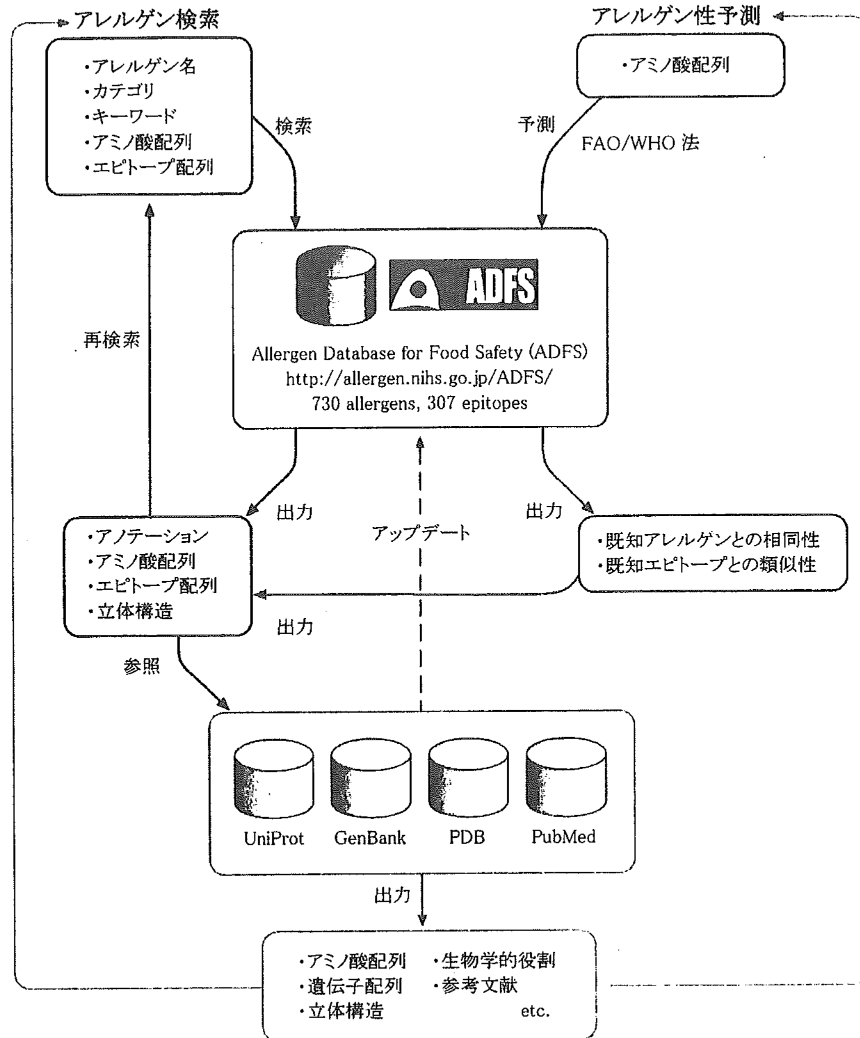

Fig.2　Schematics of ADFS
本データベースは，キーワードやアミノ酸配列など様々なクエリによりアレルゲンを検索する機能と，FAO/WHOの改変法に基づくアレルゲン性予測機能からなる．得られた出力結果は，再度ADFS内を検索したりリンクにより外部データベースを参照したりすることができる．

の予測に関し，非常に有用な情報を提供できるデータベースが構築できたと考えられる．また，既存のアレルゲンデータベースは一般にキーワード検索やアミノ酸配列検索における柔軟性が低く，また検索結果の表示が複雑で分かりにくいなどといった問題があったが，ADFSにおいてはこれらのインターフェースを改善し，より直感的な操作を可能にした．特にBLASTアルゴリズムによる配列検索は有用で，4残基からなる短いエピトープ配列も検索することができた．現在のアルゴリズムでは3残基からなるエピトープについては検索できないが，このような極端に短いエピトープはAsp f 2の^{138}HWR^{140}のみであり，全体としての検索のパフォーマンスは非常に高いと考えられる．また，本データベースの特徴の一つであるエピトープ情報の整備に関しては，今回新たに32種のアレルゲンに対してエピトープ情報を付加し，ADFSに登録されたエピトープ（立体エピトープを含む）既知のアレルゲンは43種，エピトープの配列数は307種に達した．これは，我々が知るかぎり，アレルゲンエピトープに関するデータベースとしては現時点（2005年6月）で世界最大のものである．エピトープ情報は，あるタンパク質と既知アレルゲンとの交差反応性を予測する上で極めて重要であり，バイオテクノロジー応用食品に含まれる新規タンパク質等のアレルゲン性を予測する上で，ADFSは有用な情報を提供することが可能であると思われる．そのため，今後もEntrez PubMedを通じて定期的にエピトープ情報の収集に努め，データのアップデートを行なっていきたいと考えている．

アレルゲン性予測に関しては，2001年FAO/WHO法を改変し，高いパフォーマンスの予測を可能とした．2001年FAO/WHO法と本法ではアミノ酸のウインドウ

に関する取扱いが異なるため，まれに結果が相違する場合がある．多くのタンパク質は分子内に機能的・構造的にまとまったドメイン構造を持つが，ドメインの大きさは様々であり，一概には決められない．FASTAアラインメントにより類似性の高い領域を自動的に抽出する本法は，ウインドウサイズを初めから80残基等に固定してアラインメントを行なうFAO/WHOの方法に比べ，より機能的・構造的に意味がある構造の類似性を調べることが可能であると思われる．

しかし，さらに予測率の高いアレルゲン性予測手法の開発が望まれていることもまた事実である．2003年に発表されたStadlerらによるMotif-based法[10]は，従来の予測法に比べパフォーマンスの向上が認められており，ADFSにおいては同法に基づくアレルゲン性予測のインターフェイスを現在開発中である．また，アレルゲンデータのアップデートが非常に重要となるため，年間最低1回以上のアップデートを計画している．定期的なデータのアップデートとMotif-based予測インターフェイスの確立を遂行することにより，ADFSは世界で最も洗練されたアレルゲンデータベースの一つとなることが期待される．

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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; CEDARLANE) 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 μl volume of OVA (40 μg/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 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 h at room temperature. The casein solution was removed, and each well was washed in the same manner as above. A 50 μl 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 μl 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 μl 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 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 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 μg/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 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 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 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 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.

