Vitamin A Deficiency Impairs Induction of Oral Tolerance in Mice

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Summary Oral tolerance is a phenomenon of induction of systemic unresponsiveness to antigens ingested by the oral route and loss of immune response. Studies have shown the importance of vitamin A in oral tolerance in vitro but not in an in vivo experimental model. Therefore, we carried out experiments to determine how vitamin A deficiency affects tolerance induction and the ability of mesenteric lymph node (MLN) CD11c⁺ cells to induce regulatory T cells (Tregs). Immunological tolerance was induced by oral ovalbumin (OVA) administration in vitamin A-sufficient mice. OVA-specific antibody and cytokine production were significantly reduced. On the other hand, in vitamin A-deficient mice, both OVA-specific antibody and cytokine production were not suppressed by oral OVA administration. Regarding induction of Tregs, the conversion rate of Foxp3⁺ cells from naïve CD4⁺ cell by CD11c⁺ cells was decreased in vitamin A-deficient mice. Our study indicates that vitamin A deficiency causes the breakdown of oral tolerance in vivo.

Key Words oral tolerance, vitamin A deficiency, regulatory T cells, CD11c⁺ cells, antibody

The ability of an orally administered antigen (Ag) to suppress subsequent immune responses in the gut and systemic immune system was described 100 y ago (1). Failure to induce tolerance to a food protein has been speculated to result in food allergy and celiac disease, which is the most prevalent food-induced pathology (2). Indeed, oral tolerance has been shown to suppress immunopathology in animal experimental models of autoimmune encephalitis (3), collagen-induced arthritis (4), type-I diabetes (5) and other diseases (6, 7). Thus, oral tolerance attenuates a broad range of immune responses and appears to play a central role in immune homeostasis.

Vitamin A is one of the fat-soluble vitamins and plays an important role in proliferation and differentiation of cells, visual effects and the immune system (8). It is well known that vitamin A deficiency causes various disorders such as night blindness, stunted growth, and malnutrition (9, 10). There have been many animal studies, cell culture experiments and epidemiological studies targeting a vitamin A-deficiency state to elucidate the effect of vitamin A on the immune system (11, 12). Vitamin A deficiency causes impairment of mucosal immunity and impairment of neutrophil, NK cell, helper T cell and B cell functions (8). In human studies, treatment with vitamin A has been shown to reduce mortality of infectious diseases including tuberculosis (13), measles (14), malaria (15) and HIV/AIDS (16). Thus, vitamin A is crucial for immune cell function and prevention of infectious diseases (8).

Dendritic cells (DCs) are antigen-presenting cells that are involved in the differentiation of T cells. Differentiation of naïve T cells depends on cytokines that are secreted by DCs. When DCs secrete inflammatory cytokines, interleukin (IL)-6 and IL-12, CD4⁺ naïve T cells are differentiated into type-1 helper T cells (Th1). On the other hand, anti-inflammatory cytokines promote differentiation into regulatory T cells (Tregs) (17). In addition to cytokines produced by DCs, a recent study has indicated that differentiation of CD4⁺ naïve T cells is dependent on the type of DC population. When the Ag is recognized by CD103⁺ DCs, naïve T cells preferentially differentiate into induced Tregs, which are involved in the suppression of excessive inflammatory response and development of autoimmune disease (18, 19). Tregs have also been implicated in oral tolerance. When DO11.10 mice that carry the ovalbumin (OVA)-specific T cell receptor were orally administered OVA, CD4⁺CD25⁺ T cells were expanded and suppressive ability of CD4⁺CD25⁺ cells was enhanced (20).

One of the recent topics in the oral tolerance mechanism is the involvement of vitamin A-metabolite retinoic acid (RA). Although TGF-β is a critical stimulus of the differentiation of Tregs in vitro, it was newly found that RA is also an inducer of Tregs (21). Since this finding,
it has been shown that a specialized DC population, CD103+ DCs, located in the intestine and mesenteric lymph nodes (MLNs) expresses high levels of retinaldehyde dehydrogenases and can metabolize vitamin A to RA. Development of induced Tregs by mucosal CD103+ DCs depends on both TGF-β and RA (21).

There have been many studies on differentiation of Tregs with RA in vitro, but there has been limited study on how vitamin A deficiency modulates oral tolerance induction in an in vivo model. Involvement of vitamin A in oral tolerance induction has been shown using retinal acyltransferase gene knockout mice (22). However, it has been reported oral tolerance was induced normally in VAD WT mice (23). In this study, to obtain evidence that vitamin A is essential for the oral tolerance mechanism, we orally administered Ag prior to Ag immunization in vitamin A-deficient (VAD) mice and compared the extent of tolerance induction to that in mice fed a vitamin A-sufficient (VAS) diet. We also focused on the ability of CD11c+ cells to induce CD4+CD25+Foxp3+ cells in VAD mice to determine the precise mechanism.

