Note

Suppression of Oral Tolerance by Lactococcus lactis in Mice

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Although oral ovalbumin (OVA) administration suppressed the antibody (Ab) response in OVA-immunized mice, Lactococcus lactis increased OVA-specific IgG2a in these mice. L. lactis increased the casein-specific IgG level in NC/Nga mice fed on a casein diet. The percentage of CD4+CD25+ cells was increased in DO11.10 mice orally given OVA, but this increase of CD4+CD25+ cells were suppressed in L. lactis-fed DO11.10 mice.

Key words: Lactococcus lactis; oral tolerance; ovalbumin; casein; NC/Nga mice

The accumulating evidence that some bacteria are beneficial to human health and metabolism has prompted increasing interest in foods containing live bacteria, and food manufacturers are now adding beneficial bacteria to a wide variety of foods and beverages. Probiotics are defined as live microbial food components that are beneficial for humans.1) The immunomodulatory effects of probiotics have been utilized for preventing allergic diseases. It has been shown that the administration of probiotic bacterial strains suppressed the T-helper (Th) 2 immune response and inhibited the development of allergic diseases.2–6) In the course of investigating the immunomodulatory effects of probiotic bacterial strains, we found a novel bacterial strain that suppressed the induction of oral tolerance.

Female BALB/c NC/Nga mice (Japan SLC, Shizuoka, Japan) and DO11.10 mice (The Jackson Lab., Bar Harbor, ME, USA) were maintained under specific pathogen-free conditions with a 12-h light:dark cycle at 25 ± 2°C and 55 ± 10% relative humidity. The mice were provided with food and water ad libitum. The composition of the diet was 20% casein, 44.7% α-starch (Oriental Yeast Co., Chiba, Japan), 22.3% sucrose (Mitsui Sugar Co., Osaka, Japan), 5% corn oil (Wako, Osaka, Japan), 2% cellulose, and a 5% mineral mixture and 1% vitamin mixture (Oriental Yeast Co., Tokyo, Japan). Heat-killed and lyophilized Lactococcus lactis subsp. lactis ME-426 (L. lactis) was added to the control diet at a dose 0.1% (w/w) to produce the experimental diet. All experimental procedures were approved by the Animal Research Committee of the University of Tokushima.

The mice were administered 5 mg of ovalbumin (OVA) by intragastric gavage for 5 consecutive days. Control mice were administered PBS instead of OVA. After orally administering an antigen (Ag), the mice were intraperitoneally immunized with 10 μg of OVA conjugated with 1 mg of aluminum hydroxide (HCl Biosector, Denmark) twice with a 2-week interval. Serum was collected 2 weeks after the final immunization. The serum Ag-specific antibody (Ab) levels were measured by ELISA with the standard method.

Mesenteric lymph node cells were stained with PE-conjugated anti-mouse CD4 mAb, FITC-conjugated anti-mouse CD25 mAb and APC-conjugated anti-Foxp3 mAb. The cells were analyzed with FACSscalibur and with CellQest software (Becton Dickinson, Mountain View, CA, USA) after gating out the dead cells by using forward and side light scattering. CD4+CD25+ cells (5 × 10^5 cells) were cultured in a 96-well plate with T cell-depleted and mitomycin-treated spleen cells (1 × 10^5 cells) as accessory cells, 0.5 μg/mL of CD3 mAb, and the indicated numbers of CD4+CD25+ cells for 72 h at 37 °C in 5% CO2. The cultures were pulsed with 1 μCi of ^3[H]TdR for the last 20 h of culture. Incorporated ^3[H]TdR was determined by a liquid scintillation counter.

L. lactis used in this study increased the Ag-specific IFN-γ production and decreased the Ag-specific IL-4 and IL-10 production in OVA-immunized BALB/c mouse splenocytes (data not shown). These results are consistent with those of many studies showing that probiotic bacteria possess Th1-inducing ability. The effect of L. lactis on the induction of oral tolerance was first examined. The administration of OVA for five consecutive days significantly suppressed the OVA-specific IgG, IgG1, IgG2a and IgE production. Oral administration of L. lactis increased the OVA-specific IgG2a level but not the OVA-specific IgG1 and IgE levels compared to those in mice orally administered with OVA (Fig. 1). These results indicate that L. lactis possessed a unique property and could suppress the

