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Modulation of Immunoresponse in BALB/c Mice by Oral Administration of Fag e 1−Glucomannan Conjugate

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Maillard-type glycosylation was applied to preparation of hypoallergenic agents from a major buckwheat allergen, Fag e 1. Conjugation with arabinogalactan (AG), xyloglucan (XG), or yeast glucomannan (YGM) successfully decreased *in vitro* allergenicity of Fag e 1. Determination of IgE titer in the tested allergic mice revealed that YGM was the most effective for *in vivo* allergenicity of Fag e 1 among these water-soluble polysaccharides. Real-time PCR analysis using a set of primer for IL-4 (a typical Th2 cytokine) or IFN-γ (a typical Th1 cytokine) showed that expressed mRNA for IL-4 in splenocytes drastically decreased with increasing with Fag e 1−YGM conjugate feeding. In addition, based on a flow-cytometric analysis of T cell subsets in the splenocytes, it was confirmed that the feeding led to an improvement of Th1/Th2 balance in the allergic mice where population of Th1 increased from 2.91% to 4.02%, while that of Th2 decreased from 3.75% to 2.72%. Furthermore, it was revealed that differentiation ratio of regulatory T cell (Treg) in the splenocytes increased from 14.5% to 18.7% by the oral administration. These results indicated that Fag e 1−YGM conjugate can be available for an immunomodulating agent for buckwheat allergy.

KEYWORDS: Fag e 1; buckwheat allergy; yeast glucomannan; Maillard-type glycosylation; Th1/Th2 balance; Treg; immunotherapy

1. INTRODUCTION

As the consumption of buckwheat has increased around the world, it has become recognized as an allergenic food source which causes serious anaphylactic symptoms including bronchial asthma, perennial rhinitis and dermatitis in sensitive patients (*1*−*4*). Recently it was demonstrated that 13S globulin with 22 kDa in buckwheat displayed high IgE-binding frequency with almost all sera from different patients (*5*), and thus it was considered to be a major allergenic protein of buckwheat and was termed as Fag e 1 (*6*). In the previous paper, Fag e 1 was focused on the reduction of its allergenicity as well as the improvement of its molecular surface functionalities including solubility and amphiphilicity, and applied for the naturally occurring Maillard-type glycosylation to decrease the allergenicity (*7*). As a result, it was revealed that the Maillard-type glycosylation of Fag e 1 with food-grade polysaccharides brought about a drastic reduction of the reactivity against human sera of buckwheat-allergic subjects, by using immuno dot-blotting, QCM (quartz crystal microbalance) analysis and ELISA (*7*).

Although developments of allergen-free foods or food materials using protease, heat, or chemical treatments have been desired for allergenic patients, their applications seem to be still crucial for mature patients in the case of potent allergens including buckwheat. On the other hand, there is a consideration that development of a more effective antiallergenic drug must be better than the above trials. However, indiscriminate use of synthetic chemicals usually remains a critical concern for their side effects. An alternative approach, called "allergen immunotherapy", has entered the spotlight where a small amount of antigen is orally administered for the long term to induce oral immunotolerance, which must be much safer and will be well-specific against the individual's target (*8*, *9*). However, the potential for an adverse reaction is always open for argument because this therapy involves direct intake of the allergen (*8*). Recently, instead of a small amount of administration, many attempts to modulate the allergenic response by hypoallergens aimed at modifying the tertiary structure of IgE-binding epitopes have been reported for immunotherapy (*10*). Introduction of immunotolerance has become a well-recognized approach for curing food allergy in which mature lymphocytes in the peripheral lymphoid tissues are rendered nonfunctional or hyporesponsive against the targeted allergen (*11*). Thus, establishment of more effective immunotherapeutic strategy has been awaited for long time by using attenuated allergen. In the meanwhile, it was demonstrated that hypoallergenic Fag e 1 with less than 10% allergenicity can be prepared by Maillard-type glycosylation with polysaccharide as mentioned above. Therefore, effects of oral

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administration of Fag e 1−polysaccharide conjugates on changes of *in vivo* allergenic responses were assessed by using BALB/c mice in this study. As a result, it was revealed that only yeast glucomannan conjugate exhibited a strong immunomodulatory efficacy of the allergen for desensitization in the allergic mice. In other words, Fag e 1−glucomannan conjugate can be used as a hypoallergenic agent in antigen-specific immunotherapy for buckwheat allergy. The present paper will provide a new viewpoint of the application of the water-soluble polysaccharide, i.e. yeast glucomannan hydrolysates prepared from the cell wall from *Saccharomyces cerevisiae*.

