



Communication

Oral Administration of *Lactobacillus plantarum* NRIC0380 Suppresses IgE Production and Induces CD4⁺CD25⁺Foxp3⁺ Cells *in Vivo*

Mai ENOMOTO,¹ Sayo NOGUCHI,¹ Makoto HATTORI,¹ Hisashi SUGIYAMA,² Yasuyuki SUZUKI,² Akihiro HANAOKA,² Sanae OKADA,³ and Tadashi YOSHIDA^{1,†}

¹Department of Applied Biological Science, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

²R&D Center, Toyo Suisan Kaisha Ltd., Tokyo 135-0043, Japan

³Culture Collection Center, Tokyo University of Agriculture, Tokyo 156-8502, Japan

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The influence of lactic acid bacteria (LAB) on CD4⁺Foxp3⁺ cells has not been investigated, although it has been reported that CD4⁺Foxp3⁺ cells might be involved in the inhibition of allergic symptoms. Hence we examined the effect of orally administered LAB on CD4⁺Foxp3⁺ generation. Oral administration of *Lactobacillus plantarum* NRIC0380 significantly inhibited antigen-specific IgE production and enhanced the Th1 response, as some other strains reported in previous studies. The ratio of CD4⁺CD25⁺Foxp3⁺ cells in whole CD4⁺ cells was significantly higher in both Peyer's patch and the spleen of mice that had been fed with LAB than in the control mice.

Key words: lactic acid bacteria; IgE; Th1/Th2 balance; CD4⁺CD25⁺Foxp3⁺ cell; anti-allergic activity

Lactic acid bacteria (LAB) have been reported to have several immune-regulating functions.¹⁻⁴⁾ The anti-allergic activity of LAB has in particular been well investigated.^{5,6)} Most studies of the anti-allergic activity of LAB have indicated that LAB induces a Th1 response and inhibits the Th2 response and IgE production. It is known that the immune-regulating activity of LAB depends on the strain,²⁾ and investigation of the activity of several LAB strains cannot be avoided to obtain one with strong activity. Hence we have also tested the immune-regulating functions of several LAB strains (unpublished observations). To examine the influence of LAB on cytokine production, splenocytes of ovalbumin (OVA)-specific T cell receptor transgenic mice (TCR-Tg mice; DO11.10) were incubated with OVA (Seikagaku Industry, Tokyo) and LAB. All the mice used in this study were maintained and used in accordance with the guidelines for the care and use of experimental animals of Tokyo University of Agriculture and Technology. LAB was used after autoclaving, as in previous studies.^{2,5)} The amounts of IL-4, IFN- γ , and IL-10 in the supernatants were detected by ELISA. Purified rat anti-mouse IFN- γ mAb (R4-6A2, BD Pharmingen, San Diego, CA), purified rat anti-mouse IL-4 mAb (11B11, BD Pharmingen), purified rat anti-

mouse IL-10 mAb (JES5-2A5, BD Pharmingen), biotinylated rat anti-mouse IFN- γ mAb (XMG1.2, BD Pharmingen), biotinylated rat anti-mouse IL-4 mAb (BVD6-24G2, BD Pharmingen) and biotinylated rat anti-mouse IL-10 mAb (SXC-1, BD Pharmingen) were used in ELISA. We found that a LAB strain, *Lactobacillus plantarum* NRIC0380, had comparatively strong activity. NRIC0380 was obtained from the Culture Collection Center of Tokyo University of Agriculture (NRIC, Tokyo). NRIC0380 was cultured in half-strength MRS broth under anaerobic conditions with a mixed gas (N₂:H₂:CO₂ = 8:1:1) at 30 °C until the late exponential phase of growth. The half-strength MRS broth contained (g/l) glucose, 10; Bacto proteose peptone no. 3 (Becton Dickinson, Franklin Lakes, NJ), 5; Lab-Lemco powder (Oxoid, Cambridge, UK), 5; Bacto yeast extract (Becton Dickinson), 2.5; Tween 80, 0.5; K₂HPO₄, 1; sodium acetate-3H₂O, 2.5; triammonium citrate, 1; MgSO₄-7H₂O, 0.1; and MnSO₄-4H₂O, 0.025. The pH value was adjusted to 6.5 with HCl. NRIC0380 was harvested by centrifugation, washed with sterilized deionized water, lyophilized, and then stored at -20 °C until needed. As shown in Table 1, the amount of IL-4 in the supernatant was significantly suppressed by the addition of NRIC0380 to the culture. On the other hand, production of both IFN- γ and IL-10 in the supernatant was enhanced by LAB. Differences between the control and LAB-treated groups were evaluated by Student's *t*-test, and were considered to be significant at *p* < 0.05. These effects were dependent on the LAB concentration (data not shown).

