzer (M-2000, Sysmex corp., Japan).

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.⁸ A 50 μ l volume of OVA (40 μ g/ml) in 50 mM sodium carbonate 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 μ l PBS containing 0.05% Tween 20 (PBS/Tween). To minimize the nonspecific binding of serum proteins to unoccupied solid-phase sites, 200 μ l of 0.1% casein in PBS was added, and the plates were incubated for 1 h at room temperature. The casein solution was removed, and each well was washed in the same manner as above. A 50 μ l volume of the diluted serum containing OVA-specific antibodies was then added to each well, and the plates were incubated for 20 h at 4 °C. The solutions were then removed, and each well was washed. A 50 μ l volume of rabbit anti-mouse IgE, IgG1, and IgG2a (10^{-3} dilution in PBS containing 0.1% casein [Nordic Immunology, Tilburg, the Netherlands]) was added to each well, and the plates were incubated for 1 h at room temperature. The solution in each well was then removed and washed. A 50 μ l volume of β -galactosidase-linked goat anti-rabbit Ig conjugate (10^{-3} dilution in PBS containing 0.1% casein [Amersham Pharmacia Biotech, Uppsala, Sweden]) was added to each well, and the plates were incubated for 1 h at room temperature. The antibody-enzyme conjugate solution in each well was then removed and washed. The wells were incubated for 1 h at 37 °C with 100 μ l PBS containing 0.1 mM 4-methylumbelliferyl- β -galactoside (Sigma). Finally, 25 μ l of 1 M sodium carbonate was added to each well. The fluorescence intensity of the liberated 4-methylumbelliferone was monitored at 365 nm and 460 nm for excitation and emission, respectively, with a Titertek Fluoroscan reader (Flow Laboratories Inc., Costa Mesa, CA, U.S.A.).

Cytokine Assays of Splenocytes Spleen cells were collected from the OVA-immunized mice (4–7 mice per group), and the cells (5×10^6 cells/ml) were re-stimulated with OVA *in vitro* at a final concentration of 100 μ g/ml in a 24-well culture plate at 37 °C for 3 d.²¹ The levels of IL-4, IL-5, IL-12 (p70), and IFN- γ in the culture medium (RPMI 1640) after 3 d of co-culture with OVA were measured with an OptEIA mouse cytokine ELISA set (PharMingen, San Diego, CA, U.S.A.). Absorbance was measured at 450 nm with a microplate reader (EL 340, Bio-Tek Instruments,

Winooski, VT, U.S.A.).

Induction of Active Systemic Anaphylaxis (ASA) and Measurement of Body Temperature ASA challenge was performed by i.p. injection of 1.0 mg of OVA after oral administration. The body temperature changes associated with ASA were monitored with a rectal thermometer for mice (Shibaura Electronics Co. Ltd., Japan) without general anesthesia.

Determination of Serum Histamine during the ASA Reaction Blood was collected from the eye 10 min after i.p. challenge with 1.0 mg OVA and allowed to stand for 30 min at room temperature before centrifugation to obtain the serum. Serum histamine levels were measured by the post-column HPLC method, as described previously.²²

Isolation of Mouse IELs IELs were isolated as previously described by Nagafuchi.²³ In brief, the small intestine (duodenum, jejunum, and ileum) was removed from the mice, and small intestine was turned inside-out with the help of 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 min (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 min 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 CD8 α (53-6.7; PharMingen), fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4 (H129.19; PharMingen), peridinin chlorophyll protein (PerCP)-labeled anti-mouse CD3 ϵ (145-2C11; PharMingen), FITC-labeled anti-mouse Integrin β 7 chain (M293; PharMingen), PE-labeled anti-mouse TCR $\gamma\delta$ (UC7-13D5; Santa Cruz Biotechnology, Delaware, CA), PE-labeled anti-mouse TCR $\gamma\delta$ (GL3; Immunotech, Marseille, France), FITC-labeled anti-mouse TCR $\alpha\beta$ (H57-597; Immunotech), PerCP-labeled anti-mouse CD4 (H129.19; PharMingen), and FITC-labeled anti-mouse CD8 β (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 μ l 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 10000 cells was analyzed with a FACS Calibur (Becton Dickinson, Sunnyvale, CA, U.S.A.). The data were analyzed with Cellquest[®] software.

