Effect of Cholecalciferol in Food Allergy Mouse Model Is Associated with Decrease of CD69⁺ CD4⁺ T Cells

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Summary Food allergy prevalence is increasing all over the world. Recent epidemiologic studies have shown the link between vitamin D₃ insufficiency and food allergy occurrence. In this study, we investigated the effect of supplementation with cholecalciferol, a widely used form of vitamin D₃, on food allergy using an experimental mouse model. In wild-type BALB/c mice which were sensitized and challenged with an experimental allergen, ovalbumin, a clinical symptom of food allergy, diarrhea, was significantly induced with the elevation of immunoglobulin E level and the increases of T helper 2 cytokine productions, such as interleukin-4, -5, and -13 ($p < 0.05$), whereas no change in T helper 1 cytokine production, such as interferon- γ , was observed. It was also found that cell population of CD69⁺ CD4⁺ T cells was increased slightly in spleen and significantly in the mesenteric lymphnode with the diarrheal symptom ($p < 0.05$). Treatment of cholecalciferol reduced the allergic diarrhea ($p < 0.05$) with the decreasing tendency of CD69⁺ CD4⁺ T cells, suggesting that the cell population might be associated with the attenuating effect of cholecalciferol on diarrhea occurrence, although immunoglobulin E levels and cytokine productions were not significantly altered by the treatment of cholecalciferol. When given the mice anti-CD69 mAb treatment, significant improvement of allergic diarrhea symptom was observed ($p < 0.05$), accompanying the decrease of CD69⁺ CD4⁺ T cells which suggested the contribution of these cells to the diarrhea symptom. Taken together, we suggest that administration of cholecalciferol might be useful to suppress symptomatic food allergy in association with the decrease of CD69⁺ CD4⁺ T cells.

Key Words food allergy, ovalbumin, vitamin D₃, diarrhea, CD69

The prevalence of allergic diseases including asthma, atopic dermatitis and food allergy has been increasing all over the world. Food allergy incidence was reported to exist up to 8% of children and 4% of adults to one or more food antigens (1, 2), and children with food allergies are found to be 2 to 4 times more likely to suffer from other atopic diseases (1, 2). Current standard treatment of food allergy consists of antigen avoidance as preventive care and emergency treatments as a reaction-based treatment. Oral immunotherapy research offers a promising treatment, however it has had limited success considering safety and long-term efficacy (3).

Vitamin D₃ is known to be involved not only in mineral and skeletal homeostasis but also in immune-system regulation as well as in maintaining mucosal barrier (4). Recent epidemiologic studies have shown that vitamin D₃ insufficiency is a risk factor for food allergy (1). There is suggestive prevalence in studies showing a link between vitamin D₃, sensitization to food allergen

and food allergy (5–7). In addition, there are several clinical trials evaluating vitamin D₃ supplementation for allergic diseases including food allergy (8, 9). However the effect of vitamin D₃ on food allergy has not been fully elucidated. In the current study, we investigated whether cholecalciferol, a widely used supplement form of vitamin D₃ (10), has a promising effect on the treatment of food allergy in a mouse model. The results in the present study may provide the possibility of supplementation to the management of a symptom of food allergy.

MATERIALS AND METHODS

Animals. Five-weeks old female wild-type BALB/c mice were purchased from Charles River Company (Hino Branch, Tokyo, Japan). Mice were housed in the animal facility at the Department of Microbiology and Immunology, Tokyo Women's Medical University (TWMU). During the entire period of experiments, mice were ad libitum given water and pellet diet (LABO MR stock, Nosan Corporation, Yokohama, Japan) which the diet formula was shown in Supplemental Online Material, Table S1. The study was approved by the Ethical Review Committee of Animal Experiments, TWMU, in accordance with the Guide for the Care and Use of the Laboratory Animals (Approval number: AE18-059).

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Abbreviations: Alum, aluminum hydroxide; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IgE, immunoglobulin E; IgG, immunoglobulin G; IL, interleukin; mAb, monoclonal antibody; MLN, mesenteric lymph node; OVA, ovalbumin; Th, T helper.