NRIC0380 showed comparatively strong activity modulating cytokine production toward Th1 dominance. It was thought that NRIC0380 would inhibit IgE production, as in some previous reports,^{2,5)} and so we investigated the effect of NRIC0380 on IgE production. BALB/c mice (5 to 8 mice/group) were purchased from Clea Japan (Tokyo), and were orally administered LAB; BALB/c mice with free access to a normal diet were used as a control. LAB feeding was done 3 times a week for a total number of 13 feedings. LAB was dissolved in PBS at 1 or 10 mg/ml, and 200 μ l was fed to each mouse with a feeding needle. The mice were immunized with

[†] To whom correspondence should be addressed. Tel/Fax: +81-42-367-5711; E-mail: tyoshi@cc.tuat.ac.jp

Abbreviations: β -LG, β -lactoglobulin; LAB, lactic acid bacteria; OVA, ovalbumin; TCR-Tg mice, T cell receptor transgenic mice

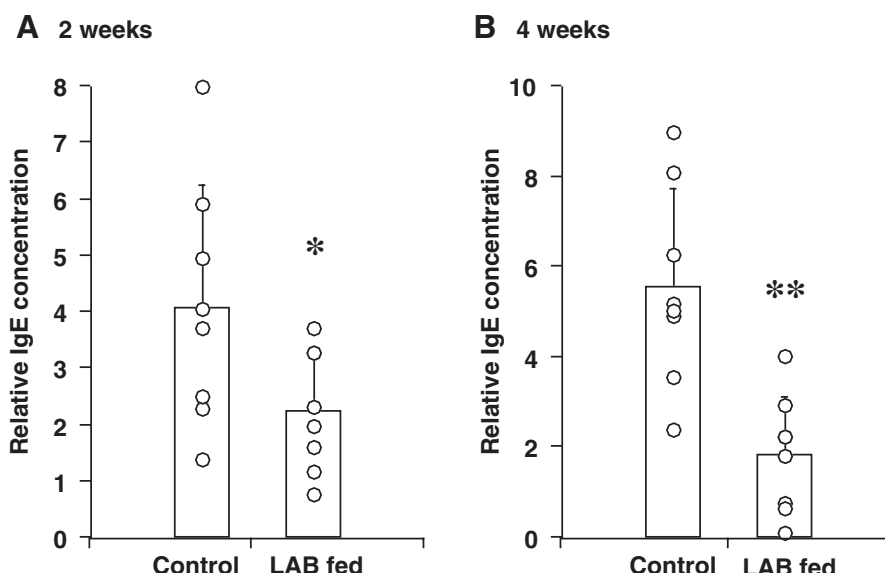


Fig. 1. Effect of Orally Administered LAB on IgE Production *in Vivo*.

BALB/c mice were fed LAB (200 μ g/feed) 13 times. The mice were immunized with β -LG after the second feeding. Two (A) and four (B) weeks after immunization sera samples were prepared, and the anti- β -LG IgE antibody titer was measured. The results are representative of three independent experiments. *Significantly different as compared with the control value ($p < 0.05$). **Significantly different as compared with the control value ($p < 0.01$).

Table 1. Effects of *L. plantarum* NRIC0380 on Cytokine Production *in Vitro*

Stimulus	IL-4	IFN- γ	IL-10
NRIC0380	1.81 $\pm$ 0.18*	109 $\pm$ 18.5*	1.20 $\pm$ 0.16*
No LAB	3.81 $\pm$ 0.42	37.4 $\pm$ 6.28	0.65 $\pm$ 0.16

Splenocytes of DO11.10 were cultured with OVA (10 μ M) and LAB. Three d later the supernatants were collected, and the amounts of IFN- γ , IL-4, and IL-10 in each supernatant were measured by ELISA. The data are shown as the amount of each cytokine produced (ng/ml) when the cells were stimulated by LAB at 0.1 μ g/ml or the control (without LAB). The results are representative of four independent experiments. *Significantly different as compared with the control value ($p < 0.05$).

100 μ g of bovine β -lactoglobulin (β -LG; genotype AA) dissolved in saline (100 μ l) mixed with an equal amount of an alum adjuvant (20 mg/ml) after the secondary feeding. Two and 4 weeks after immunization the mice were bled, and serum samples were prepared. The amount of the β -LG-specific IgE antibody was also measured by ELISA. The bound antibody was detected with biotin-labeled rat anti-mouse IgE (R35-118, BD Pharmingen). The titer of β -LG-specific IgE in the sample sera was calculated relative to that of β -LG-specific IgE in the standard serum (obtained from β -LG-immunized mice). The concentration of total IgE in the sera of the control group obtained 4 weeks after the immunization was 6.77 ± 1.75 μ g/ml, and that of the mice fed NRIC0380 was 3.49 ± 2.00 μ g/ml. The antigen-specific IgE titer of the mice orally administered NRIC0380 was significantly lower than that of the control mice (Fig. 1). This inhibitory effect of LAB was observed within 2 weeks of immunization (Fig. 1A), and was maintained for 4 weeks (Fig. 1B). Figure 1 shows the results for the mice fed 200 μ g of NRIC0380 at each feeding, and similar results were obtained for the mice fed 2 mg of LAB (data not shown). No differences were apparent between the two groups.