Statistical Analysis All values are expressed as means $\pm$ standard deviation of the mean. Statistical comparisons were performed with Student's *t*-test for hematological and flow cytometric analysis, or Mann Whitney's *U*-test for the analysis of serum antibody titer.

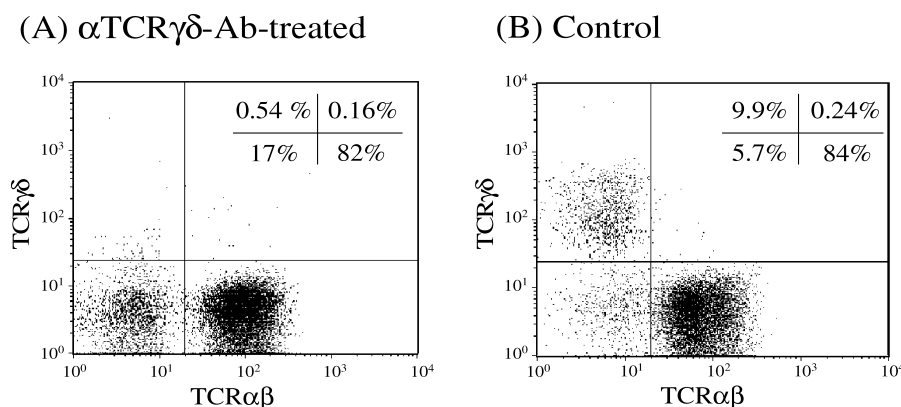


Fig. 1. Expression of TCR $\alpha\beta$ and TCR $\gamma\delta$ on IELs from Anti-TCR $\gamma\delta$ -Antibody-Treated WBB6F1-(+/+) Mice

IELs were isolated from (a) anti-TCR $\gamma\delta$ -antibody-treated and (b) control WBB6F1-(+/+) mice orally sensitized with 1.0 mg of OVA. Cells were stained with the antibodies as described in Materials and Methods. The histograms presented are representative of five independent experiments.

Table 1. Serum OVA-Specific Antibody of WBB6F1-(+/+) Mice Orally Sensitized with OVA

OVA-specific antibody	WBB6F1-(+/+) mice		
	Normal	Control	α TCR $\gamma\delta$ -Ab-treated
IgG1	<50	628 $\pm$ 731 ^{a)}	2394 $\pm$ 1147*†
IgE	<50	<50	61 $\pm$ 21
IgG2a	<50	<50	<50

OVA-specific antibody titers of the sera from normal (non-sensitized) mice, control (control hamster-IgG-treated and OVA-sensitized) mice, and α TCR $\gamma\delta$ -Ab-treated (α TCR $\gamma\delta$ -Ab-treated and OVA-sensitized) mice were examined. a) Mean $\pm$ S.D., $n=4$. * Significant difference from normal at $p<0.05$. † Significant difference from the control (hamster-IgG-treated) at $p<0.05$.

RESULTS

Effect of Anti-TCR $\gamma\delta$ Antibody Treatment on Oral Sensitization of Wild-Type Mice The congenic wild-type (+/+) mice were sensitized by oral administration of 1.0 mg OVA by gavage every day for 9 weeks after the hamster anti-TCR $\gamma\delta$ antibody or purified hamster IgG (control) treatment.

First, the lymphocyte subset composition of spleen and IELs after oral sensitization was analyzed by FCM. In the anti-TCR $\gamma\delta$ -antibody-treated mice, there was a decreasing trend in the number of TCR $\gamma\delta$ -T cells of the spleen compared with the control mice (data not shown). No changes in the composition of other splenic subsets were found. As shown in Fig. 1, the proportion of $\gamma\delta$ -IELs in the anti-TCR $\gamma\delta$ -antibody-treated mice (0.54%) was much lower than that in the control-antibody-treated mice (9.9%). In addition, the proportion of CD8 $\alpha\alpha$ + T cells, including TCR $\gamma\delta$ -T cells, which were thought to be a thymus-independent T cell subset of IELs, showed a decreasing tendency in the anti-TCR $\gamma\delta$ -antibody-treated mice (data not shown).