2. MATERIALS AND METHODS

2.1. Materials. Common buckwheat (*Fagopyrum esculentum* Moench) was obtained from the Education and Research Center of Alpine Field Science in Shinshu University. Arabinogalactan (AG) and xyloglucan (XG) were from MRC Polysaccharide Inc. (Toyama, Japan) and Dainippon Sumitomo Seiyaku Inc. (Osaka, Japan), respectively. Human sera were obtained from buckwheat-allergic subjects, consisting of 2 males and 4 females ranging in age from 12 to 45 years old, as previously reported (*7*). Superdex 200 and Q Sepharose FF were purchased from GE Healthcare in Japan (Tokyo, Japan). Shodex SUGAR-SH1011 column was from Showa Denko K.K (Tokyo, Japan). Goat antimouse IgE labeled with HRP was purchased from Southern Biotech Associates Inc. (Birmingham, AL). BALB/c mice were purchased from Charles River Ltd. (Tokyo, Japan). Reagents and antibodies used for flow cytometric analysis were purchased from BD Biosciences Co., Ltd. (Tokyo, Japan). Primers and SYBR Premix Ex *Taq*II were obtained from Takara Bio Inc. (Tokyo, Japan). All other reagents were of biochemical levels.

2.2. Preparation of Fag e 1−Polysaccharide Conjugates. Fag e 1 was prepared from common buckwheat flour as described previously (*7*), and conjugated with polysaccharides according to Nakamura et al. (*12*). Yeast cell wall prepared from *Saccharomyces cerevisiae* was digested using a commercial glucanase (Onoduka 3S, Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) for 1 h at 37 °C. The supernatant was dialyzed against distilled water and freeze-dried. The lyophilized powder was applied for SEC using Superdex 200 column to obtain a uniform fraction with 15 kDa average molecular mass. The fraction was collected, pooled, and freeze-dried. The resulting powder consisted of glucose and mannose according to SEC+IEC using Shodex SUGAR-SH1011 column, named as yeast glucomannan (YGM). Fag e 1 and YGM, AG, and XG were individually dissolved in 50 mM phosphate buffer (pH 7.0) with a given weight ratio of 1:1 (weight base) and freeze-dried. The resulting powder was put into an incubator and stored at 60 °C for one week under a relative humidity of 65% in a container saturated with KI solution to generate a naturally occurring Maillard-linkage. Fag e 1 conjugate with polysaccharide was separated from free protein and carbohydrate using a Superdex 200 column. To confirm the formation of Maillard-type conjugation between protein and polysaccharide, SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was conducted according to the method of Laemmli (*13*) using 15% (w/w) acrylamide separating gel with 5% (w/w) stacking gel containing 1% (w/v) sodium dodecyl sulfate (SDS). After electrophoresis, the gels were stained for protein and carbohydrate with 0.025% (w/v) Coomassie Brilliant Blue R-250 solution and 0.5% (w/v) periodic acid−Fuchsin solution (*14*), respectively.

2.3. Preparation of Allergic Mouse. Four-week-old female BALB/c strain mice (Charles River Ltd., Tokyo, Japan) were maintained under hygienic conditions with free access to food and water. The ambient temperature was maintained at 20 ± 2 °C with a 12 h light/dark cycle. Every experimental manipulation was undertaken in accordance with the institutional guidelines for the care and use of laboratory animals of Shinshu University. Mice were immunized with 50 μg of Fag e 1 dissolved in 50 μL of PBS and alum adjuvant by intraperitoneal (ip) injection for the initial immunization. Boosters were held on 14 and 28 days later from initial immunization with 25 μg of Fag e 1 and alum adjuvant by ip injection.