Type I allergy is caused by imbalance of the Th1/Th2 response, because IL-4 produced by Th2 induces IgE isotype switching. IgE is a critical factor in inducing the type I allergic reaction. It is known that almost all patients with allergic diseases have a high level of the serum IgE titer, although there are some people who do not develop any allergic symptoms in spite of having a high level of IgE. Orihara *et al.* recently found that the percentage of CD4⁺Foxp3⁺ cells in sera from healthy people having a high level of IgE without showing any allergic symptoms was significantly higher than that in sera from allergic patients having a similarly high level of IgE.⁷⁾ In addition, it has been reported that IPEX (the syndrome of immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance) patients, who lack Foxp3⁺ cells, had severe eczema and food allergy.⁸⁾ Furthermore, the regulatory activity of Foxp3⁺ cells obtained from allergic patients has been found to be lower than that from control healthy donors.^{9,10)} These reports strongly suggest that the CD4⁺Foxp3⁺ cell is another critical factor in the development of allergic diseases, in addition to IgE. Foxp3 is a transcription factor that has been reported to be critical for the induction and function of nTreg cells.¹¹⁾ Although the mechanisms and roles of Foxp3⁺ cells in the inhibition and the development of immune diseases have not been identified, the involvement of Foxp3⁺ cells in such disorders is assumed.¹²⁾ It has been reported that LAB inhibited some inflammatory diseases in murine models, such as autoimmune diabetes and inflammatory bowel disease.^{13,14)} This evidence suggests that LAB induces such regulatory T cells as CD4⁺Foxp3⁺ cells. However, the activity of LAB to induce CD4⁺Foxp3⁺ cells has hardly been investigated yet.

Next we examined the effect of orally administered LAB (1 mg/feed) on CD4⁺CD25⁺Foxp3⁺ generation. BALB/c mice were fed NRIC0380 and immunized with β -LG, as described above for the IgE assay. Twenty-

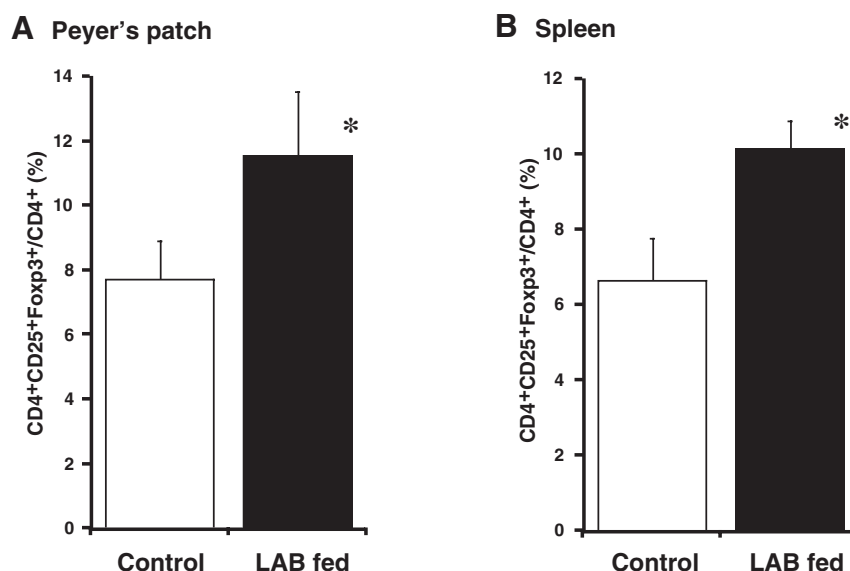


Fig. 2. Effect of Orally Administered LAB on CD4⁺CD25⁺Foxp3⁺ Cell Generation *in Vivo*.