**MATERIALS AND METHODS**

*Mice and diets.* BALB/c mice (Japan SLC, Shizuoka, Japan) and D011.10 mice on a BALB/c background (The Jackson Laboratory, Bar Harbor, ME) were maintained under specific pathogen-free conditions with a 12-h light : dark cycle at 25 ± 2 °C and 55 ± 10% relative humidity. Mice were given free access to water and food throughout the experiment. All studies were performed in accordance with the ethical guidelines for animal experimentation by the Institution of Health Bioscience, The University of Tokushima, Japan, and were approved by the institution review board of the animal ethics committee.

The mice were maintained on either a control diet or vitamin A-free diet. The composition of the control diet was 20% casein, 44.5% gelatinized-starch (Oriental Yeast Co. Ltd., Chiba, Japan), 22% sucrose (Mitsui Sugar Co. Ltd., Osaka, Japan), 5% corn oil (Wako, Osaka, Japan), 2% cellulose, 5% mineral mixture, 1% vitamin mixture (Oriental Yeast Co. Ltd.), 0.3% dl-methionine and 0.2% cholin bitarate (Wako). Composition of the vitamin A-free diet was the same as that of the control diet except for the vitamin A content. Vitamin A-free vitamin mixture was purchased from Oriental Yeast Co. We defined mice fed the control diet and mice fed the vitamin A-free diet as VAS and VAD mice, respectively.

VAD mice were generated by long-term feeding of the vitamin A-free diet. Dams were given the vitamin A-free diet between gestation and lactation periods. Offspring began to have the vitamin A-free diet from weaning.

*Oral Ag administration and immunization.* Mice at 8 wk old were given 1 mg OVA or 1 mg BSA orally and daily administration was continued for 1 wk. After oral Ag administration, VAD mice were given the vitamin A sufficient diet for 4 wk and then immunization was performed. Since vitamin A deficiency is known to diminish systemic immune function (8), we avoided the effect of vitamin A deficiency on induction of cellular and humoral immune responses followed by OVA immunization.

Mice were immunized intraperitoneally with 500 μL of OVA solution containing 10 μg of OVA (Sigma Chemical Co., St. Louis, MO) absorbed in 2 mg of aluminium hydroxide gel adjuvant (HCl Biosector, Frederikssund, Denmark) at 5 and 7 wk after starting oral Ag administration.

*Measurement of retinol content in the liver.* Because it has been shown that VAD mice showed reduction of retinol contents in the serum and the liver (24). Retinol levels in the liver were determined by high-performance liquid chromatography (HPLC). The liver from mice at 8 wk old was homogenized in a chloroform/methanol solution (2 : 1, v/v). The chloroform/methanol solution was added to the homogenate up to 50 mL. Twelve percent volume of water was added to the homogenate and then left overnight. After removing the upper layer, methanol was added to the lower layer fraction up to 40 mL. The extracted solution was dried, and then 3% pyrogallol and 4 N sodium hydroxide were added and the solution was incubated at 70°C for 30 min. Extraction was performed three times with hexane. Extracts were dried and dissolved with methanol. Twenty microliters of the sample solution was injected into an HPLC system (LC-10A: Shimadzu Corporation, Kyoto, Japan) equipped with UV detection (325 nm). The separation of the compounds was carried out by isocratic reversed-phase condition used with a COSMOSIL 5C18-MS-II column (4.6 x 150 mm; Nacalai Tesque Inc., Kyoto, Japan) with methanol and water (92 : 8) as the mobile phase at 1.0 mL/min. We detected the peak area of standard retinol and generated a standard curve. Then retinol content of the liver was calculated from the peak area of the sample.

*Measurement of Ag-specific antibody (Ab).* OVA-specific IgG, IgG1, IgG2a and IgE were measured by ELISA. Blood was collected from mice and centrifuged at 4,000 rpm for 10 min at 4°C. The sera were stored at −40°C until used. OVA was dissolved with a coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6) at 10 μg/mL. Fifty microliters of OVA solution was added to each well and incubated overnight at 4°C. After blocking with PBS solution containing 1% BSA, the diluted sera were incubated for 2 h. Subsequently, each well was washed three times with PBS containing 0.05% Tween 20. Incubation was performed for 2 h after addition of alkaline phosphatase (AP)-conjugated anti-mouse IgG, IgG1 or IgG2a Ab. For IgE Ab measurement, biotin-labeled anti-mouse IgE monoclonal (m) Ab was added to each well and left for 1 h at room temperature (RT). After washing, 3,000-fold diluted AP-labeled streptavidin solution was added and incubated for 1 h at RT. p-Nitrophenyl phosphate was dissolved in 10% diethanolamine to prepare a 10% diethanolamine buffer, and the solution was added to each well. After color development, the reaction was stopped by addition of 3 N NaOH and the absorbance was measured at a wavelength of 405 nm.