2.4. Oral Administration and Measurement of Induced Specific IgE Antibody to Fag e 1. Sera were collected from caudal vein of the model mice, and applied for the measurement of specific IgE antibody Fag e 1. After confirmation for the elevation of specific IgE titer and typical allergic symptoms such as scraping behavior and diarrhea by allergen challenge, the mice were fed at liberty with homemade feed containing 5 mg of protein/100 g of Fag e 1−polysaccharide conjugates which was prepared using MF pellet (Oriental Yeast Co. Ltd., Tokyo, Japan). Then, the treatment with the forage was continued for 8 weeks, and the changes of specific IgE level were monitored. Enzyme-linked immunosorbent assay (ELISA) was performed to determine specific IgE level. Microplate was first coated with rabbit anti-Fag e 1 antibody by overnight incubation at 4 °C. The plate was blocked by incubation for a further 90 min at room temperature with 0.1% skim milk and follows a further incubation for 90 min at 37 °C with 1 mg/mL of Fag e 1 diluted in acetate buffer solution. Twenty-time dilutions of mouse serum samples were added and reacted to Fag e 1 for overnight at 37 °C. Antimouse IgE labeled with HRP was further incubated for 90 min at room temperature. Substrate containing *o*-phenylenediamine and H_2O_2 was applied for coloring form followed by 2 N H_2SO_4 to stop the enzyme−substrate reaction. Finally, absorbance at 490 nm was measured by a Microplate Reader (Model 680, Bio-Rad Laboratories, Tokyo, Japan). The microplate was washed 5 times by PBST between each process using a microplate washer (ImmunoWash, Bio-Rad Laboratories, Tokyo, Japan). The absorbance at 490 nm in the control mice sera with normal feeding was 0.310, and we expressed those of Fag e 1−polysaccharides conjugate as a relative amount. After treatment with prepared forage, splenocytes were collected from allergic mice, and used for cytokine quantified ELISA, quantitative polymerase chain reaction (qPCR) and flow cytometric analyses. Mice were euthanized humanely by CO_2 gas, and spleen cells were harvested and washed with Hanks' balanced salt solution, and then used for further experiments.

2.5. Determination of Expressed Cytokines. The washed cells were resuspended in RPMI-1640 media containing 10% FBS to prepare a cell suspension with 1.0×10^6 cells/mL. Then, the cell suspension was transferred into 10% FBS-RPMI-1640 media containing 7 mM 2-mercaptoethanol, 2 μg/mL concanavalin A, and 250U- penicillin/streptomycin. Subsequently, the cells were cultured for 72 h at 37 °C under the 5% CO_2 condition to release interferon-γ (IFN-γ) and interleukin-4 (IL-4). Microplates were coated with cell culture supernatant and further incubated for overnight at 4 °C. The plate was blocked with 0.1% BSA for 90 min at room temperature, followed by a further incubation for 90 min at room temperature with biotin conjugated anti-IFN-γ (Funakoshi, Tokyo, Japan) or biotin conjugated anti-IL-4 antibody (Genetex Inc., San Antonio, TX). Streptavidin-HRP (Sigma-Aldorich Japan, Tokyo, Japan) was added to induce biotin−avidin complex formation. The following coloring process was performed as previously described (*15*). In addition, to determine expressed cytokines' levels in splenocytes, qPCR was performed according to guidances (*16*, *17*). Total RNA solution was prepared from the above splenocytes using RNA-isoplus (Takara Bio Inc., Tokyo, Japan) according to the manufacturer's instruction. mRNAs were reverse-transcribed to cDNAs, and resulting cDNAs were stored at −80 °C until use. Commercial universal primers for cytokines (Takara Bio Inc., Kyoto, Japan) were used for amplified β-actin, IFN-γ, and IL-4. The qPCR mixture was prepared using 9 μL of sample cDNA and 10 μL of SYBR Premix Ex *Taq*II and 1 μL of appropriate primers. The PCR reaction was performed by Thermal Cycler Dice Real Time System (Takara Bio Inc., Kyoto, Japan).

2.6. Determination of T Cell Differentiation. Flow cytometric analysis was performed to estimate the T cell differentiation. Splenocytes obtained from allergic mice were stimulated with 10 ng/mL phorbol 12-myristate 13-acetate and 1 μg/mL ionomycin for cytokine releasing. BD GolgiPlug (BD Biosciences Co., Ltd., Tokyo, Japan), a protein transport inhibitor containing brefeldin A, was added to be released cytokines in the tested cell. The cells were incubated for 12 h at 37 °C with 5% CO_2, and then transferred into staining buffer containing 1% fetal bovine albumin-Dulbecco's PBS (pH 7.2). The tested splenocytes were stained with FITC-conjugated anti-CD4 for surface staining for 30 min at 4 °C in the dark condition. Cells were resuspended with the fixation/permeabilization solution (BD Biosciences Co., Ltd.) for 20 min at 4 °C to fix them and increase permeability. Intracellular staining for IFN-γ and IL-4 were done with APC-conjugated anti-IFN-γ and PE-conjugated anti-IL-4, respectively, by incubation for 30 min at 4 °C in the dark. Resulting cells were analyzed using BD FACSCalibur (BD Bioscience; Tokyo, Japan) equipped with cell sorter (BD FACSAria) and CellQuest software