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Abbreviations: Ab, antibody; Ag, antigen; L. lactis, Lactococcus lactis subsp. lactis ME-426; OVA, ovalbumin; Th, T-helper
induction of oral tolerance by some Ab classes in this model. The suppressive effect of \textit{L. lactis} on the induction of oral tolerance was then examined by using another model. It has been shown in a previous study that NC/Nga mice produced an Ab to a food-derived Ag and could provide a new animal model of food allergy.\textsuperscript{9)} We used this model to investigate how \textit{L. lactis} could affect the production of an Ab against a food-derived Ag. NC/Nga mice were fed a diet containing casein as a protein source and were given \textit{L. lactis} for 4 weeks. The serum casein-specific IgG and IgG1 levels were significantly higher in the \textit{L. lactis}-fed mice than in the control mice (Fig. 2). These results obtained by using two models show that \textit{L. lactis} possessed the ability to suppress oral tolerance induction.

\textit{L. lactis} increased the Ag-specific IgG2a class in the OVA oral tolerance model (Fig. 1) and increased the Ag-specific IgG1 class in the NC/Nga model (Fig. 2). Although it is difficult to identify the reason for this, it is speculated that different experimental systems (different Ag and Ag dose, use with or without an adjuvant, mouse strain, etc.) might have contributed. It is now clear that oral tolerance is an active immunological process and is mediated by more than one mechanism. The administration of a low dose of Ag favors the induction of active cellular regulation, whereas a higher dose favors the induction of anergy or deletion.\textsuperscript{10,11)} CD4\textsuperscript{+}CD25\textsuperscript{+} T cells are naturally occurring regulatory T cells that are anergic and have suppressive properties.\textsuperscript{12)} The experiment using DO11.10 mice showed that the oral administration of OVA induced CD4\textsuperscript{+}CD25\textsuperscript{+} cells in the spleen and lymph nodes. Furthermore, CD4\textsuperscript{+}CD25\textsuperscript{+} cells from OVA-fed DO11.10 mice exhibited more suppressive activity than did cells from control diet-fed DO11.10 mice in a previous study.\textsuperscript{13)} The oral administration of OVA increased the percentage of CD4\textsuperscript{+}CD25\textsuperscript{+} cells in BALB/c mice fed \textit{L. lactis} by determining the suppressive activity of CD4\textsuperscript{+}CD25\textsuperscript{+} cells in response to anti-CD3 mAb stimulation. Serially diluted CD4\textsuperscript{+}CD25\textsuperscript{+} cells from control diet- and \textit{L. lactis}-fed mice were added to a culture of CD3 mAb-stimulated CD4\textsuperscript{+}CD25\textsuperscript{+} cells. As
shown in Fig. 3B, CD4⁺CD25⁺ cells from the control mice suppressed the proliferation response to anti-CD3 mAb stimulation in a dose-dependent manner. The ability of naturally occurring CD4⁺CD25⁺ cells from *L. lactis*-treated mice to suppress CD4⁺CD25⁺ cell activation was the same as that of CD4⁺CD25⁺ cells from the control mice.

Studies on the effects of probiotics on the induction of oral tolerance have been limited. Jae-Seon et al. have reported that the co-administration of *Lactobacillus casei* with type II collagen significantly suppressed type II collagen-reactive T cell proliferation and reduced the level of Th-1 type IgG isotypes, while it increased the Foxp3 expression level and the population of Foxp3⁺CD4⁺ T cells. The results of that study indicate that *L. casei* could up-regulate Ag-specific oral tolerance and then suppress the Th-1 immune response against arthritic inflammation. Intestinal microflora have been thought to play a critical role in the induction of oral tolerance. It has been suggested that the acquisition of susceptibility to oral tolerance induction was dependent on the microflora. Our study is the first to show a bacterial strain that can break the induction of oral tolerance. The mechanism of its action, however, is presently unknown. We determined the cytokine profile in mesenteric lymph node cells from *L. lactis*-treated mice in response to anti CD3 mAb and found that the level of IL-10 production was not changed when compared to that in control mice. Interestingly, the production of both IFN-γ and IL-4 from T cells was increased (data not shown), but the contribution of this event to oral tolerance is not clear. Elucidation of the mechanism for breakdown of oral tolerance by *L. lactis* will contribute to the successful application of mucosal tolerance for treating human diseases.

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**References**