*Cytokine production.* To prepare single-cell suspen-
sions, the spleen was squeezed with two slide glasses in RPMI-1640 medium (Sigma Chemical Co.) and filtered through mesh. To remove red blood cells, the cells were treated with 0.84% NH₄Cl (pH 7.5) at RT for 10 min. Then the cells were washed two times with RPMI-1640 medium and suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 nmol/L 2-mercaptoethanol, 100 µg/mL streptomycin, and 100 U/mL penicillin. Splenocytes (5×10⁶ cells/mL) were stimulated with 1 mg/mL OVA in a 24-well plate at 37°C under 5% CO₂ for 72 h. After culture, culture supernatants were collected and stored at −40°C until used. Concentrations of interferon (IFN)-γ (BioLegend, San Diego, CA), interleukin (IL)-4 (BioLegend), IL-6 (eBioscience, San Diego, CA), and IL-10 (eBioscience) in the supernatants were determined using a mouse cytokine ELISA kit according to the manufacturer’s instructions.

Differentiation of CD4⁺Foxp3⁺ cells by MLN CD11c⁺ cells. CD11c⁺ cells were purified from MLNs of VAD mice using CD11c microbeads and a MACS MS column (Miltenyi Biotec Inc., Auburn, CA) according to
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the manufacturer’s instructions. CD4⁺CD62L⁺ T cells were purified from DO11.10 transgenic mice using a CD4⁺CD62L⁺ T cell isolation kit (Miltenyi Biotec Inc.). CD4⁺CD62L⁺ T cells (2×10⁵ cells/well) from DO11.10 mouse MLNs were cultured in a 48-well plate with CD11c⁺ DCs (2.5×10⁵ cells/well) in the presence of 0.2 μg/mL OVA₃₂₃–₃₃₉ peptide for 5 d at 37°C/5% CO₂.

Flow cytometry. For DC population analysis, the cells were stained with biotin-conjugated anti-mouse CD103 (eBioscience) mAb for 30 min on ice in the dark. After staining, cells were washed with FACS buffer twice. Cells were stained with FITC-conjugated anti-mouse CD11c mAb (eBioscience), APC-conjugated anti-mouse CD8α mAb (BioLegend), PE-conjugated anti-mouse I-A mAb (eBioscience), and PerCP-labeled streptavidin (BD Pharmingen, San Diego, CA) for 30 min on ice in the dark. Cells were washed twice with FACS buffer and re-suspended in FACS buffer for flow cytometric analysis. Stained cells were analyzed with a Guava easyCyte and guava InCyte software (Merck-Millipore, Darmstadt, Germany).

For Tregs analysis, CD11c⁺ DCs and CD4⁺CD62L⁺ T cells were co-cultured with OVA, and the cells were collected and stained with an anti-Mouse/Rat Foxp3 staining set (eBioscience).

Data analysis. All data are shown as means±SD. For comparison between the VAS group and VAD group, statistical analysis was carried out using Student’s t test. Comparisons of multiple values were performed by one-way ANOVA and Scheffe’s post hoc test. Differences with p<0.05 were considered significant. All statistical analyses were performed using SPSS ver. 18.0J for windows (SPSS Inc., Tokyo, Japan).

RESULTS

Hepatic retinol is depressed in VAD mice

To evaluate the extent of the vitamin A-deficient state, we determined retinol content in the liver by HPLC. Content of retinol in the liver of VAS mice was 11.7±8 μg/g, while that of VAD mice was less than 2.7 μg/g, indicating that long-term feeding of a vitamin A-free diet caused a severe vitamin A-deficient state.

Vitamin A deficiency could not suppress Ab production induced by oral tolerance

Mice that had been treated with OVA orally showed a significantly lower level of Ab production than did mice that had been treated with BSA, indicating that oral Ag administration induced tolerance in VAS mice. In contrast to the control diet, a significant difference in OVA-specific IgG, IgG2a and IgE responses was not observed between BSA- and OVA-treated VAD mice (Fig. 1). In the case of OVA-specific IgG1, mice that had been treated with OVA orally produced more Ab than did mice that had been treated with BSA orally in VAD mice (Fig. 1B). Tolerance-induced suppression of Ag-specific cytokine production was not observed in VAD mice.

Administration of oral OVA prior to immunization significantly reduced OVA-specific IFN-γ, IL-4, IL-6 and IL-10 in VAS mice, indicating that oral Ag administration induced tolerance in mice fed the control diet. In contrast to the VAS mice, a significant difference in OVA-specific IFN-γ, IL-6 and IL-10 responses was not observed between oral BSA- and OVA-treated mice. In VAD mice, oral administration of OVA could not reduce cytokine production induced by OVA immunization except for OVA-specific IL-4 (Fig. 2). In the case of OVA-specific IL-4 production, mice treated with OVA produced less IL-4 than did mice treated with BSA in VAD mice (Fig. 2B).