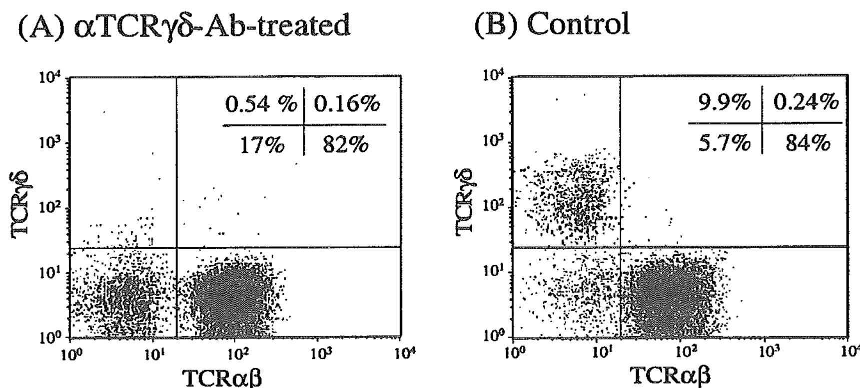

Fig. 1. Expression of TCR$\alpha\beta$ and TCR$\gamma\delta$ on IELs from Anti-TCR$\gamma\delta$-Antibody-Treated WBB6F1-(+/+) Mice

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	αTCR$\gamma\delta$-Ab-treated
IgG1	<50	628±731[a]	2394±1147*†
IgE	<50	<50	61±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 αTCR$\gamma\delta$-Ab-treated (αTCR$\gamma\delta$-Ab-treated and OVA-sensitized) mice were examined. *a*) Mean±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

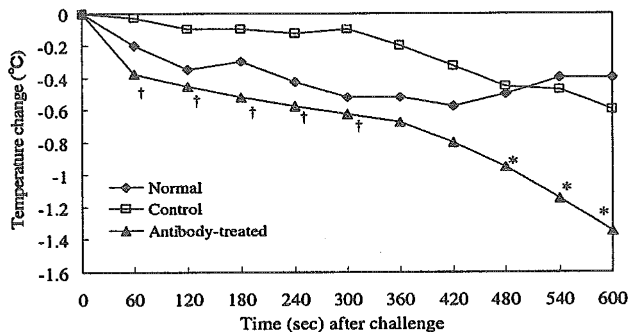

Fig. 2. Changes in Body Temperature of Anti-TCR$\gamma\delta$ Antibody-Treated WBB6F1-(+/+) Mice after Systemic Immunization

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-

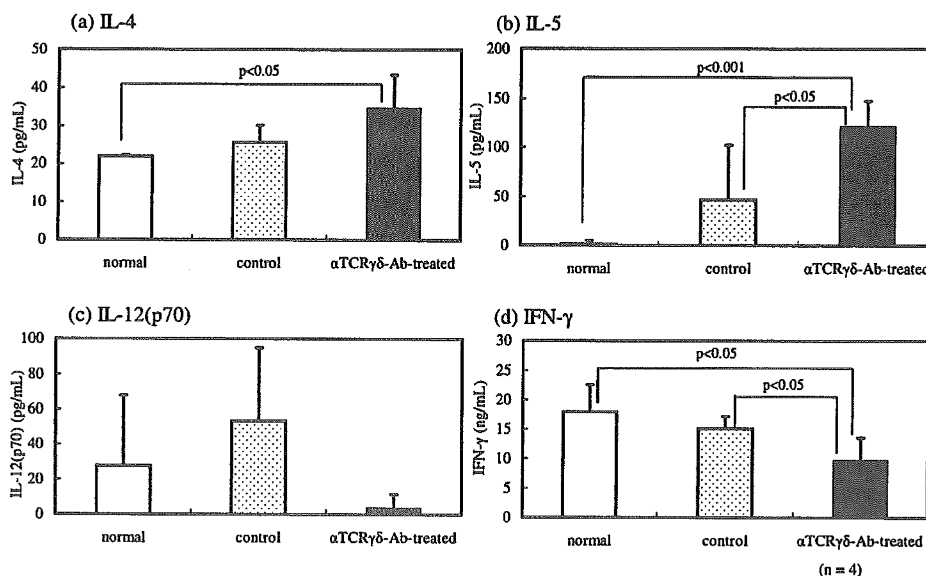

Fig. 3. Cytokine Production by Splenocytes from Anti-TCR$\gamma\delta$-Antibody-Treated WBB6F1-(+/+) Mice

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-TCR$\gamma\delta$ 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 TCR$\gamma\delta$-T cells caused by anti-TCR$\gamma\delta$ 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 TCR$\gamma\delta$-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±1.177[a)]	2.102±1.235*	9.920±4.932*	8.850±6.071	14.62±4.360	17.26±7.650

a) Mean±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±18199**	<50	2251±5662
IgE	<50	274±319	<50	<50
IgG2a	<50	100±109	<50	<50

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

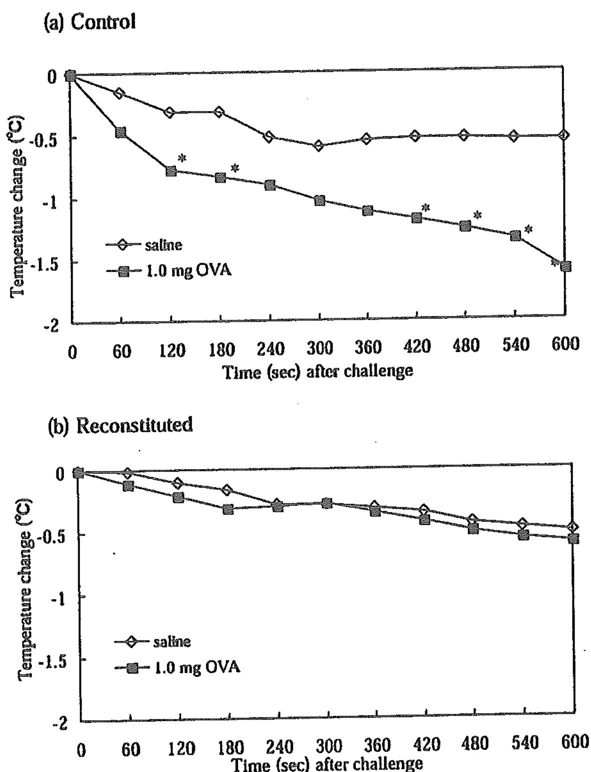

Fig. 4. Changes in Body Temperature of the Bone-Marrow-Cell-Reconstituted W/W^v Mice after Systemic Immunization

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 CD8α+ 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 claify the direct role of $\gamma\delta$-IELs in oral sensitization.