Fig. 1. Effect of cholecalciferol administration in food allergy model. (A) Protocol of OVA sensitization, OVA challenge and cholecalciferol administration to mice. (B) Diarrhea occurrence during the 7 d of challenge was monitored in mice which were not sensitized but challenged with OVA (control group, $n=5$), sensitized and challenged with OVA (OVA group, $n=5$), or sensitized and challenged with OVA plus treatment with cholecalciferol (OVA + cholecalciferol group, $n=5$). (C) Total IgE levels in sera 24 h after the final OVA challenge were measured by ELISA. The data for each group were expressed as means $\pm$ SE. * $p<0.05$, OVA-group compared with control group and OVA + cholecalciferol group compared with control group (Mann-Whitney U test).

Food allergy mouse model. Allergen sensitization to mice was performed by intraperitoneal injection of 50 μ g of ovalbumin (OVA) (Sigma-Aldrich, Burlington, MA, USA) with 2 mg of aluminum hydroxide (Alum) in 200 μ L of PBS, on days 0, 7 and 21. Allergen challenge was performed by oral gavage inoculation of 50 mg of OVA in 200 μ L of PBS administered to mice for seven consecutive days from day 28 to day 34 ($n=5$). Control mice ($n=5$) were those which were not sensitized with OVA and Alum. The food allergy reaction was diagnosed by the clinical sign of diarrhea which was a typical symptom of an experimental food allergy model. The diarrhea was assessed by visually monitoring feces of mice within 60 min after each OVA challenge. Mice showing profuse liquid form feces were recorded as diarrhea-positive as shown in Supplemental Online Mate-

rial, Fig. S1. The number of diarrhea positive mice was counted after each OVA challenge, and the percentage of diarrhea occurrence was calculated by dividing number of diarrhea-positive mice by number of mice per group. Mice were sacrificed on day 35 by intraperitoneal injection of 75 mg/kg pentobarbital with 10 mg/kg xylazine. Sera, spleens and mesenteric lymph nodes (MLNs) were collected for further analyses.

Cholecalciferol and anti-CD69 antibody treatments in food allergy model mice. Cholecalciferol treatment was undertaken by oral administration of 0.2 μ g cholecalciferol together with OVA challenge in 200 μ L of PBS ($n=5$). The effect of cholecalciferol was investigated by monitoring of diarrhea occurrence. Mice were sacrificed on day 35, and cytokine levels were measured in the culture supernatants of 4×10^5 cells derived from

Fig. 2. Effect of oral cholecalciferol administration on cytokine productions in spleen and MLN. Experiments were performed as in Fig. 1A. Twenty-four hours after final OVA challenge, spleen and MLN derived cells were collected from the mice which were not sensitized but challenged with OVA (control group, $n=5$), sensitized and challenged with OVA (OVA group, $n=5$), or sensitized and challenged with OVA plus treatment with cholecalciferol (OVA+cholecalciferol group, $n=5$), and stimulated with OVA in vitro. Cytokine levels in the culture supernatants were measured by ELISAs. The data for each group were expressed as means $\pm$ SE. * $p < 0.05$ (Mann-Whitney U test).

spleens and MLNs in 200 μ L of complete-RPMI medium per well incubated with re-stimulation of OVA (100 μ g/mL) at 37°C in 5% CO₂ for 5 d.

In another experiment, 50 μ g of anti-CD69 monoclonal antibody (mAb, clone H1.2F3, BioLegend, San Diego, CA, USA) was administered by intraperitoneal injection in 200 μ L of PBS on day 27 as the first treatment, and at 3–4 h prior to OVA challenge on day 28 as the second treatment ($n=5$). Fifty micrograms of hamster immunoglobulin G (IgG) (BioLegend) was used as control IgG corresponding to the IgG of anti-CD69 mAb ($n=5$). The effect of anti-CD69 antibody was investigated by monitoring of diarrhea occurrence and mea-

surements of cytokine levels in the culture supernatants of cells derived from spleens and MLNs, under the same cell culture condition as the cholecalciferol treatment experiment.