Second, antigen-specific antibody production in OVA-sensitized wild-type (+/+) mice was examined as described in the Materials and Methods section. As shown in Table 1, there was a significant increase in specific IgG1 antibody titer in the anti-TCR $\gamma\delta$ -antibody-treated mice as compared with the control-antibody-treated mice. The OVA-specific IgE antibody titer also increased slightly in the anti-TCR $\gamma\delta$ -antibody-treated group. By contrast, no increase in OVA-specific IgG2a antibody titer was observed in any of the groups.

Third, ASA induction in the orally sensitized mice was assessed as described in the Materials and Methods section. As

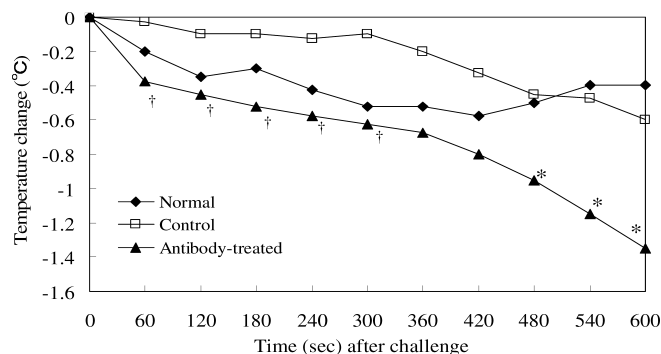


Fig. 2. Changes in Body Temperature of Anti-TCR $\gamma\delta$ Antibody-Treated WBB6F1-(+/+) Mice after Systemic Immunization

Body temperature was monitored at 1-min intervals after OVA challenge. Each value represents the mean for 10 mice. * Significant difference from normal at $p<0.05$. † Significant difference from the control at $p<0.05$.

shown in Fig. 2, a decrease in body temperature (hypothermia) was observed in the anti-TCR $\gamma\delta$ -antibody-treated mice, but hypothermia did not occur in the control antibody-treated mice. The serum histamine concentration after ASA induction slightly increased in the anti-TCR $\gamma\delta$ -antibody-treated group, but that there was no significant change in the control-antibody-treated groups (data not shown).

Fourth, *in vitro* production of cytokines by re-stimulated splenocytes from the orally sensitized +/+ mice was examined. As shown in Fig. 3, there were significant increases in the production of IL-4 and IL-5 by splenocytes in the anti-TCR $\gamma\delta$ -antibody-treated mice as compared with the OVA-non-sensitized mice. On the other hand, the Th1-type cytokine (IL-12 (p70) and IFN- γ) levels in the splenocyte cul-

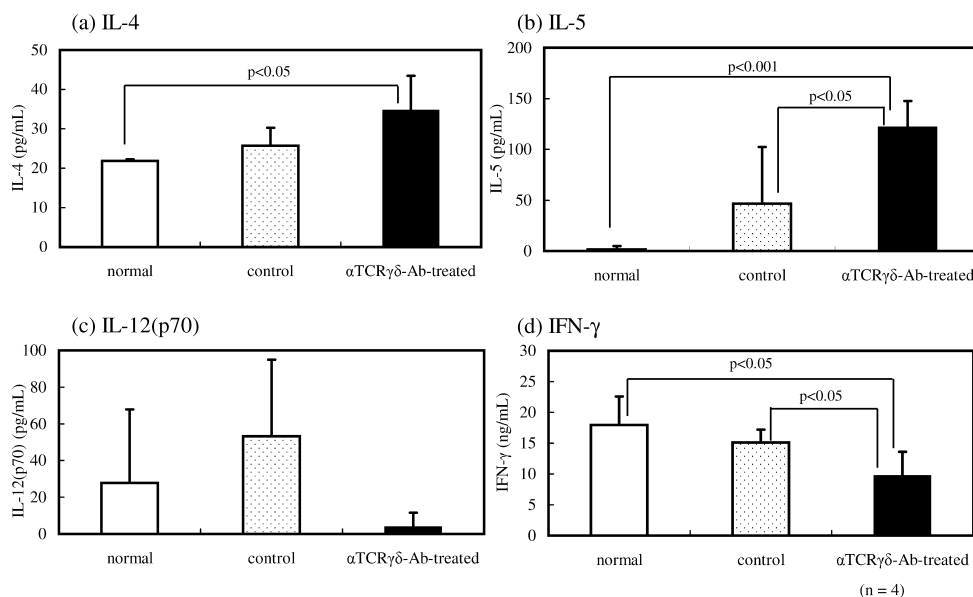


Fig. 3. Cytokine Production by Splenocytes from Anti-TCRγδ-Antibody-Treated WBB6F1-(+/+) Mice

Splenocytes were isolated from WBB6F1-(+/+) mice orally sensitized with 1 mg of OVA or saline alone (white) after i.p. sensitization with 1 mg of OVA. (a) IL-4, (b) IL-5, (c) IL-12(p70), and (d) IFN-γ cytokine production by splenocytes was measured as described in Materials and Methods. Each value represents the mean ± S.D. for 4 mice.