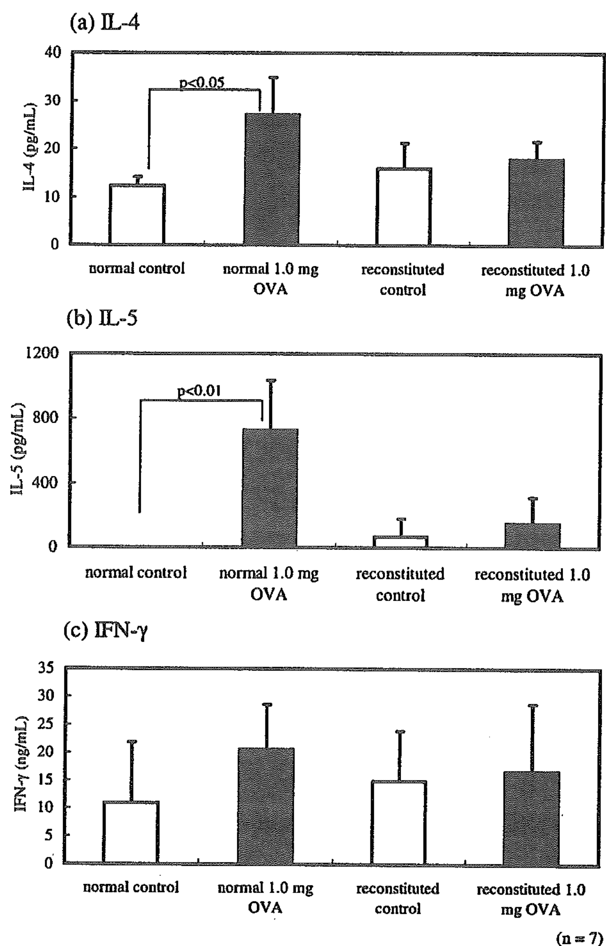

Fig. 5. Cytokine Production by Splenocytes from the Reconstituted W/W^v Mice

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±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 cytolysis.[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 enteral 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 TCRδ-deficient mice.[26)] Notably, a lack of tolerance was found when TCRδ-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-β1 (TGF-β1) *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-β1 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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Kinetic Analysis of Pepsin Digestion of Chicken Egg White Ovomucoid and Allergenic Potential of Pepsin Fragments

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Key Words

Ovomucoid · Allergen · Digestion · Simulated gastric fluid · Fragment, pepsin-digested · Human serum IgE

Abstract

Background: The allergenic potential of chicken egg white ovomucoid (OVM) is thought to depend on its stability to heat treatment and digestion. Pepsin-digested fragments have been speculated to continue to exert an allergenic potential. OVM was digested in simulated gastric fluid (SGF) to examine the reactivity of the resulting fragments to IgE in sera from allergic patients. ***Methods:*** OVM was digested in SGF and subjected to SDS-PAGE. The detected fragments were then subjected to N-terminal sequencing and liquid chromatography/mass spectrometry/mass spectrometry analysis to confirm the cleavage sites and partial amino acid sequences. The reactivity of the fragments to IgE antibodies in serum samples from patients allergic to egg white was then determined using Western blotting (n = 24). ***Results:*** The rate of OVM digestion depended on the pepsin/OVM ratio in the SGF. OVM was first cleaved near the end of the first domain, and the resulting fragments were then further digested into smaller fragments. In the Western blot analysis, 93% of the OVM-reactive sera also bound to the 23.5- to 28.5-kDa fragments, and 21% reacted with the smaller 7- and 4.5-kDa fragments. ***Conclusion:*** When the digestion of OVM in SGF was kinetically analyzed, 21% of the examined patients retained their IgE-binding capacity to the small 4.5-kDa fragment. Patients with a positive reaction to this small peptide fragment were thought to be unlikely to outgrow their egg white allergy. The combination of SGF-digestibility studies and human IgE-binding experiments seems to be useful for the elucidation and diagnosis of the allergenic potential of OVM.

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Introduction

Chicken egg white is one of the strongest and most frequent causes of food allergies among young children [1–5]. Egg white contains several allergens, including ovalbumin, ovotransferrin, lysozyme and ovomucoid (Gal d 1, OVM). OVM accounts for about 11% of all egg white proteins [6] and has a molecular weight of 28 kDa, containing a carbohydrate content of 20–25% [7]. OVM is known to be stable to digestion and heat, and cooked eggs can cause allergic reactions in OVM-specific allergic patients [8–11]. One possible reason for this is that OVM contains linear epitopes that are only slightly affected by conformational changes induced by heat denaturation.

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OVM consists of 186 amino acids divided into three domains of about 60 amino acids each; the third domain has been reported to be the most important domain with regard to allergenicity [12]. In a previous report, N-glycans in the third domain were suggested to be essential for allergenicity [13]; however, a recent report found that the deletion of the N-glycans did not affect the allergic reactivity.

We previously reported the digestibility of 10 kinds of food proteins in simulated gastric fluid (SGF) [8, 14]. OVM was digested relatively rapidly, but several fragments were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue (CBB) staining. The reactivity of these fragments with IgE antibodies from the sera of patients with egg white allergy is very important to understanding the mechanism of OVM allergy.

A few previous reports have described the reactivity of IgE in sera from patients with egg white allergies with OVM-derived fragments. Kovacs-Nolan et al. [15] separated pepsin-digested fragments of OVM using high-performance liquid chromatography (HPLC) and examined the IgE-binding activities of each fragment using an enzyme-linked immunosorbent assay (ELISA). Besler et al. [16] investigated the reactivity of pepsin-digested fragments with patient IgE using Western blotting and showed that the fragments retain their binding capacity to human IgE in some serum samples from OVM-allergic patients. However, little attention has been paid to the digestive conditions, and the number of serum samples has been somewhat small in these studies. Urisu et al. [17] reported that the sera of subjects that tested positive or negative during an oral egg white challenge exhibited a significant difference in their reactivity with pepsin fragments.

In the present report, kinetic data for different generations of SGF-stable OVM fragments were obtained, and the reactivity of the fragments with serum IgE from patients with egg white allergies was investigated using Western blotting.

Materials and Methods

Pepsin (catalog number P6887) and chicken egg white OVM (T2011, Trypsin Inhibitor, Type III-O) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). The concentration of the OVM test solution was 5 mg/ml of water. The gels and reagents used for the SDS-PAGE analysis were purchased from Invitrogen (Carlsbad, Calif., USA).

Serum Specimens

Sera from 24 patients with egg white allergies and a healthy volunteer were used after obtaining informed consent from the patients and ethical approval by the Institutional Review Board of the National Institute of Health Sciences. Twenty-two of the patients had been diagnosed as having an egg white allergy at hospitals in Japan, based on their clinical histories and positive IgE responses to egg white proteins by radioallergosorbent test (RAST), while the remaining 2 allergen-specific sera were purchased from Plasma Lab International (Everett, Wash., USA); the commercial sera originated from adult Caucasians who had been diagnosed as having several food allergies, including egg white, based on their clinical history and skin tests. The commercial sera also showed positive IgE responses to egg white proteins when examined using RAST.

Preparation of SGF

Pepsin (3.8 mg; approximately 13,148 units of activity) was dissolved in 5 ml of gastric control solution (G-con; 2 mg/ml NaCl, pH adjusted to 2.0 with distilled HCl), and the activity of each newly prepared SGF solution was defined as the production of a $\Delta A280$ of 0.001/min at pH 2.0 and 37°C, measured as the production of trichloroacetic acid-soluble products using hemoglobin as a substrate. The original SGF was prepared at a pepsin/OVM concentration of 10 unit/μg, and this solution was diluted with G-con for the experiments performed at pepsin/OVM concentrations of 1 and 0.1 unit/μg. The SGF solutions were used within the same day.