Enzyme-linked immunosorbent assay (ELISA). ELISA (eBioscience, San Diego, CA, USA) was used to measure the levels of cytokines, including T helper 2 (Th2) cytokine such as interleukin (IL)-4, IL-5, IL-13, and T helper 1 (Th1) cytokine, such as interferon (IFN)- γ , in cell culture supernatants of spleen and MLN cells. Total immunoglobulin E (IgE) levels in sera collected 24 h after the final OVA challenge were measured by ELISA (eBioscience).

Fig. 3. Effect of cholecalciferol administration on CD4⁺ T cell population in spleen and MLN. Experiments were performed as in Fig. 1A. Twenty-four hours after final OVA challenge, spleen and MLN derived cells were collected from the mice which were not sensitized but challenged with OVA (control group, $n=5$), sensitized and challenged with OVA (OVA group, $n=5$), or sensitized and challenged with OVA plus treatment with cholecalciferol (OVA + cholecalciferol group, $n=5$), and stained with flow cytometry antibodies. Flow cytometry were carried out using FACSCalibur and the percentages of cell populations were calculated based on the data. The data for each group were expressed as means $\pm$ SE. * $p < 0.05$ (Mann-Whitney U test).

Flow cytometry. Cell population analysis was performed by flow cytometry using FACSCalibur (BD Biosciences, San Diego, USA). PE-conjugated anti-CD69, FoxP3, GATA-3 and T-bet mAbs, and APC- or FITC-conjugated anti-CD4 mAbs (BD Biosciences) were used. FoxP3, GATA-3 and T-bet staining were performed using staining kits (Thermo Fisher Scientific Co., Waltham, MA, USA). Flow cytometry data were analyzed by using the FlowJo software (FlowJo LLC, Ashland, OR, USA).

Statistical analysis. Statistical analyses were performed using Mann-Whitney U test. p value < 0.05 was considered as statistically significant difference.

RESULTS

Effect of cholecalciferol administration on clinical sign of OVA-induced food allergy

The object of the study was to assess the effect of cholecalciferol on food allergy using an experimental model. In the current study, mice were non-sensitized but challenged with OVA (control group), sensitized and challenged with OVA (OVA group), or sensitized and challenged with OVA plus cholecalciferol treatment (OVA + cholecalciferol group) as shown in Fig. 1A. When given the challenges with OVA to mice for 7 d, OVA

Fig. 4. Effect of anti-CD69 mAb treatment in food allergy model. (A) Protocol of OVA sensitization, OVA challenge and control IgG or anti-CD69 mAb treatment to mice. (B) Diarrhea occurrence during the 7 d of OVA challenge was monitored in mice which were challenged with OVA (OVA group, $n=5$), challenged with OVA plus treatment with control IgG (OVA+control IgG group, $n=5$) or anti-CD69 mAb (OVA+anti-CD69 mAb group, $n=5$). (C) Total IgE levels in sera 24 h after the final OVA challenge were measured by ELISA. The data for each group were expressed as means $\pm$ SE. * $p<0.05$, OVA+anti-CD69 mAb group compared with OVA+control-IgG group, and OVA+anti-CD69 mAb group compared with OVA group (Mann-Whitney U test).

group developed a food allergic reaction, diarrhea, from days 3 to 6. Treatment with cholecalciferol significantly decreased the percentage of diarrhea occurrence during the OVA challenges ($p<0.05$, diarrhea occurrence percentage of OVA group compared with OVA+cholecalciferol group, on days 3, 4, 5 and 6, respectively, Fig. 1B). Although diarrhea occurrence of 33% was observed on day 5 in OVA+cholecalciferol group, the percentage of diarrhea occurrence was significantly less than OVA group. Control group did not show signs of diarrhea during the entire 7 d of OVA challenge (Fig. 1B).