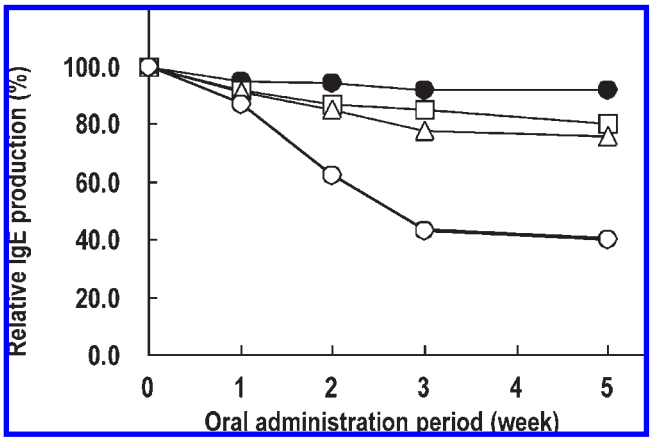

Figure 1. Changes of IgE level in tested mice fed with Fag e 1−polysaccharide conjugates. □, Fag e 1−AG conjugate feeding; △, Fag e 1−XG conjugate feeding; ○, Fag e 1−YGM conjugate feeding; ●, normal feeding. The data are from a representative experiment repeated three times with similar results.

(Becton & Dickinson Japan, Tokyo). To determine differentiation level of the regulatory T cell (Treg), obtained splenocytes were stained with their surface by FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 for 30 min at 4 °C in the dark. Resulting cells were also analyzed using the BD FACSCalibur. The percentage of Treg was calculated according to the rate of $CD25^+$ cell gating with $CD4^+$ as follows:

$$\text{Treg}\ (\%) = (CD4^+CD25^+)/(CD4^+CD25^- + CD4^+CD25^+) \times 100)$$

All antibodies were purchased from BD Biosciences (Tokyo, Japan).

2.7. Statistical Analysis. All experiments were conducted at least in triplicate. Data were analyzed using the Scheffe method after analysis of variance (ANOVA) for comparing differences between treatment means.

3. RESULTS AND DISCUSSION

3.1. Production of Hypoallergenic Fag e 1 by the Conjugation with Polysaccharides. A major allergen of buckwheat, Fag e 1, was prepared from common buckwheat (*Fagopyrum esculentum* Moench), and applied for the naturally occurring Maillard-type glycosylation with water-soluble polysaccharides, arabinogalactan (AG), xyloglucan (XG) or yeast gluccomannan (YGM). According to the determination of changes of SDS−PAGE profiles, it was confirmed that free amino groups in the protein were covalently linked to the reducing ends of the polysaccharides by the controlled dry-heating at 60 °C under 65% relative humidity without any chemical reagent (data not shown). Effects of Maillard-type glycosylation with YGM on the allergic immune response of Fag e 1 were investigated by ELISA using human sera mixture collected from 6 buckwheat-allergic patients. As a result, it was demonstrated that the introduction of carbohydrate chains onto the molecular surface of the protein yielded more than 90% reduction of the *in vitro* allergenicity of Fag e 1. The allergenicity of the protein drastically decreased by the conjugation with the water-soluble polysaccharide, in that the reactivity of Fag e 1−YGM conjugate was 7.1 ± 0.5% (n = 5), while that of AG and XG was 8.2 ± 0.7% (n = 5) and 5.5 ± 0.5% (n = 5), respectively. The *in vitro* assay indicated the importance of polysaccharide moiety on the molecular surface of Fag e 1, where IgE antibody must be not easy to react with epitopes on the specific allergen due to the steric hindrance produced by carbohydrate chains. Thus, hypoallergenic Fag e 1 conjugates were successfully produced by the Maillard-type glycosylation. This neoglycoprotein might be used for developing a desensitization of buckwheat allergic patients.