Digestion in SGF

SGF (1,520 μl) was incubated at 37°C for 2 min before the addition of 80 μl of OVM solution (5 mg/ml). The digestion was started by the addition of OVM. At each scheduled time point (0.5, 2, 5, 10, 20, 30, and 60 min), 200 μl of the reaction mixture was transferred to a sampling tube containing 70 μl of 5 × Laemmli buffer (40% glycerol, 5% 2-mercaptoethanol, 10% SDS, 0.33 *M* Tris, 0.05% bromophenol blue, pH 6.8) and 70 μl of 200 m*M* Na_2CO_3. For the zero-point samples, the OVM solution (10 μl) was added to neutralized SGF (190 μl of SGF, 70 μl of 5× Laemmli buffer, and 70 μl of 200 m*M* Na_2CO_3). All neutralized samples were then boiled at 100°C for 3 min and subjected to SDS-PAGE.

SDS-PAGE Analysis and Staining Procedure

Samples (15 μl/lane) were loaded onto a 10–20% polyacrylamide Tris/Tricine gel (Invitrogen, Carlsbad, Calif., USA) and separated electrophoretically. The gels were fixed for 5 min in 5% trichloroacetic acid, washed for 2 h with SDS Wash (45.5% methanol, 9% acetic acid), stained for 10 min with CBB solution (0.1% Coomassie Brilliant blue R, 15% methanol, 10% acetic acid), and destained with 25% methanol and 7.5% acetic acid. The stained gel images were then analyzed using Image Gauge V3.1 (Fuji Film, Tokyo, Japan), and the density of each band was quantified. Periodic acid-Schiff (PAS) staining [18] was used to detect the glycosylated fragments.

N-Terminal Sequence Analysis

OVM (1.5 mg) was digested in SGF containing 1 unit/ml pepsin, concentrated by centrifugation using Centriprep YM-3 (Millipore Corporation, Bedford, Mass., USA) and subjected to SDS-PAGE followed by electrical transblotting to a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad, Richmond, Calif., USA) and CBB staining. The detected fragment bands were then cut out and sequenced using a Procise 494HT Protein Sequencing System (Applied Biosys-

tems, Foster City, Calif., USA) or an HP G1005A Protein Sequencing System (Hewlett-Packard, Palo Alto, Calif., USA); each fragment was analyzed for 5 cycles.

Carboxymethylation and Peptide Mapping Using Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (LC/MS/MS)

The digested OVM sample was separated electrophoretically as described above, stained with CBB, and the stained bands were cut out. The gel pieces were homogenized in 20 m*M* Tris-HCl (pH 8.0) containing 0.1% SDS and the proteins were extracted. The extracts were concentrated and purified by acetone precipitation. The acetone precipitates were incubated with 2-mercaptethanol (92.5 m*M*) in 72 µl of 0.5 *M* Tris-HCl buffer (pH 8.6) containing 8 *M* guanidine hydrochloride and 5 m*M* EDTA at room temperature for 2 h. To this solution, 1.5 mg of monoiodoacetic acid was added, and the mixture was incubated at room temperature for 2 h in the dark. The reaction mixture was desalted using a MicroSpin G-25 column (Amersham Bioscience, Uppsala, Sweden) and lyophilized. Reduced and carboxymethylated proteins were digested with trypsin (50 ng/µl in 50 m*M* NH_4HCO_3).

Tandem electrospray mass spectra were recorded using a hybrid quadruple/time-of-flight spectrometer (Qstar Pulsar i; Applied Biosystems, Foster City, Calif., USA) interfaced to a CapLC (Magic 2002; Michrom BioResources, Auburn, Calif., USA). Samples were dissolved in water and injected into a C18 column (0.2 × 50 mm, 3 µm, Magic C18, Michrom BioResources). Peptides were eluted with a 5–36% acetonitrile gradient in 0.1% aqueous formic acid over 60 min at a flow rate of 1 µl/min after elution with 5% acetonitrile for 10 min. The capillary voltage was set to 2,600 V, and data-dependent MS/MS acquisitions were performed using precursors with charge states of 2 and 3 over a mass range of 400–2,000.

Western Blotting of Digested Fragments with Human Serum IgE

The digested OVM samples were applied to a 10–20% polyacrylamide Tris/Tricine 2D gel, followed by electrical transfer to a nitrocellulose membrane. The membrane was then blocked with 0.5% casein-PBS (pH 7.0) and cut into 4-mm strips. The strips were incubated with diluted human serum (1/4 to 1/5) in 0.2% casein-PBS (pH 7.0) at room temperature for 1 h and then at 4°C for 18 h. After washing with 0.05% Tween 20-PBS, the strips were incubated with rabbit anti-human IgE (Fc) antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands) at room temperature for 1 h, and then with horseradish peroxidase-conjugated donkey anti-rabbit Ig antibodies (Amersham Biosciences, Little Chalfont, UK) at room temperature for 1 h. Finally, the strips were reacted with Konica ImmunoStain HRP-1000 (Konica, Tokyo, Japan), according to the manufacturer's protocol.

Results

Kinetics of OVM Digestion by Pepsin

OVM was digested in SGF containing various concentrations of pepsin, and the fragments were separated by SDS-PAGE and stained with CBB (fig. 1). The molecular weight of OVM, based on its amino acid sequence, is about 20 kDa, but a broad band representing intact OVM appeared at about 34–49 kDa in the SDS-PAGE gel because of the presence of five N-linked sugar chains. The pepsin band was detected at 39 kDa, overlapping with the intact OVM band, and lysozyme (14 kDa) contamination was detected in the OVM sample that was used. Intact OVM rapidly disappeared within 0.5 min in SGF (pepsin/OVM = 10 unit/µg), and a fragment band was detected at 7 kDa. When the pepsin content in SGF was reduced to 1 and 0.1 unit/µg, the digestion rate markedly decreased. Intact OVM was still detected after 30 min when the pepsin/OVM ratio was 0.1 unit/µg. The fragment bands were clearer (fig. 2) when a concentrated SGF-digested OVM solution (pepsin/OVM = 1 unit/µg, digestion times 5 and 30 min) was used, followed by SDS-PAGE. As shown in figure 2, a strong 23.5- to 28.5-kDa band (FR 1) was detected at 5 min, while 10- (FR 2), 7- (FR 3) and 4.5- to 6-kDa (FR 4) bands were detected after 30 min. FR 1 and FR 2 were both positively stained by PAS, suggesting that the FR 1 and FR 2 fragments have high carbohydrate contents. The time courses for the amounts of intact OVM and the four fractions are plotted in figure 3, where the pepsin/OVM ratio is 1 unit/µg. FR 1 rapidly increased but slowly disappeared after 2 min. FR 2 and FR 3 also rapidly reached maximum values at 5 min and then slowly disappeared. On the other hand, FR 4 gradually increased throughout the entire period of the experiment.

Preheating (at 100°C for 5 or 30 min) of the OVM solution (5 mg/ml in water) did not influence the digestion pattern (fig. 1).