Effect of cholecalciferol administration on IgE production

OVA-sensitized and -challenged mice increased IgE level in sera compared to those of the control mice ($p<0.05$, OVA group compared with the control group, Fig. 1C). It was seemed that treatment of cholecalciferol

slightly decreased total IgE levels to approximately 82% of that of OVA group, although the difference between the two groups was not statistically significant (Fig. 1C). *Effect of cholecalciferol administration on cytokine productions in OVA-induced food allergy*

Effects of cholecalciferol on cytokine productions were analyzed in the OVA-induced food allergy model. OVA group showed the increased cytokine productions of IL-4, IL-5, IL-13 in spleen and MLN, compared to those of control group ($p<0.05$, control group compared with OVA group, Fig. 2 A–F), whereas production of IFN- γ was not changed (Fig. 2 G, H). Although IL-4, IL-5 and IL-13 productions in spleen were slightly lower in OVA+cholecalciferol group approximately reduced to 80, 73 and 74%, respectively, compared to those of OVA group, the effects of cholecalciferol treatment on

Fig. 5. Effect of anti-CD69 mAb treatment on cytokine productions in spleen and MLN. Experiments were performed as in Fig. 4A. Twenty-four hours after final OVA challenge, spleen or MLN derived cells were collected from the mice which were sensitized and challenged with OVA (OVA group, $n=5$), sensitized and challenged with OVA plus treatment with control IgG (OVA + control IgG group, $n=5$) or anti-CD69 mAb (OVA + anti-CD69 mAb group, $n=5$), and stimulated with OVA in vitro. Cytokine levels in the culture supernatants were measured by ELISAs. The data for each group were expressed as means $\pm$ SE. * $p < 0.05$ (Mann-Whitney U test).

the cytokine productions were not statistically significant. In addition, cytokine productions in MLN were not altered by the treatment of cholecalciferol.

Effect of cholecalciferol administration on CD4⁺ T cell population in OVA-induced food allergy

The effect of cholecalciferol on CD4⁺ T cell population was investigated in the food allergy model. OVA group showed increasing tendency of CD69⁺ CD4⁺ T cells in spleen ($p=0.07$, control group compared with OVA group, Fig. 3A) and significantly increased the cell population in MLN ($p < 0.05$, control group compared with OVA group, Fig. 3B). OVA + cholecalciferol group showed decreasing tendency of CD69⁺ CD4⁺ T cells in spleen ($p=0.34$, OVA group compared with OVA + cho-

lecalciferol group) and significantly decreased the cell population in MLN ($p < 0.05$, OVA group compared with OVA + cholecalciferol group). Among three groups of control, OVA, and OVA + cholecalciferol, there were not significant differences on cell populations of T-bet⁺, GATA-3⁺, or FoxP3⁺ CD4⁺ T cells in the spleen and MLN (Fig. 3 C–H).

Effect of anti-CD69 mAb treatment on clinical sign of OVA-induced food allergy

Since it was suggested that the administration of cholecalciferol was capable of decreasing the population of CD69⁺ CD4⁺ T cells with the reduction of food allergic reaction such as diarrhea, the cell population seemed to be involved in the regulation of diarrhea occurrence.

Fig. 6. Effect of anti-CD69 mAb treatment on CD4⁺ T cell population in spleen and MLN. Experiments were performed as in Fig. 4A. Twenty-four h after final OVA challenge, spleen and MLN derived cells were collected from the mice which were sensitized and challenged with OVA (OVA group, $n=5$), sensitized and challenged with OVA plus treatment with control IgG (OVA+control IgG group, $n=5$) or anti-CD69 mAb (OVA+anti-CD69 mAb group, $n=5$) and stained with flow cytometry antibodies. Flow cytometry were carried out using FACSCalibur and the percentages of cell populations were calculated based on the data. The data for each group were expressed as means $\pm$ SE. * $p<0.05$ (Mann-Whitney U test).

Therefore, to confirm whether the presence of CD69⁺ CD4⁺ T cells affects on the allergic diarrhea, anti-CD69 mAb was administered to the food allergy model for the assessment of diarrhea occurrence as shown in Fig. 4A. Mice sensitized and challenged with OVA (OVA group) started to develop diarrhea from day 4, while mice sensitized and challenged with OVA plus control IgG treatment (OVA+control IgG group) and mice sensitized and challenged with OVA plus anti-CD69 mAb treatment (OVA+anti-CD69 mAb group) had no diarrhea. On day 6, OVA group reached the highest diarrhea occurrence of 70%, while that of OVA+control IgG group

and OVA+anti-CD69 mAb group showed only of an average of 38% and 13%, respectively. OVA+anti-CD69 mAb group had significantly lower levels of diarrhea than the other groups with the delay of start of diarrhea ($p<0.05$, OVA+control IgG group compared with OVA+anti-CD69 mAb group, Fig. 4B).