BALB/c mice were fed LAB (1 mg/feed) 13 times. The mice were immunized with β -LG after the second feeding. Twenty-one d after the last feeding, the profiles of CD4⁺CD25⁺Foxp3⁺ cells in Peyer's patch (A) and the spleen (B) were analyzed by flow cytometry. The results are representative of two independent experiments. *Significantly different as compared with the control value ($p < 0.05$).

one d after the last feeding, the profiles of CD4⁺CD25⁺Foxp3⁺ cells in the spleen and Peyer's patch were analyzed by flow cytometry. Fluorescein isothiocyanate (FITC) anti-mouse CD4 (GK1.5), biotinylated anti-mouse CD25 (PC61.5), streptavidine-labeled PE-Cy5, and a PE anti-mouse/rat Foxp3 staining set (FJK-16s) were purchased from eBioscience (San Diego, CA) and used in flow cytometry. As shown in Fig. 2, the ratio of CD4⁺CD25⁺Foxp3⁺/CD4⁺ in the mice fed NRIC0380 was significantly higher in both Peyer's patch (Fig. 2A) and the spleen (Fig. 2B) than in the control mice. The total number of CD4⁺ cells in Peyer's patch and the spleen was not notably different between the LAB-fed and control groups (data not shown). This result suggests that the increase in the percentage of CD4⁺CD25⁺Foxp3⁺ cells was not due to any decrease in the other phenotypes of CD4⁺ cells. This is the first study to report CD4⁺Foxp3⁺ cell induction by oral administration of a LAB strain.

IgE is a key molecule in type I allergic diseases. Many previous studies have therefore attempted to inhibit IgE production to treat allergy.^{2,5,15} LAB has been reported to have strong activity inhibiting IgE production.^{2,5} This effect of LAB is considered to be partly mediated by the activity of LAB to enhance the Th1 response and inhibit Th2.^{2,5,16} Furthermore, it has been found that the intensity of such LAB activity depends on the strain.² Our preliminary study suggested that the intensity of activity to induce CD4⁺Foxp3⁺ cells might also depend on the strain (data not shown), although the mechanism of induction of CD4⁺Foxp3⁺ cells has not been clarified. These results strongly suggest that NRIC0380 can be a useful strain for anti-allergic food products. NRIC0380 is considered to suppress an allergy by its IgE-inhibiting activity and CD4⁺Foxp3⁺ cell-inducing activity. These two activities work synergistically to suppress allergic symptoms.

Several mechanisms involved in the inhibition of IgE production by LAB have been advocated. The most

Table 2. Effects of *L. plantarum* NRIC0380 on Cytokine Production *in Vivo*

Fed	IL-4	IFN- γ	IL-10	Relative ratio of IFN- γ /IL-4
NRIC0380	112.3 $\pm$ 28.8	74.6 $\pm$ 11.7*	3.73 $\pm$ 0.43	2.58
Control diet	152.3 $\pm$ 25.3	39.2 $\pm$ 20.6	2.21 $\pm$ 1.51	1

BALB/c mice were orally administered LAB 5 times every 2 or 3 d. The mice were i.p. immunized with β -LG/alum after the second feeding. Ten d later, the splenocytes of the mice were cultured with β -LG (1 mg/ml). After a 3-d culture the supernatants were collected, and the amounts of IFN- γ , IL-4, and IL-10 in each supernatant were measured by ELISA. The data are shown as the amount of each cytokine produced (pg/ml for IL-4, ng/ml for IFN- γ and IL-10). The relative ratios of IFN- γ /IL-4 are shown as an index of the Th1/Th2 balance, IFN- γ /IL-4 being calculated relative to the ratio of the control group. The results are representative of two independent experiments. *Significantly different as compared with the control value ($p < 0.05$).

important one is to induce IL-12 from macrophages and dendritic cells, although it has not been clarified how LAB is recognized by these cells.^{2,16} NRIC0380 might also induce IL-12 production, resulting in Th1 dominance. This is considered to be the major mechanism for IgE inhibition by NRIC0380. The spleen cells obtained from the mice fed LAB 5 times produced significantly higher levels of IFN- γ than those from the control mice although the level of IL-4 hardly changed, suggesting a Th1 dominant cytokine environment (Table 2). On the other hand, it cannot be excluded that CD4⁺Foxp3⁺ cells induced by LAB are involved in the inhibition of IgE, although the mechanism is not understood at all.

The increase in CD4⁺Foxp3⁺ cells was apparent in both Peyer's patch and the spleen. This indicates that orally administered LAB affected not only mucosal immune responses, but also systemic responses. Several previous studies have demonstrated that LAB inhibited allergic symptoms in the eyes and nose, as well as at the intestine.⁶

We found that oral administration of NRIC0380 significantly increased CD4⁺CD25⁺Foxp3⁺ cells. However, the regulatory activity of CD4⁺CD25⁺Foxp3⁺ cells induced by NRIC0380 has not yet been demonstrated. Foligne *et al.* reported that the protective effect of LAB on TNBS-induced colitis was abrogated by anti-CD25 monoclonal antibody.¹⁷⁾ It is suggested based on this observation and our results that LAB induced CD4⁺CD25⁺Foxp3⁺ regulatory cells, and that those cells were essential to the anti-inflammatory effect of LAB. We are currently examining the anti-allergic function of these cells and the mechanism of it in our laboratory.

The evidence obtained in our study should contribute to the use of LAB strains, including NRIC0380, in the treatment of allergies and inflammatory diseases.

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