Table 2. Hematological Changes in W/W^v and Wild Type Mice

	W/W ^v				WBB6F1-(+/+)	
	Control		Reconstitution		Saline	1.0 mg OVA
	Saline	1.0 mg OVA	Saline	1.0 mg OVA		
RBC (×10 ⁴ /μl)	563.9 ± 40.1 ^{a)}	549.6 ± 36.0	722.2 ± 170.7*	748.3 ± 135.5**	986.3 ± 50.7	976.9 ± 49.9
HGB (g/dl)	12.03 ± 0.91	11.77 ± 1.09	11.54 ± 1.19	11.43 ± 1.19	15.13 ± 0.73	14.79 ± 0.95
HCT (%)	38.23 ± 2.41	37.35 ± 2.32	41.20 ± 4.84*	40.60 ± 5.72	50.84 ± 2.46	50.34 ± 2.64

a) Mean ± S.D. *,** Significantly different from control WBB6F1-W/W^v saline at $p < 0.05$ and 0.01 , respectively.

ture medium were decreased. These finding suggested that oral sensitization of WBB6F1-(+/+) mice after the anti-TCRγδ antibody results in a state of Th2-dominant helper T cell activation. By contrast, there was no significant change in the control-antibody-treated group as compared with OVA-non-sensitized group, although the production of IL-5 increased slightly.

The above findings suggested that the decrease in the number of TCRγδ-T cells caused by anti-TCRγδ antibody treatment has increased oral sensitization of wild-type WBB6F1-(+/+) mice.

Effect of Reconstitution with Bone Marrow Cells on Oral Sensitization of W/W^v Mice to OVA Bone marrow cells from C57BL/6J mice were injected i.v. into 4-week-old W/W^v mice, and the mice were allowed to reconstitute for 5 months as described in Materials and Methods.

According to the Harrison's paper,²⁰⁾ lymphocyte and erythroid repopulation in W/W^v mice was relatively low in unirradiated recipient.

Therefore, we first carried out hematologic analysis to confirm reconstitution of the bone marrow after 5 month inoculation. As shown in Table 2, the RBC count of the W/W^v mice was about half that of the wild-type mice. However, it recovered to about 80% of the wild-type in the reconstituted W/W^v mice. Significant rises in HCT were observed in the

reconstituted W/W^v mice compared to the control W/W^v mice. The PLT and WBC counts were within the normal range in both groups (data not shown).

Next, the lymphocyte subset composition of splenocytes and IELs was analyzed by FCM. The splenocytes showed an increasing trend in the CD4⁺ T cell subset in the antigen-sensitized control mice as compared with the sensitized reconstituted mice (data not shown). As shown in Table 3, the population of TCRγδ-T cells in the IELs was much higher in the reconstituted W/W^v mice than in the control W/W^v mice.

The above results indicated that bone marrow cells from C57BL/6J mice were successfully grafted in the W/W^v mice.

After oral administration of OVA for 9 weeks in the reconstituted W/W^v mice, we examined the following three parameters to know the oral sensitization of the mice. First, antigen-specific antibody production was examined. As shown in Table 4, there were marked increases in the OVA-specific IgG1 and IgE antibody titers in the OVA-oral-sensitized W/W^v mice. By contrast, the rise in OVA-specific IgG1 titer was not significant, and no rise in OVA-specific IgE and IgG2a titers was observed in the reconstituted mice.