Effect of anti-CD69 mAb treatment on IgE production

Treatment with control IgG or anti-CD69 mAb slightly but significantly decreased IgE level in the sera compared to OVA group. However the difference of IgE level between OVA+control IgG group and OVA+anti-CD69 mAb group was not statistically significant

(Fig. 4C).

Effect of anti-CD69 mAb treatment on cytokine productions in OVA-induced food allergy

Effects of anti-CD69 mAb treatment on cytokine productions were analyzed in the OVA-induced food allergy model. No significant effects of anti-CD69 mAb treatment on IL-4, IL-5, and IL-13 production were observed in spleen or MLN (Fig. 5A–F). Although only control IgG treatment showed increased production of IFN- γ in spleen but not in MLN ($p < 0.05$, OVA group compared with OVA+control IgG group, Fig. 5G), totally anti-CD69 mAb treatment did not induce any modifications on cytokine producibilities in spleen and MLN.

Effect of anti-CD69 mAb on CD4⁺ T cell population in OVA-induced food allergy

The effect of anti-CD69 mAb treatment on CD4⁺ T cell population was investigated in the food allergy model. OVA+anti-CD69 mAb group significantly decreased the CD69⁺ CD4⁺ T cells in spleen and MLN ($p < 0.05$, OVA group compared with OVA+anti-CD69 mAb group, and OVA+control IgG group compared with OVA+anti-CD69 mAb group, Fig. 6A, B).

However, there were not significant differences on cell populations of T-bet⁺, GATA-3⁺, or FoxP3⁺ CD4⁺ T cells in the spleen and MLN among three groups of OVA, OVA+control IgG, and OVA+anti-CD69 mAb (Fig. 6C–H).

DISCUSSION

Our experimental mouse model provided the opportunity to study the effect of cholecalciferol in food allergy. In a previous mouse model study, vitamin D₃ was indicated to inhibit asthma-associated inflammatory responses (11). In the present study, we demonstrated significant inhibitory effect of cholecalciferol on a symptom of food allergy when given to mice during allergen challenges.

In our food allergy model, OVA-sensitized and challenged mice developed a typical food allergic reaction, diarrhea, with the increase of CD4⁺ T cells expressing CD69, an early activation marker of inflammation (12), slightly in spleen and significantly in MLN. Treatment with cholecalciferol improved the diarrhea with decrease of the CD69⁺ CD4⁺ T cell population especially seen in MLN. Therefore, it seemed that the CD69⁺ CD4⁺ T cell population could be involved in the occurrence of diarrhea. It was also found that mice which decreased the CD69⁺ CD4⁺ T cell population by the treatment with anti-CD69 mAb improved the diarrhea, suggesting the decrease of CD69⁺ CD4⁺ T cells contributed to the down-regulation of food allergic reaction. From these results, thereby, the decreasing effect of cholecalciferol on the CD69⁺ CD4⁺ T cell population was presumed to elicit the reduction of diarrhea occurrence. However, since it has remained to be clarified how cholecalciferol directly or indirectly was linked to down-regulation of the cell population and diarrhea occurrence in our model, further investigations would be necessary to know the detailed mechanism.

Despite the suppression of allergic diarrhea in chole-

calciferol- or anti-CD69 mAb-treated mice, both treatments did not significantly lower serum IgE levels, and had no significant effects on allergen-induced cytokine productions including IL-4, IL-5 and IL-13. We speculate these results might be due to non-IgE mediated association in food allergies (13).

CD4⁺ FoxP3⁺ Treg cell populations both in spleen and MLN were not significantly changed by the cholecalciferol treatment, indicating that cholecalciferol was not effective in creating Treg-induced tolerance for reducing diarrhea in our mouse model. This result was also shown elsewhere that vitamin D₃ did not affect Treg cells (14). In anti-CD69 mAb treated mice, we did not observe any changes in FoxP3⁺ CD4⁺ T cell population. Based on our data, we suggest that neither cholecalciferol treatment nor anti-CD69 mAb treatment induced Treg-mediated immune tolerance to down-regulate the allergic reaction. Moreover, cholecalciferol treatment to OVA-challenged mice did not affect type 1 helper T cells (Th1) specific transcription factor T-bet, and type 2 helper T cells (Th2) specific transcription factor GATA-3. Additionally, anti-CD69 mAb treatment also did not change Th1/Th2 specific transcription factors. These results mean that Th1/Th2 specific transcriptions might not be crucial factors in the reduction of diarrhea in our food allergy model.