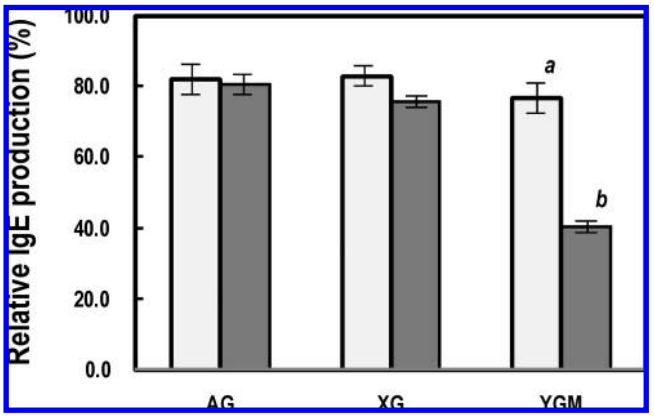

Figure 2. Desensitizing effects of Fag e 1−polysaccharide conjugates. Vertical bars represent standard errors of the mean (n = 3). Means labeled with a different letter within a rootstock are significantly different ($p < 0.05$). □, Fag e 1−polysaccharide mixture; ■, Fag e 1−polysaccharide conjugate.

3.2. Desensitizing Effects of Fag e 1−Polysaccharide Conjugates. Since hypoallergenic agents were successfully prepared, desensitizing effects of Fag e 1−polysaccharide conjugates were assessed using BALB/c mice. Fag e 1 sensitized mice came to exhibit high concentrations of specific IgE antibody and anaphylaxis reactions including diarrhea with response to Fag e 1 stimulation. Resulting allergic mice prepared from BALB/c mice were fed at liberty with homemade feed containing 5 mg of protein/100 g of Fag e 1 or its polysaccharide conjugates. According to the feeding record, it was shown that the average of sample intake was 6 mg of protein/kg body-weight/day. As shown in **Figure 1**, specific IgE titer caused by Fag e 1 stimulation decreased with increasing feeding with Fag e 1−polysaccharide conjugates, compared to normal feeding. Among them, the results demonstrated that Fag e 1−YGM conjugate was the most effective. After 5 week feeding, the specific IgE titer in the allergic mice fed with Fag e 1−YGM conjugate fell to 40.2 ± 2.1% (n = 3) of the initial anti-Fag e 1−IgE, whereas that of Fag e 1−AG conjugate and Fag e 1−XG conjugate was 80.1 ± 3.1% (n = 3) and 75.8 ± 2.9% (n = 3), respectively. Since it is believed that some polysaccharides could exhibit somewhat immunomodulation actions, desensitization effects were investigated in the control Fag e 1−polysaccharide mixtures without dry-heating and purification processes. As a result, we found that all Fag e 1−polysaccharide mixtures were not effective for the reduction of the specific IgE titer in the allergic mice after 5-week feeding (**Figure 2**). It is noteworthy that conjugate formation with YGM only caused fruitful desensitization of the allergenic mice. Fag e 1−YGM conjugate caused a substantial reduction of IgE level in the allergic mice for 3-week feeding. In addition, it was observed that 5-week feeding with Fag e 1−YGM conjugate yielded a reduction of typical allergenic symptoms including scraping behavior and diarrhea. This phenomenon should be occurring due to oral immune tolerance through the small intestine (*9*). It is well-known that macrophage mannose receptor (MR) is an integral membrane protein expressed on the surface of tissue macrophages. After ligation of mannose-rich glycoconjugates or pathogens, the receptor mediates endocytosis and phagocytosis of the bound ligands by macrophages (*18*). MR is expressed on tissue macrophages and on subsets of vascular and lymphatic endothelial cells (*19*). Recently it was reported that macrophages accumulate in gastrointestinal intestine of mice during infection and these cells are known to express, which may act as a pattern recognition receptor (*20*). MR also facilitates the uptake of