Table 1. N-Terminal sequences of pepsin fragments

Digestion period	Fraction	Fragment	Residues	Sequence	Ratio %[a]
5 min	FR 1	1-1	50–54	FGTNI	73.1
		1-2	51–55	GTNIS	11.6
		1-3	1–5	AEVDC	6.9
5 min	FR 2	2-1	1–5	AEVDC	68.8
		2-2	134–138	VSVDC	28.2
5 min	FR 3	3-1	1–5	AEVDC	48.4
		3-2	134–138	VSVDC	24.3
		3-3	104–108	NECLL	9.6
		3-4	85–89	VLCNR	6.5
30 min	FR 4	4-1	134–138	VSVDC	30.6
		4-2	104–108	NECLL	24.0
		4-3	19–23	VLVCN	20.6

[a] Molar ratios of the fragments to the total amount in each fraction.

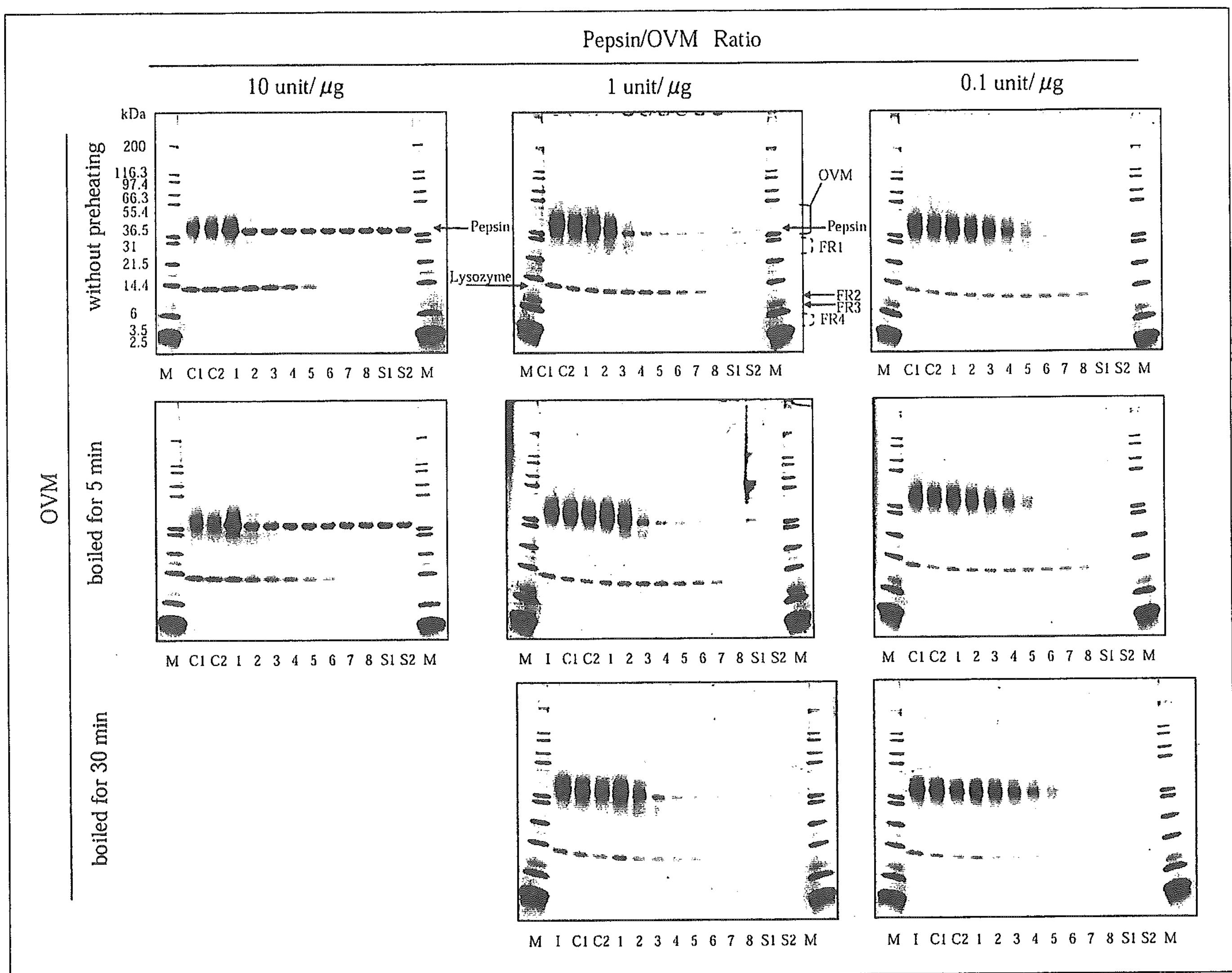

Fig. 1. Kinetic patterns of OVM digestion in SGF-containing pepsin. Digested samples were analyzed by SDS-PAGE followed by CBB staining. The digestion patterns of OVM without preheating (upper panels), preheated at 100°C for 5 min (middle panels), and preheated at 100°C for 30 min (lower panels) are shown. The ratio of pepsin to OVM was 10 unit/1 μg (left), 1 unit/1 μg (middle), and 0.1 unit/1 μg (right). Lane M = Molecular weight markers; lanes C1 and C2 = OVM without pepsin at 0 (C1) and 60 (C2) min; lanes 1–8 = SGF-digested OVM at 0, 0.5, 2, 5, 10, 20, 30 and 60 min, respectively; lanes S1 and S2 = SGF alone at 0 (S1) and 60 (S2) min; lanes I = OVM without preheating; FR 1 = fraction 1 containing a fragment at 23.5–28.5 kDa; FR 2 = fraction 2 containing a 10-kDa fragment; FR 3 = fraction 3 containing a 7-kDa fragment; FR 4 = fraction 4 containing 4.5- to 6-kDa fragments.

Sequence Analysis of OVM Fragments

The sequences of the five N-terminal residues in each fragment were analyzed, and the data are summarized in table 1. Figure 4 schematically depicts the identified fragments; the arrows in the upper panel indicate the sites of pepsin cleavage.

The internal sequences of the FR 1, FR 3, and FR 4 fragments were also identified by LC/MS/MS and are shown in table 2 and in the upper panel of figure 4.