Second, ASA induction in orally sensitized mice was examined. As shown in Fig. 4, a significant decrease in body temperature was observed in the OVA-sensitized and OVA-challenged W/W^v mice. However, no significant decrease in

Table 3. Flow Cytometric Analysis of the IELs of W/W^v and Wild Type Mice

	W/W ^v mice				WBB6F1-(+/+)	
	Control		Reconstituted		Saline	1.0 mg OVA
	Saline	1.0 mg OVA	Saline	1.0 mg OVA		
TCR $\gamma\delta$ (TCR $\alpha\beta$ - $\gamma\delta$ +))	3.802 $\pm$ 1.177 ^{a)}	2.102 $\pm$ 1.235*	9.920 $\pm$ 4.932*	8.850 $\pm$ 6.071	14.62 $\pm$ 4.360	17.26 $\pm$ 7.650

a) Mean $\pm$ S.D. (%(gated)), n=7. * Significant difference from the W/W^v control saline group at $p<0.05$.

Table 4. Serum OVA-Specific Antibody of W/W^v Mice Orally Sensitized with OVA

OVA-specific antibody	W/W ^v mice			
	Control		Reconstituted	
	Saline	1.0 mg OVA	Saline	1.0 mg OVA
IgG1	<50	39407 $\pm$ 18199**	<50	2251 $\pm$ 5662
IgE	<50	274 $\pm$ 319	<50	<50
IgG2a	<50	100 $\pm$ 109	<50	<50

a) Mean $\pm$ S.D., n=7. * Significant difference from the control saline group at $p<0.001$.

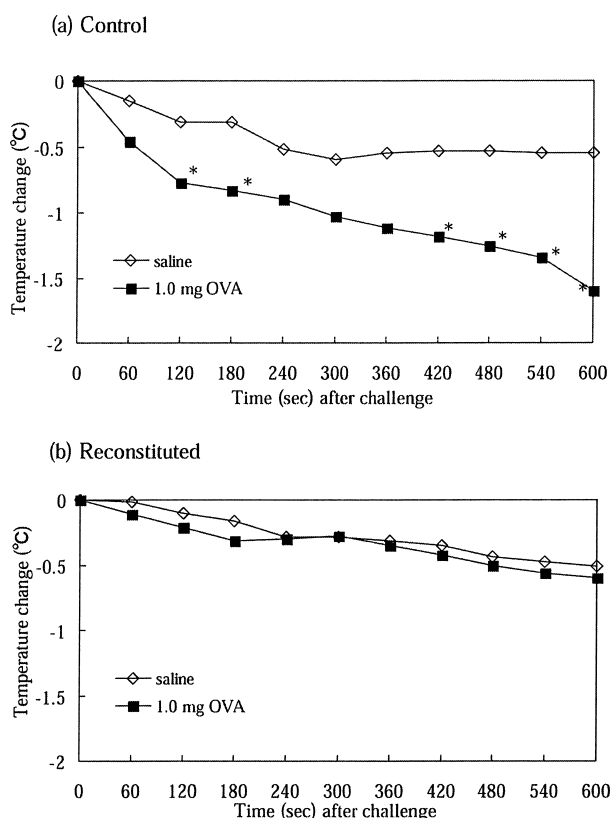


Fig. 4. Changes in Body Temperature of the Bone-Marrow-Cell-Reconstituted W/W^v Mice after Systemic Immunization

The body temperature of (a) control and (b) the reconstituted W/W^v mice was monitored at 1-min intervals after OVA challenge. Each value represents the mean for 10 mice. * Significant difference from the control saline group at $p<0.05$.

body temperature was observed in the OVA-sensitized and OVA-challenged bone marrow-reconstituted W/W^v mice. Measurement of the serum histamine concentration after the ASA reaction revealed that the histamine levels of the control W/W^v mice were below the detection limit and that the

histamine level was lower in the reconstituted groups (about 1/10 the level in the wild-type mice; data not shown).

Third, the *in vitro* production of cytokines by splenocytes re-stimulated with antigen was measured. As shown in Fig. 5, there were significant rises in the production of Th2-type cytokine (IL-4, IL-5) in the OVA-sensitized W/W^v mice, which showed Th2-dominant helper T cell activation. However, there was no significant increase in the production of Th2-type cytokines in the OVA-sensitized and bone marrow-reconstituted W/W^v mice. Th1-type (IL-12 (p70) and IFN- γ) cytokine levels were also measured in the splenocyte culture medium but no changes in concentration were observed in either the W/W^v mice or the reconstituted W/W^v mice.

The proportion of $\gamma\delta$ -IELs in the reconstituted W/W^v mice was much higher than in the control W/W^v mice. Thus, the increase in $\gamma\delta$ -IELs seemed to cause the decreased oral sensitization in the reconstituted W/W^v mice.