Expression of MHC class II and CD103 molecules in the MLN CD11c⁺ cell population

We investigated DC phenotype because DCs have been shown to play an important role in induction of Tregs (25). Percentages of MHC class II⁺ and CD103⁺ cells in the CD11c⁺CD8⁺ population were higher than those in the CD11c⁺CD8⁻ population. VAD mouse CD11c⁺CD8⁺ DCs showed a significantly lower percentage of MHC class II-positive cells than did VAS mice. Increment in
the percentage of CD103+ cells was observed in VAD CD11c+CD8- DCs (Table 1).

**Vitamin A deficiency affects induction of Foxp3+ cells by MLN CD11c+ cells**

We determined the ability of MLN DCs to induce Tregs. D011.10 mouse CD4+CD62L+ cells were stimulated with OVA in the presence of MLN CD11c+ cells from VAS and VAD mice. The conversion rate to Foxp3+ cells in VAD mice was significantly reduced compared to that in VAS mice (Fig. 3).

**DISCUSSION**

Oral tolerance refers to suppression of systemic immune responses against oral Ag administration. Several mechanisms are known. A high dose of an oral Ag induces anergy and deletion (26, 27), whereas a low dose Ag induces active suppression (28, 29). Several laboratories revealed a critical role for RA in directing intestinal CD4+ T cell development (20, 21, 30). In those studies, it was shown that RA is a critical factor for Treg differentiation. A specialized DC subset can metabolize vitamin A to RA, which synergizes with the cytokine TGF-β to induce Foxp3+ Tregs from naïve T cells. Results of many in vitro studies support a critical role of the vitamin A-metabolite RA in differentiation of Tregs and contribution of vitamin A to induction of oral tolerance. However, the crucial role of vitamin A for oral tolerance is controversial. Our results support that vitamin A deficiency impairs Ag-induced tolerance in vivo.

As shown in Figs. 1 and 2, oral OVA administration induced tolerance against OVA Ag, which was demonstrated as suppression of Ag-specific Ab responses and cytokine production in VAS mice. In the case of VAD mice, a significant decrease in the levels of OVA-specific Abs was not observed, while the OVA-specific IgG1 level in OVA-treated mice was significantly increased compared to that in BSA-treated mice (Fig. 2B). After oral OVA treatment, vitamin A-deficient mice were given the VAS diet for 4 wk. Vitamin A and RA generally promote differentiation toward Th2 cells and the production of Abs (31–36). It is possible that supplementation of vitamin A in the period of vitamin A recovery causes a pronounced effect of vitamin A on Ab responses. However, this remains a matter for debate because IgG2a and IgG1 responses reflect physiological Th1 and Th2 parameters, respectively. In OVA-specific cytokine production, only Ag-specific IL-4 was decreased in VAD mice treated orally with OVA (Fig. 2B). Differentiation of IL-4-producing CD4+ T cells might be insensitive to vitamin A deficiency. Alternatively, dependency of vitamin A on tolerance induction might be different depending on cell type. However, these two mechanisms are speculation. Further study is needed to elucidate mechanisms.

Vitamin A deficiency reduced the expression of MHC class II molecules in CD11c+CD8+ cells, which are important molecules in the Ag-presenting pathway. Unexpectedly, the percentage of CD103+ cells both in CD11c+CD8- and CD11c+CD8+ cells was higher in VAD mice than in control mice (Table 1). In addition, a significant reduction in the number of CD11c+CD103+ cells in MLNs was not observed in VAD mice (data not shown). These results suggest that vitamin A deficiency does not change the number or percentage of CD11c+CD103+ cells. As shown in Fig. 3, induction of Foxp3 was significantly weak in VAD mice, suggesting that vitamin A contributes to the generation of Tregs by CD11c+ cells. Our results clearly showed that the function of DCs from VAD mice for induction of Tregs was impaired. It has been shown that DCs isolated from the gut of mice fed a retinoid-free diet have reduced levels of RA-producing enzymes (37, 38). Considering the results of these studies and our results, the number of CD103+ DCs does not decrease in mice fed a vitamin A-deficient diet, but induction of Treg function is attenuated.

A number of different mechanisms have been implicated in oral tolerance, including active regulation of Tregs as well as clonal deletion and clonal anergy of T cells. We could not evaluate the effect of vitamin A on clonal deletion or clonal anergy. Administration of single high doses of Ag favors clonal deletion or anergy, whereas multiple low doses of Ag were linked to T cell-mediated suppression (26–29). Because we used treatment of multiple low doses of Ag in this study, the contribution of vitamin A to clonal deletion and anergy induction might be limited.

Our data indicate the crucial role of vitamin A in oral tolerance induction in vivo.

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