Reactivity of the Fragments with Serum IgE from Patients with Egg White Allergy

Western blot analysis using patient sera as the source of the primary antibodies was performed to identify sera that reacted with intact OVM and the SGF fragments. Representative blotting data are shown in figure 5, and all the results are listed in table 3. Ninety-two percent of the serum samples from allergic patients reacted with OVM, and 93% of the OVM-positive sera reacted with FR 1

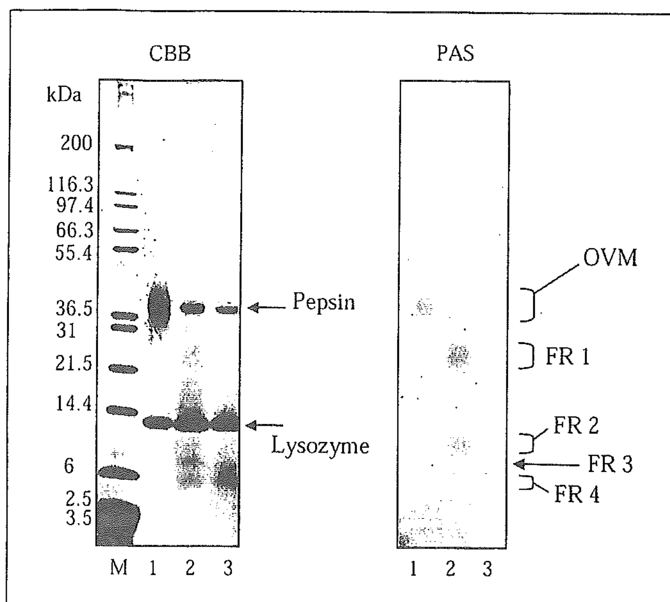

Fig. 2. CBB and PAS staining of OVM fragments following digestion in SGF (pepsin/OVM = 1 unit/μg) for 5 and 30 min. Lane M = Molecular weight markers; lane 1 = original OVM (2.5 μg/lane); lanes 2 and 3 = OVM digested for 5 and 30 min, respectively, and concentrated (12 μg, equivalent to the original OVM/lane). Samples were applied to two SDS-PAGE gels and electrophoresed. One plate (left panel) was stained with CBB reagent, and the other (right panel) was stained with PAS reagent.

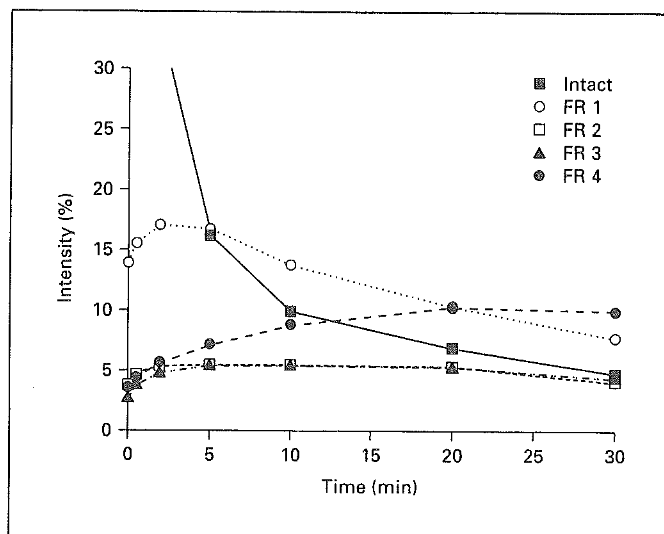

Fig. 3. Quantification of the SGF-digestion pattern of intact OVM and the digestion fragments at a pepsin/OVM ratio of 1 unit/μg. The intensity of each band was calculated using the ratio of the band's density to the total density of the originally detected band at t = 0. Values are the mean of duplicate analyses. Similar results were observed in another set of experiments.