DISCUSSION

The W/W^v mice are a good model for studying induction of food allergy, since antigen-specific antibody responses are easily induced by oral antigen administration.^{8,12)} In this paper, we have attempted to clarify the cause of high susceptibility of the W/W^v mice to oral sensitization in order to elucidate the mechanism of food allergy induction.

Most TCR $\gamma\delta$ -T cells in IELs ($\gamma\delta$ -IELs) are CD8 α + and c-kit+ cells, and they develop thymus-independently from CPs, which are small clusters of c-kit+ immature lymphocytes located in the lamina propria.^{14,24)} Impaired development of $\gamma\delta$ -IELs in W/W^v mice has been considered to be caused by the mutation in the c-kit gene. We speculated that the decrease in $\gamma\delta$ -IELs and impairment of the function of $\gamma\delta$ -IELs was involved in the oral sensitization of W/W^v mice in a previous paper,¹²⁾ and in this study, we have performed two different experiments to clarify the direct role of $\gamma\delta$ -IELs in oral sensitization.

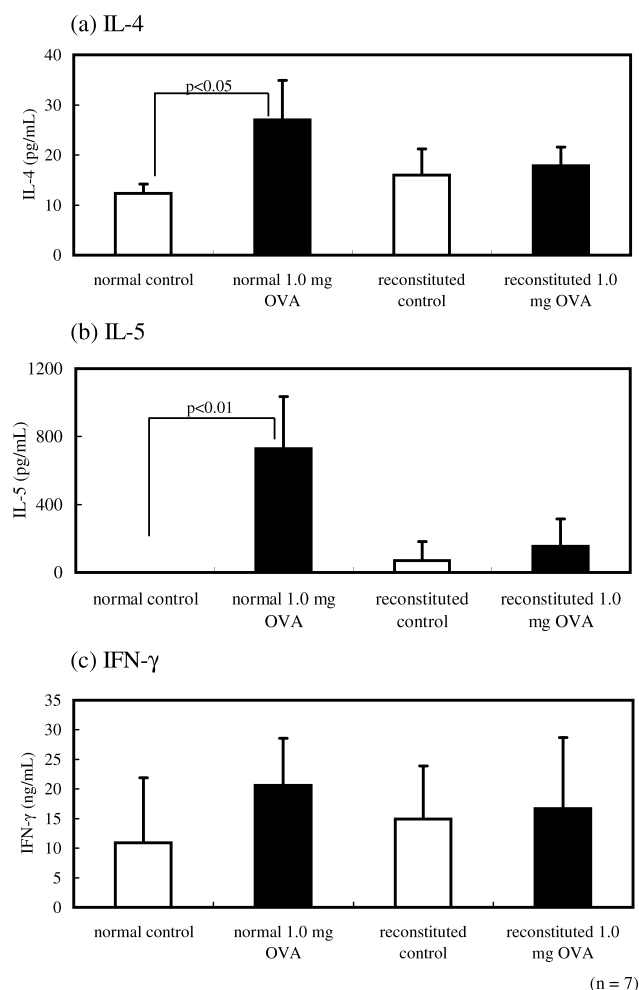


Fig. 5. Cytokine Production by Splenocytes from the Reconstituted W/W^v Mice

Splenocytes were isolated from W/W^v mice orally sensitized with 1 mg of OVA or saline alone (white) after i.p. sensitization with 1 mg of OVA. (a) IL-4, (b) IL-5, and (c) IFN- γ cytokine production by splenocytes was measured as described in Materials and Methods. Each value represents the mean $\pm$ S.D. for 7 mice.

Firstly, congenic wild-type (+/+) mice were treated with anti-TCR $\gamma\delta$ antibody before oral administration of OVA. The efficacy of treatment was sustained for 5 weeks after the final anti-TCR $\gamma\delta$ antibody treatment, because FCM showed a striking reduction in the population of TCR $\gamma\delta$ -T cells in the antibody-treated mice even though OVA was administered daily throughout the period (Fig. 1). The mechanism for the decrease of TCR $\gamma\delta$ -T cells by the antibody treatment seems to be down regulation of TCR expression on TCR $\gamma\delta$ -T cells or the depletion of those cells by complement-dependent cytotoxicity.¹⁷⁾

Oral sensitization of WBB6F1(+/-) mice after the anti-TCR $\gamma\delta$ antibody treatment yielded the following findings: (i) increases in antigen-specific antibody titers in the anti-TCR $\gamma\delta$ antibody-treated mice (Table 1), (ii) hypothermia after i.p. antigen challenge, demonstrating ASA induction (Fig. 2), and (iii) Th2-dominant T cell cytokine production (Fig. 3). These results demonstrate that the decrease in number of TCR $\gamma\delta$ -T cells caused by anti-TCR $\gamma\delta$ antibody treatment increased oral sensitization.