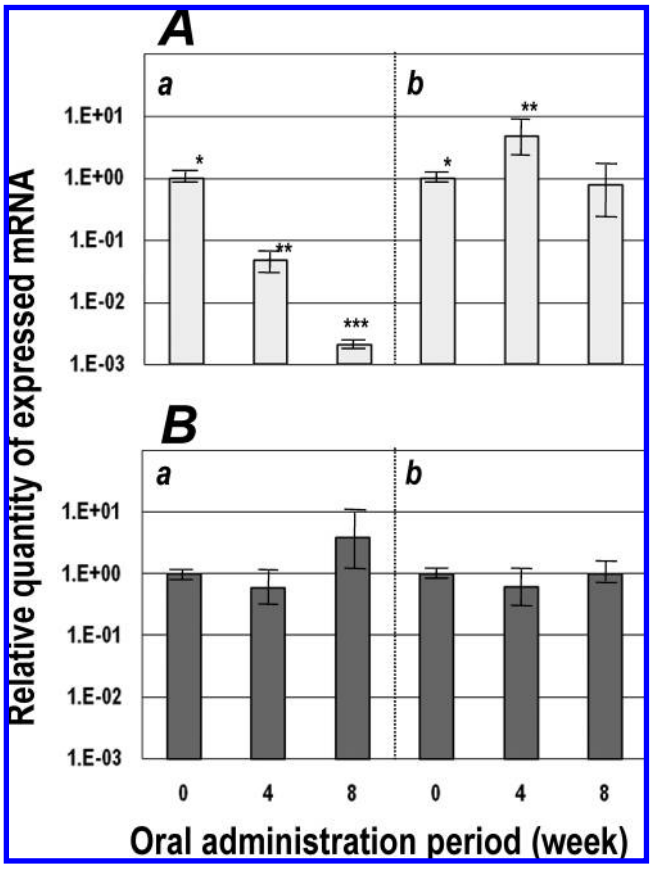

Figure 3. Changes of mRNA levels expressed in splenocytes of tested mice fed with Fag e 1−YGM conjugate (**a**) and normal feed (**b**). All data obtained were converted into relative values based on data from 0-week sample. Vertical bars represent standard errors of the mean ($n = 3$). Means labeled with a different symbol within individual feeding systems are significantly different ($p < 0.05$). **A**, IL-4; **B**, IFN-γ.

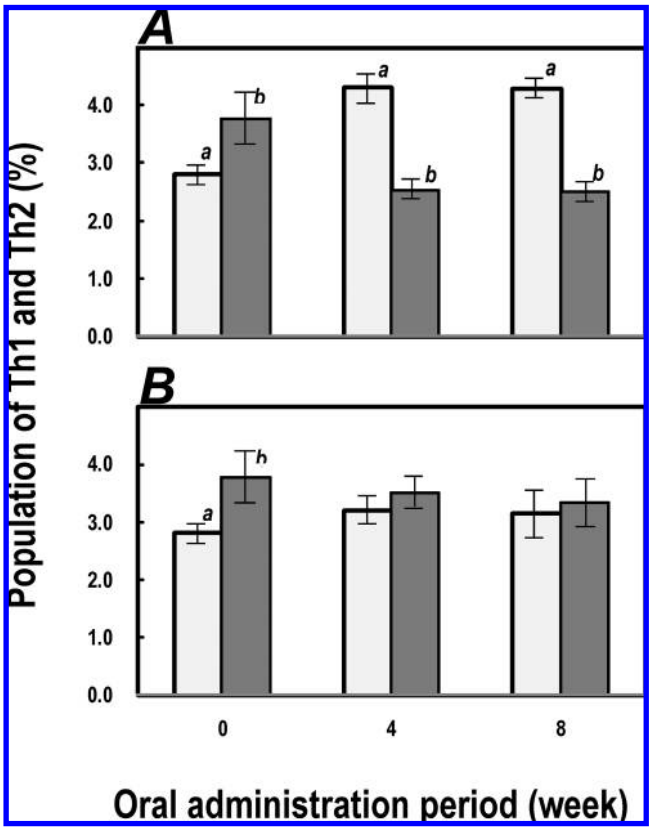

Figure 4. Changes of the population of Th1 and Th2 in splenocytes of tested mice fed with Fag e 1−YGM conjugate (**A**) and normal feed (**B**). Vertical bars represent standard errors of the mean ($n = 3$). Means labeled with a different letter within a rootstock are significantly different ($p < 0.05$). □, Th1; ■, Th2.

mannosylated antigens by dendritic cells in vitro for presentation to the acquired immune system (*21*). The coating of liposomes with oligomannose or yeast mannan drastically enhanced their ability to induce an ovalbumin-specific delayed-type footpad swelling response (*22*). Mannosylated niosomes coated with a modified polysaccharide *O*-palmitoyl mannan enhanced their affinity toward the antigen presenting cells of Peyer's patches (*23*). It seems reasonable to expect that the conjugation with mannose residues will cause the acceleration of the hypoallergenic Fag e 1 intake through lymphatic endothelial cells of small intestine. Therefore, Fag e 1−YGM conjugate was used for further experiments to assess if the conjugate can realize an oral immune-tolerance condition in the allergic mice.