Table 2. Identified inside sequences in pepsin- and trypsin-digested OVM

Pepsin digestion	Fraction	Residues	Sequence
5 min	FR 1	83–89	VMVLCNR
		90–103	AFNPVCGTDGVTYD
		90–112	AFNPVCGTDGVTYDNECLLCAHK
		90–122	AFNPVCGTDGVTYDNECLLCAHKVEQGASVDKR
		113–122	VEQGASVDKR
5 min	FR 3	90–112	AFNPVCGTDGVTYDNECLLCAHK
		90–122	AFNPVCGTDGVTYDNECLLCAHKVEQGASVDKR
		104–111	NECLLCAH
		104–112	NECLLCAHK
		104–121	NECLLCAHKVEQGASVDK
		104–122	NECLLCAHKVEQGASVDKR
		113–122	VEQGASVDKR
		134–159	VSVDCSEYPKPDCTAEDRPLCGSDNK
		165–185	CNFCNAVVESNGTLTLSHFGK
30 min	FR 4	90–112	AFNPVCGTDGVTYDNECLLCAHK
		104–111	NECLLCAH
		104–112	NECLLCAHK
		104–122	NECLLCAHKVEQGASVDKR
		112–122	KVEQGASVDKR
		113–121	VEQGASVDK
		113–122	VEQGASVDKR
		165–185	CNFCNAVVESNGTLTLSHFGK

```
      1          11         21         31         41         51
      |          |          |          |          |          |

  1 AEVDCSRFPN ATDKEGKDVL VCNKDLRPIC GTDGVTYTND CLLCAYSIEF GTNISKEHDG   60

 61 ECKETVPMNC SSYANTTSED GKVMVLCNRA FNPVCGTDGV TYDNECLLCA HKVEQGASVD  120

121 KRHDGGCRKE LAAVSVDCSE YPKPDCTAED RPLCGSDNKT YGNKCNFCNA VVESNGTLTL  180

181 SHFGKC
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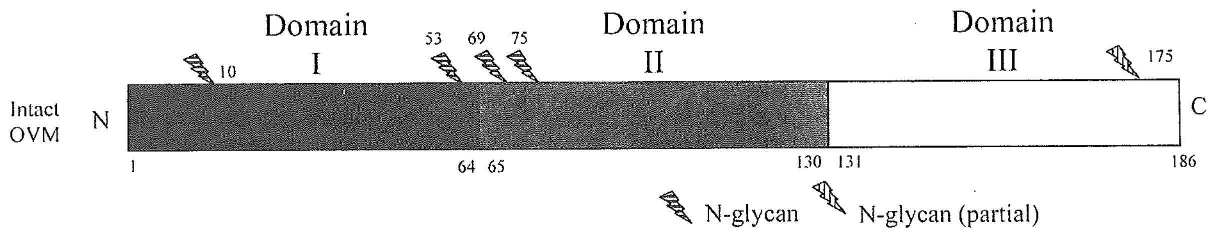

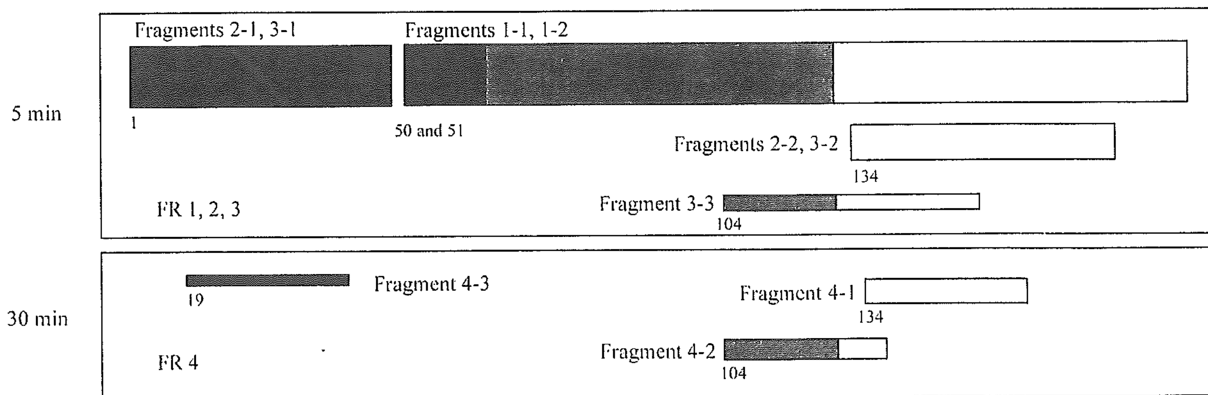

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after 5 min of digestion. Three of the serum samples also reacted with FR 2, FR 3, and FR 4 after 30 min of digestion.

The three samples that react with FR 2, FR 3, and FR 4 were obtained from patients who exhibited persistent allergies to egg white. One of these serum samples, No.4, was obtained from a 3-year-old girl who is presently 6 years old; her total IgE level has decreased slightly to 4,450 IU/ml, but the specific IgE level for egg white remains at more than 100 IU/ml, and the patient has not outgrown her hypersensitivity to eggs. Another patient, No. 13, was a 1-year-old boy; 7 years later, his total and egg white-specific IgE levels had been reduced to 947 and 6.85 IU/ml, respectively, but eating raw eggs still caused allergic symptoms. The third FR 4-positive patient, No. 19, was an 11-year-old boy whose total IgE level decreased to 3,940 IU/ml and whose egg white-specific IgE decreased to 13.5 IU/ml after a period of about 2 years; however, this patient has also not outgrown his allergies. These cases and our previously reported data [17] indicate that the induction of egg white tolerance may be difficult in patients whose serum IgE exhibits binding activity to digested small fragments of OVM.

Discussion

In the SGF-digestion system, preheating the OVM (100 °C for 5 or 30 min) did not affect the OVM digestion pattern (fig. 1), consistent with the results of previous reports [9] in which heat treatment did not markedly decrease the allergenicity of OVM. On the other hand, a decrease in the pepsin/OVM ratio dramatically reduced the digestion rate, suggesting that digestibility may vary depending on the amount of OVM intake and the conditions of the individual's digestion system. In its native state, OVM possesses serine protease inhibitor activity. Fu et al. [11] and our group [10] previously reported that intact OVM was stable for 60 min in simulated intestinal fluid. Kovacs-Nolan et al. [15] also reported that pepsin-

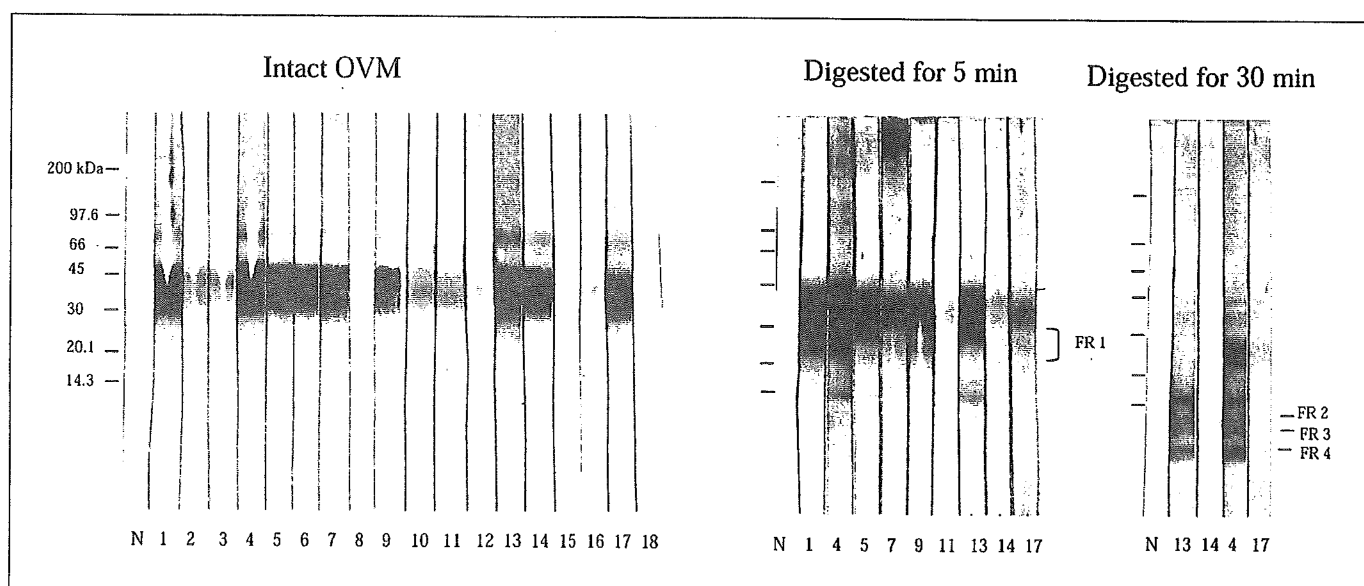

Fig. 4. Amino acid sequence and schematic representation of the SGF-digestion pattern of OVM. The amino acid sequence of OVM is shown in the upper panel. The arrows indicate the SGF-digested points according to the results of an N-terminal analysis of the OVM fragments (table 1), and the underlined regions indicate sequences identified by LC/MS/MS. Solid line = FR 1; dotted line = FR 3; dashed line = FR 4. Shaded areas represent reported human IgE epitopes [16]. The lower panel is a summary of the OVM digestion pattern according to N-terminal analysis.

Fig. 5. Western blot analysis of intact OVM and the fragments with serum IgE from egg white allergic patients and a normal volunteer. The fragments were prepared as described in the legend of figure 2. The number of each strip corresponds to the sample numbers in table 2.

Table 3. Reactivity of OVM and pepsin fragments with patient serum IgE

No.	IgE content, IU/ml		Reactivity with patient IgE[1]				
	total	egg white-specific	intact OVM	FR 1	FR 2	FR 3	FR 4
1	3,700	>100	+++	++	–	–	–
2	402	3.74	+	n.d.	n.d.	n.d.	n.d.
3	251	6.85	+	n.d.	n.d.	n.d.	n.d.
4	6,510	>100	+++	+++	+	+	++
5	2,060	>100	++	++	–	–	–
6	1,240	12.4	++	n.d.	n.d.	n.d.	n.d.
7	4,180	31.3	++	++	–	–	–
8	56	20.1	±	n.d.	n.d.	n.d.	n.d.
9	1,355	50.7	++	++	–	–	–
10	22,810	2.11	+	n.d.	n.d.	n.d.	n.d.
11	1,463	4.65	+	–	–	–	–
12	14,230	0.70–3.49	±	n.d.	n.d.	n.d.	n.d.
13	8,000	>100	+++	+++	+	+	++
14	22,490	1.05	+++	±	–	–	–
15	934	66.3	+	n.d.	n.d.	n.d.	n.d.
16	345	20.1	+	n.d.	n.d.	n.d.	n.d.
17	1,500	80	++	+	–	–	–
18	3,300	>10	–	n.d.	n.d.	n.d.	n.d.
19	20,500	26.8	+++	++	±	±	±
20	138	45.4	++	+	–	–	–
21	940	2.44	+	+	–	–	–
22	91	0.70–3.49	+	±	–	–	–
23	828	0.9	++	+	–	–	–
24	21	3.50–17.4	–	n.d.	n.d.	n.d.	n.d.
	positive/tested		22/24 (92%)[2]	13/14 (93%)[3]	3/14 (21%)[3]	3/14 (21%)[3]	3/14 (21%)[3]

n.d. = Not done.
[1] Intensity of the reactivity of each band was evaluated by the ratio to normal serum: – = <1; ± = 1–2; + = 2–5; ++ = 5–10; +++ = >10.
[2] Percent of egg white-positive samples.
[3] Percent of intact OVM-positive samples.

digested OVM retains its trypsin inhibitor activity. Therefore, OVM and its pepsin-digested fragments were thought to be stable in the small intestine.

At a pepsin/OVM ratio of 1 unit/μg, FR 1 reached a maximum level after 2 min of digestion, while both FR 2 and FR 3 reached maximum levels after 5 min of digestion; thereafter, FR 1, FR 2, and FR 3 gradually decreased. However, FR 4 increased continuously throughout the 30-min period of digestion and the major fragments were seen after 30 min of digestion (fig. 3). FR 4 was mainly composed of three fragments whose N-terminals were 134V, 104N and 19V (table 1). A C-terminal sequence, 165N–185C, was also identified in FR 4 (table 2). These fragments contain known IgE epitopes [19] and therefore may cause allergic responses. Three of the OVM-positive sera from patients with egg white allergy reacted positively with the FR 4 fragments (table 3).