Based on the review by Kimura et al., there has been no anti-CD69 mAb treatment model reported on food allergy to date (15). We used two-day treatment of anti-CD69 mAb with 50 μ g/mouse which was lower dose than other asthma mouse models (16), and the dose was similar to the asthma model established by Wang et al. (17). As for our experiment, we compared the effects of control IgG and anti-CD69 mAb in the assessment of diarrhea occurrence. Unexpectedly, the slight reduction of allergic diarrhea was observed in the mice treated with control IgG corresponding to the IgG of anti-CD69 mAb. We speculate it might be due to the ratio change of IgE and IgG during the allergen challenge, as it has been reported that reduction of allergic reaction was mostly induced with the increase of IgG (18). In our experiment, such the unexpected factor of IgG antibodies seemed to be appeared. However, because the influence of the IgG antibodies was added equally to both groups of control IgG and anti-CD69 mAb treatment, it could be suggested that the decrease of CD69⁺ CD4⁺ T cells resulted in the diarrhea reduction with considering the influence of IgG injection.

In terms of immune modification effects of cholecalciferol and anti-CD69 mAb, we did not observe any significant reductions of IgE levels in sera and cytokine producibilities in spleen and MLN when the mice were treated with cholecalciferol or anti-CD69 mAb, whereas the diarrhea was improved in both experiments. Future studies will need to investigate the mechanism how the decrease of CD69⁺ CD4⁺ T cell population resulted in the reduction of diarrhea occurrence.

On the other hand, it has been reported that 1 α ,25-dihydroxyvitamin D₃, which was activated form of cholecalciferol, had protective effects against intestinal

tissue barrier disruption and high permeability in the intestinal mucosa induced by exogenous stimulants (19–21). Therefore, by such protective effect, vitamin D₃ seems likely to maintain integrity of mucosal barrier, leading to attenuation of allergen exposure to intestinal tissue and reduction of allergen permeability in intestinal mucosa. As a consequence, the migration of CD4⁺ CD69⁺ T cells into the local inflamed site might be down-regulated in the presence of cholecalciferol, implying that the regulation is presumed to be one of the mechanisms underlying the inhibitory effect of cholecalciferol on food allergic reaction.

We also suggest another possible inhibitory effect of cholecalciferol on the food allergy symptom through a different type of cell such as mast cells. Mast cells are well known to be required in allergen-induced diarrhea (22–24). As it has been reported that vitamin D₃ suppressed the mast cell activation (25, 26), we presume that cholecalciferol might also possibly control the allergic diarrhea through the suppressive effect on mast cells in addition to that on CD4⁺ T cells in our model. In addition, several studies reported that mast cells could express CD69 on the cell surface in some situations (27, 28). Actually, Gaudenzio et al. demonstrated that CD69 expression on the activated mast cells was induced in the presence of allergen with immune cytokines (28). In our study, therefore, anti-CD69 mAb treatment might affect the cell number or function of mast cells for reducing the diarrhea occurrence. The possible involvement of the mast cells in our food allergy model also has to be further investigated in future studies.

In current study, it was suggested that cholecalciferol treatment exhibited reduction in food allergy symptom of diarrhea with the decrease of CD69⁺ CD4⁺ T cells. Our data could provide a novel possibility of cholecalciferol administration for controlling a symptom of food allergy.

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Author contributions

E.Z performed the experiments, analyzed the data and wrote the manuscript. S.A. analyzed the data and wrote the manuscript. T.O. contributed in the interpretation of the results. N.Y. analyzed the data and wrote the manuscript. J.Y. interpreted the results, wrote the manuscript and supervised the entire study.

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Supporting Information

Supplemental online material is available on J-STAGE.

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