3.3. Changes of Expressed Cytokines in Allergic Mice Fed with Fag e 1−YGM Conjugate. After feeding with Fag e 1−YGM conjugate, splenocytes in allergic mice were harvested and used for ELISA. Rereleased cytokines from the tested cells, interferon-γ (IFN-γ) which is a typical helper T cell type 1 (Th1) cytokine and interleukin-4 (IL-4) which is a typical helper T cell type 2 (Th2) cytokine were determined. As a result, it was demonstrated that feeding with Fag e 1−YGM conjugate for 5 weeks caused an outstanding reduction of IL-4 production compared with normal feeding. IL-4 value of conjugate feeding was 61.2 ± 2.3% compared to that of normal feeding. In addition, the change of IFN-γ production in allergic mice fed with the conjugate feeding also indicated a desensitization symptom, in which the IFN-γ value significantly ($p < 0.05$) increased to 121.5 ± 4.7% of normal feeding. Since Th2 is assumed to produce IgE in the body (*17*, *24*), these data were well-coincided with the depletion of the specific IgE in the allergic mice fed with Fag e 1−YGM conjugate. Therefore, the development of expressed cytokines in allergic mice fed with Fag e 1−YGM conjugate was investigated in detail by 8 week feeding. Splenocytes were collected from allergic mice administrated with Fag e 1−YGM conjugate at the interval of 0, 4, 8 weeks, and a qPCR was performed. As shown in **Figure 3A**, we demonstrated that expressed mRNA for IL-4 drastically decreased by the treatment with Fag e 1−YGM conjugate for 8 weeks. A significant ($p < 0.05$) increment was observed in the expression level of IFN-γ in the allergic mice treated with Fag e 1−YGM conjugate for 8 weeks, whereas there was no change for 4 week feeding (**Figure 3B**). Gene expression was thoroughly correlated with phenotypic changes as described above.

3.4. T Cell Differentiation in Desensitized Mice. Changes of populations of Th1 and Th2 in helper T cell in the allergic mice fed with Fag e 1−YGM conjugate were investigated based on flow-cytometric analysis. The results revealed that T cell differentiation toward Th1 and Th2 took a turn for the better balance in the desensitized mice with conjugate feeding, as shown in **Figure 4**. Fag e 1−YGM conjugate treatment caused marked differentiations for increase of Th1 and decrease of Th2. Populations of Th1 and Th2 in the allergic mice orally administered with Fag e 1−YGM conjugate for 8 weeks were 4.02 ± 0.38% and 2.72 ± 0.21%, respectively, while those at the beginning of the treatment (0-week feeding) were 2.91 ± 0.24% and 3.75 ± 0.51%, respectively. On the other hand, populations of Th1 and Th2 in the control system with normal feeding were 3.08 ± 0.43% and 3.33 ± 0.44%, respectively. Because cell-mediated immune response and humoral immune