The present results are consistent with the previous finding that pediatric subjects with a higher IgE-binding activity to pepsin-treated OVM were unlikely to outgrow their egg allergy [17]. For peanut allergies, differences in IgE-binding epitopes have been reported between the patients with clinically active peanut allergies and those who developed a tolerance, regardless of the presence of high or low peanut-specific IgE levels [20].

The N-terminal residue of the major fragment (4-1) of FR 4 was Val-134 (30%; table 1). This fragment retains most of domain III, which has been reported to have significantly higher human IgG- and IgE-binding activities than those of domains I and II [12]. A domain-III OVM

variant has also been reported to cause a reduction in immunogenicity and allergenicity [21].

Domains I, II, and III contain one, three, and one N-glycosylation sites, respectively [7]. The possible relation between the carbohydrate chain in domain III and allergenicity is interesting. One report suggested that this carbohydrate chain may play an important role in allergenic determinants against human IgE antibody [13], and another report suggested that the carbohydrate chains of OVM may protect against peptic hydrolysis [22]. However, the carbohydrate moieties have been shown to have only a minor effect on allergenicity [23]. As shown in figure 2, intact OVM, FR 1, and FR 2 fragments were detected using PAS staining, suggesting the presence of carbohydrate chains, but FR 4 was not stained with the PAS reagent, despite being clearly detected with CBB. Therefore, FR 4 might contain little or no carbohydrate chains. Since FR 4 seems to maintain its allergenic potential, as described above, the absence of the carbohydrate chains in FR 4 suggests that they are not necessary for OVM allergenicity. Since the minimum peptide size capable of eliciting significant clinical symptoms of allergic reactions is thought to be 3.1 kDa [24], FR 4 may be able to trigger mast cell activation and elicit clinical symptoms.

In this report, the SGF-digestion kinetic pattern of OVM was investigated in detail, and the partial sequences of the fragments in the 4 fractions separated by SDS-PAGE were determined. Furthermore, the reactivity of the fragments with a number of serum samples from patients with egg white allergies was detected using Western blotting. The four fractions were separated according to their molecular weight and consisted of more than one fragment, as determined by N-terminal analysis. The identified sequences that started at Asn-104 and Val-134 in FR 3, as determined using LC/MS/MS (table 2), coincided with the 3-2 and 3-3 fragments in the N-terminal analysis (table 1), and the sequence that started at Asn-104 in FR 4 coincided with fragment 4-2. Moreover, the LC/MS/MS analysis indicated that FR 3 and FR 4 contained other parts of domain II and the C-terminal sequence N165–C185, which are thought to be minor components of these fractions. The combination of SGF digestion and patient IgE may provide useful information for the diagnosis and prediction of potential OVM allergenicity.

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Applicability of the Quantification of Genetically Modified Organisms to Foods Processed from Maize and Soy

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The applicability of quantifying genetically modified (GM) maize and soy to processed foods was investigated using heat treatment processing models. The detection methods were based on real-time quantitative polymerase chain reaction (PCR) analysis. Ground seeds of insect resistant GM maize (MON810) and glyphosate tolerant Roundup Ready (RR) soy were dissolved in water and were heat treated by autoclaving for various time intervals. The calculated copy numbers of the recombinant and taxon specific deoxyribonucleic acid (DNA) sequences in the extracted DNA solution were found to decrease with time. This decrease was influenced by the PCR-amplified size. The conversion factor (C_f), which is the ratio of the recombinant DNA sequence to the taxon specific DNA sequence and is used as a constant number for calculating GM% at each event, tended to be stable when the sizes of PCR products of two DNA sequences were nearly equal. The results suggested that the size of the PCR product plays a key role in the quantification of GM organisms in processed foods. It is believed that the C_f of the endosperm ($3n$) is influenced by whether the GM originated from a paternal or maternal source. The embryos and endosperms were separated from the F1 generation seeds of five GM maize events, and their C_f values were measured. Both paternal and maternal GM events were identified. In these, the endosperm C_f was lower than that of the embryo, and the embryo C_f was lower than that of the endosperm. These results demonstrate the difficulties encountered in the determination of GM% in maize grains (F2 generation) and in processed foods from maize and soy.

KEYWORDS: ***Zea mays*; *Glycine max*; genetically modified; MON810; Roundup Ready; processed food; endosperm; embryo; heat treatment; GMO detection; quantitative analysis**

INTRODUCTION

A rapid increase in the growth of genetically modified organisms (GMO) as well as dependence on foodstuffs including these materials have occurred in recent years (*1*). Some consumers are concerned about the use of advanced technology and potential health and environmental risks associated with GMOs (*2*). One particular matter of concern to consumers has been the proper labeling of food products that have been produced using GM technology. Labeling systems have been introduced for GM foods in the European Union (EU), Korea, Japan, Australia, and other countries; however, the labeling of GM foods is not compulsory in the United States and Canada. Under current legislation, the presence of GM material in conventional food products does not have to be labeled, provided that such use can be shown to be adventitious and technically unavoidable. The governments of the following countries have announced the following threshold levels for the unintentional mixing of GMOs with food products: 0.9% in the EU (*3*), 3% in Korea (*4*), and 5% in Japan (*5*).

Polymerase chain reaction (PCR) is a widely used technique to confirm the existence of GMOs and to ensure the reliability of labeling systems (*6−18*). For initial use in GMO screening, qualitative PCR, which determines whether more than a particular detectable limit of GMOs is present in a sample, is considered the method of first choice (*6−12*); real-time PCR

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