Secondly, bone marrow cells from C57BL/6J mice, which are one of the parents of WBB6F1 mice, were grafted into

W/W^v mice.^{18,19)}

Oral sensitization of the reconstituted W/W^v mice gave the following results: (i) reduced antigen-specific antibody titers in the reconstituted mice (Table 3), (ii) no hypothermia after i.p. antigen challenge (Fig. 4), and (iii) no Th2-dominant cytokine production (Fig. 5). These results suggest that the increase in $\gamma\delta$ -IELs causes the decrease in oral sensitization in the reconstituted W/W^v mice.

TCR $\gamma\delta$ -T cells are known to be committed to protection against enteric infection by parasites and viruses, and also recently shown to be involved in the regulation of allergy development.^{25–27)} Interestingly, a recent report has shown that TCR $\gamma\delta$ -T cells regulate the induction and maintenance of systemic unresponsiveness induced by oral immunization.²⁸⁾ Moreover, Fujihashi *et al.* reported on a role of TCR $\gamma\delta$ -T cell in the induction of oral tolerance at a low- or high-dose antigen using TCR δ -deficient mice.²⁶⁾ Notably, a lack of tolerance was found when TCR δ -deficient mice were orally sensitized with low-dose antigen, which is consistent with our present results.

The site of induction of oral tolerance is still a matter of controversy. Since the oral antigen is taken up through the intestine, the gut-associated lymphoid tissue (GALT) seems to be involved with the induction of oral tolerance.²⁹⁾

It has been reported that TCR $\gamma\delta$ -T cells produce the regulatory cytokine transforming growth factor- β 1 (TGF- β 1) *in vitro* and induce oral tolerance *in vivo*,³⁰⁾ suggesting that the TCR $\gamma\delta$ -T cells among the IELs produce the regulatory cytokines (TGF- β 1 and/or IL-10) upon stimulation and function as regulatory T cells which inhibit antigen-specific T cells.³¹⁾

In conclusion, the decrease in the number of $\gamma\delta$ -IELs caused by anti-TCR $\gamma\delta$ antibody treatment increased oral sensitization of wild-type (+/+) mice. The increase in the number of $\gamma\delta$ -IELs seems to be involved in the production of oral tolerance. Thus, the susceptibility of W/W^v mice to oral sensitization seems to be associated with the decreased numbers of $\gamma\delta$ -IELs. Moreover, our findings indicate that W/W^v mice are a good model for studying the mechanism of induction of food allergy, including the role of TCR $\gamma\delta$ -T cells in food-antigen-induced hypersensitivity.

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REFERENCES

- Knippels L. M., Penninks A. H., Spanhaak S., Houben G. F., *Clin. Exp. Allergy*, **28**, 368–375 (1998).
- Knippels L. M., Penninks A. H., Smit J. J., Houben G. F., *Toxicol. Appl. Pharmacol.*, **156**, 161–169 (1999).
- Li X. M., Serebrisky D., Lee S. Y., Huang C. K., Bardina L., Schofield B. H., Stanley J. S., Burks A. W., Bannon G. A., Sampson H. A., *J. Allergy Clin. Immunol.*, **106**, 150–158 (2000).
- Melamed D., Friedman A., *Eur. J. Immunol.*, **24**, 1974–1981 (1994).
- DePaolo R. W., Rollins B. J., Kuziel W., Karpus W. J., *J. Immunol.*, **171**, 3560–3567 (2003).
- Weiner H. L., *Immunol. Rev.*, **182**, 207–214 (2001).
- Akiyama H., Teshima R., Sakushima J., Okunuki H., Goda Y., Sawada