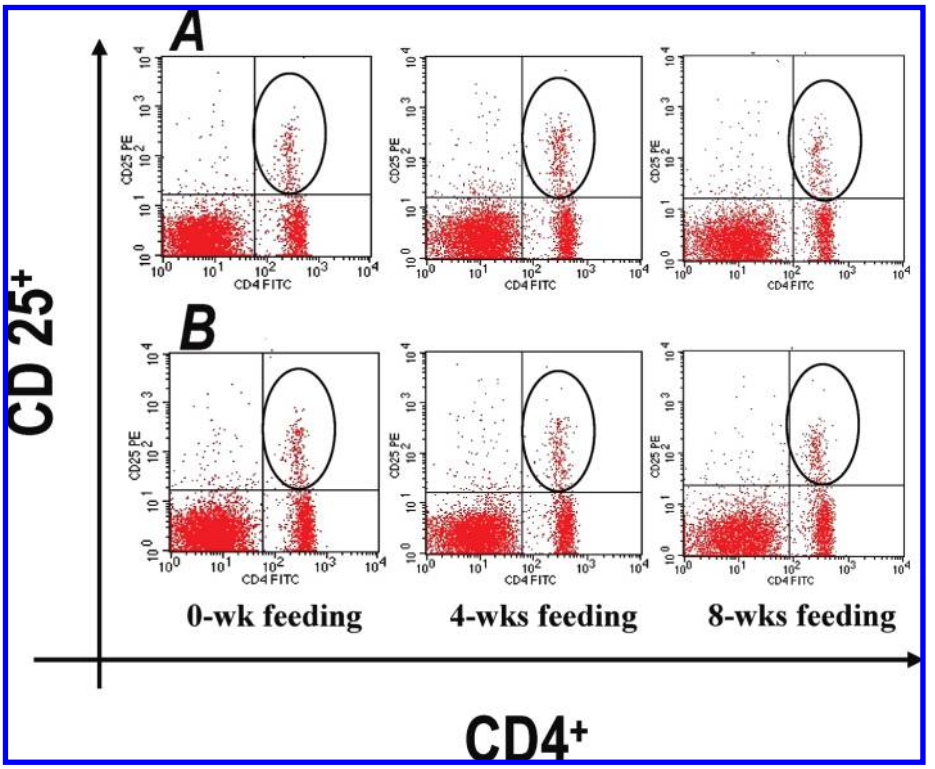

Figure 5. Changes of Treg in splenocytes of tested mice fed with Fag e 1−YGM conjugate (**A**) and normal feed (**B**). The circles indicate populations of Treg subset.

response including allergy are closely correlated with Th1 and Th2, respectively, (*25*−*27*), these results indicated that allergic constitution of allergic mice was improved through continuously taking the Fag e 1−YGM conjugate.

Differentiation level of regulatory T cell (Treg) was also evaluated based on the flow-cytometric analysis in the Fag e 1−YGM conjugate feeding system. Treg is assumed as a specialized subpopulation of T cells that act to regulate both Th1 and Th2 immune responses to antigens. In addition, a recent report has demonstrated that antigen-specific Treg cells play a role in the development of clinical tolerance to food antigens (*28*). In this paper, $CD4^+CD25^+$ T cells were counted as Treg (*29*). As shown in **Figure 5**, the existence of Treg increased 1.3 times compared with that at the beginning of the treatment. After Fag e 1−YGM conjugate feeding for 8 weeks, the population of Treg become 18.7 ± 1.3% from 14.5 ± 0.8%, whereas that of normal feeding was 14.7 ± 1.1%. This result suggests that augmented Treg population might induce the development of immune tolerance in Fag e 1 allergen. In this experiment, Treg appears to not only suppress Th2 differentiation but also affect Th1 immune response because the expression level of IFN-γ was not so drastic increase during 8 week administration of Fag e 1−YGM conjugate (**Figure 3B-a**). Additionally, in this experiment, immunotherapeutic effects of Fag e 1 or YGM itself using Fag e 1−YGM mixture were also investigated. As a result, the population differentiated to Treg was 15.1 ± 0.8%, and subsequently, it was confirmed that Fag e 1−YGM mixture was not effective for the improvement of immune responses. While it has been considered that Treg is associated with Th1-inducible autoimmune diseases such as rheumatoid arthritis or multiple sclerosis, and has little relation to allergenic syndromes (*30*), the T cell subset may suppress the onset of allergic disease by downregulating other types of immune cells besides Th1 and Th2 cells (*31*). The identification of novel helper T cell subsets, i.e., IL-17-producing Th cells (Th17) and regulatory T cells (Treg), provided new insight into our understanding of the molecular mechanisms involved in the development of infectious and autoimmune diseases as well as immune responses, and thus led to revision of the classic Th1/Th2 paradigm (*32*). Recent advances in the characterization of this cell population have firmly established their existence and their critical role in regulating the immune response. A role of Th1, Th2, Th17, and Treg in allergic diseases by using the balancing square model has been proposed by Orihara et al. (*31*, *33*). Our observation of the behavior of Treg in the splenocytes indicated that increase in the Treg population may be correlated with the improvement of allergenic symptoms. The excess state with both Th17 and Th2 is assumed as the worst situation for allergenic response. In addition, it is reported that Treg cells may be upregulated in the excess condition for Th2 in asymptomatic atopic individuals, where mast cells are therefore not activated, despite the presence of high levels of IgE antibodies and constant exposure to common allergens (*33*). Since our results showed that treatment with Fag e 1−YGM conjugate emerged both Th1 and Treg excess condition, it was predicted that the total immune system in the allergy mice was restored by the oral administration, indicating the possibility of immunotherapeutic utilization of the conjugate. Thus, it can be concluded that the Fag e 1−YGM conjugate having an immunoregulatory/modulatory or immunosuppressive (tolerizing) response might be useful for curing allergenic symptoms caused by buckwheat. We suggested that Fag e 1−YGM conjugate prepared through Maillard-type linkage can be available for a food-grade immunomodifier for patients suffering from buckwheat allergy.

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