- J., Toyoda M., *Immunol. Lett.*, **78**, 1—5 (2001).
- 8) Okunuki H., Teshima R., Sakushima J., Akiyama H., Goda Y., Toyoda M., Sawada J., *Immunol. Lett.*, **74**, 233—237 (2000).
- 9) Nocka K., Majumcer S., Chabot B., Ray P., Cervone M., Bernstein A., Besmer P., *Genes Dev.*, **3**, 816—826 (1989).
- 10) Nocka K., Tan J. C., Chiu E., Chu T. Y., Ray P., Traktman P., Besmer P., *EMBO J.*, **9**, 1805—1813 (1990).
- 11) Kitamura Y., Go S., Hatanaka K., *Blood*, **52**, 447—452 (1978).
- 12) Okunuki H., Teshima R., Harikai N., Sakai S., Akiyama H., Maitani T., Sawada J., *Biol. Pharm. Bull.*, **26**, 1260—1265 (2003).
- 13) Laky K., Lefrancois L., von Freeden-Jeffry U., Murray R., Puddington L., *J. Immunol.*, **161**, 707—713 (1998).
- 14) Oida T., Suzuki K., Nanno M., Kanamori Y., Saito H., Kubota E., Kato S., Itoh M., Kaminogawa S., Ishikawa H., *J. Immunol.*, **164**, 3616—3626 (2000).
- 15) Brandtzaeg P., Baekkevold E. S., Farstad I. N., Jahnsen F. L., Johansen F. E., Nilsen E. M., Yamanaka T., *Immunol. Today*, **20**, 141—151 (1999).
- 16) Goodman T., Lefrancois L., *Nature (London)*, **333**, 855—858 (1988).
- 17) Mengel J., Cardillo F., Aroeira L. S., Williams O., Russo M., Vaz N. M., *Immunol. Lett.*, **48**, 97—102 (1995).
- 18) Nakano K., Sonoda T., Hayashi C., Yamatodani A., Kanayama Y., Yamamura T., Asai H., Yonezawa T., Kitamura Y., Galli S. J., *J. Exp. Med.*, **162**, 1025—1043 (1985).
- 19) Lefrancois L., Olson S., *J. Immunol.*, **159**, 538—541 (1997).
- 20) Harrison D. E., Astle C. M., *Exp. Hematol.*, **19**, 374—377 (1991).
- 21) Akiyama H., Hoshino K., Tokuzumi M., Teshima R., Mori H., Inakuma T., Ishiguro Y., Goda Y., Sawada J., Toyoda M., *Biol. Pharm. Bull.*, **22**, 551—555 (1999).
- 22) Kawasaki M., Toyoda M., Teshima R., Sawada J., Saito Y., *Biol. Pharm. Bull.*, **17**, 1321—1325 (1994).
- 23) Nagafuchi S., Totsuka M., Hachimura S., Goto M., Takahashi T., Yajima T., Kuwata T., Kaminogawa S., *Biosci. Biotechnol. Biochem.*, **64**, 1459—1465 (2000).
- 24) Kanamori Y., Ishimaru K., Nanno M., Maki K., Ikuta K., Nariuchi H., Ishikawa H., *J. Exp. Med.*, **184**, 1449—1459 (1996).
- 25) Kokkonen J., Holm K., Karttunen T. J., Maki M., *Scand. J. Gastroenterol.*, **35**, 1137—1142 (2000).
- 26) Fujihashi K., Dohi T., Kweon M. N., McGhee J. R., Koga T., Cooper M. D., Tonegawa S., Kiyono H., *Int. Immunol.*, **11**, 1907—1916 (1999).
- 27) Zuany-Amorim C., Ruffie C., Haile S., Vargaftig B. B., Pereira P., Pretolani M., *Science*, **280**, 1265—1267 (1998).
- 28) Ke Y., Pearce K., Lake J. P., Ziegler H. K., Kapp J. A., *J. Immunol.*, **158**, 3610—3618 (1997).
- 29) Bienenstock J., Befus D., *Am. J. Anat.*, **170**, 437—445 (1984).
- 30) Perez-Machado M. A., Ashwood P., Thomson M. A., Latcham F., Sim R., Walker-Smith J. A., Murch S. H., *Eur. J. Immunol.*, **33**, 2307—2315 (2003).
- 31) Kapp J. A., Kapp L. M., McKenna K. C., Lake J. P., *Immunology*, **111**, 155—164 (2004).