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ARTICLE

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Effect of *Lactobacillus pentosus* ONRIC b0240 on Intestinal IgA Production in Mice Fed Differing Levels of Protein

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ABSTRACT: Protein-energy malnutrition (PEM) is frequently associated with the occurrence of infection due to a decline in immune function. Here, an experiment was conducted with the objective of enhancing mucosal immunity by administration of *Lactobacillus pentosus* ONRIC b0240 (b240) in PEM model mice. Three groups of male C3H/HeN mice aged approximately 12 weeks were caged in groups of five or six and received various treatments. The mice were fed 4 (low-protein diet; PEM model), 20 (standard-protein diet), or 40% (high-protein diet) ovalbumin (OVA) with or without 0.05% b240. Five weeks later, all mice were sacrificed, and the organs were extracted for analysis of the immune response. Acute toxicity was not observed in this study. The addition of b240 showed no influence on body weight; however, body weight decreased with increasing protein level. Interestingly, intestinal total IgA was significantly increased ($p < 0.05$) in all test diets with b240. The in vitro study showed that the number of B cells and type 2 helper T (Th2) cells were significantly increased in mouse spleen cells with b240 treatment, whereas no differences were found in the number of Th1 cells. b240 also has the ability to augment IgA and IgG production in mouse Peyer's patch cells. These results suggest that b240 enhances IgA production and helps recover the intestinal immune system in PEM model mice via augmentation of humoral immunity.

KEYWORDS: *Lactobacillus pentosus*, protein-energy malnutrition, IgA, mucosal immunity

INTRODUCTION

Protein-energy malnutrition (PEM), caused by low intake of both protein and calories, is especially common in children in underdeveloped countries, because children require more protein per kilogram of body weight than adults to support the rapid growth and development that occurs during childhood.[1] PEM is frequently associated with the occurrence of infection due to a decline in immune function.[2] Especially, children with PEM show consistent abnormalities in immune responses, such as thymic atrophy, reduced frequency and magnitude of delayed cutaneous hypersensitivity responses, decreased number of rosette-forming T lymphocytes, particularly cluster of differentiation (CD) 4^+ helper T (Th) cells, and decreased natural killer cell (NK cell) activity.[2] Protein deprivation also leads to a reversible reduction in the immunoglobulin (Ig) A response to antigens encountered at the intestinal mucosa.[3] The enhancement of the secretory immune response and, in particular, intestinal IgA production could be helpful in protecting against infection.[4] However, intestinal immune function and the mechanisms of immunodysfunction have not been sufficiently clarified.

Some studies have shown that certain milk components, probiotics, and prebiotics may potentially modify immunity.[5−7] In patients, probiotics and prebiotics reduce diarrhea, promote a healthy gut flora, reduce pathogenic gut bacteria, and directly or indirectly modulate the immune system.[8] This should also be beneficial in severe acute malnutrition when impaired gut function is a problem, manifested as diarrhea and malabsorption, small bowel overgrowth, increased intestinal permeability, enteropathy, Gram-negative (enteric) bacteremia, and suboptimal immune response.[9−11] However, few data are available on the efficacy of probiotics in mucosal immunity in PEM.[12] Particularly, the species *Lactobacillus pentosus* is a major colonizer of the human intestine,[13] and the numbers of IgA-positive cells in the lamina propria increased in mice after oral administration.[14−16] The aim of the present study was to investigate intestinal immune regulation in PEM model mice fed low-, standard-, and high-protein diets with or without *L. pentosus* ONRIC b0240 (b240).

MATERIALS AND METHODS

Materials. Phycoerythrin (PE)-labeled antimouse interleukin (IL)-4 and IL-6 monoclonal antibodies (mAb), PE-labeled antimouse interferon (IFN)-γ mAb, biotin-labeled antimouse CD4, CD8, CD19, CD49, and CD11b mAb, and PE/cyanine 5 (PE/Cy5)-labeled streptavidin were purchased from BioLegend (San Diego, CA). Horseradish peroxidase (HRP)-labeled antimouse IgA and IgG were obtained from Bethyl Laboratories (Montgomery, TX). Brefeldin A (BFA), ionomycin, streptomycin, and phorbol 12-myristate 13-acetate (PMA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). IntraPrep was obtained from Beckman Coulter (Marseille, France). Defined fetal bovine serum (FBS) was obtained from HyClone Laboratories (Road Logan, UT). Penicillin was purchased from MP Biomedicals (Costa Mesa, CA). RPMI-1640 was purchased from Nissui Pharmaceutical (Tokyo, Japan). TMB was purchased from KPL (Gaithersburg, MD). All chemicals used in this study were of the highest analytical grade commercially available.

Bacterium. *L. pentosus* strain b240 (ONRIC b0240: b240) was isolated by Okada et al. from fermented tea leaves.[17] Strain b240 was grown in MRS broth (Beckton Dickinson, Franklin Lakes, NJ) at 33 °C

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Table 1. Experimental Groups[a] and Composition of the Test Diets (%)

low protein diet	1	4% OVA (n = 6)
	2	4% OVA + 0.05% b240 (n = 6)
standard protein diet	3	20% OVA (n = 5)
	4	20% OVA + 0.05% b240 (n = 6)
high protein diet	5	40% OVA (n = 5)
	6	40% OVA + 0.05% b240 (n = 6)

	without 0.05% b240			with 0.05% b240		
OVA(%)	4%	20%	40%	4%	20%	40%
cornstarch	55.75	39.75	19.75	55.70	39.70	19.70
sucrose	13.20	13.20	13.20	13.20	13.20	13.20
α-cornstarch	10.00	10.00	10.00	10.00	10.00	10.00
soybean oil	7.00	7.00	7.00	7.00	7.00	7.00
mineral mix[b]	3.50	3.50	3.50	3.50	3.50	3.50
vitamin mix[c]	1.00	1.00	1.00	1.00	1.00	1.00
L-cystine	0.30	0.30	0.30	0.30	0.30	0.30
choline bitartrate	0.25	0.25	0.25	0.25	0.25	0.25

[a] Each experimental group contains five or six mice. [b] American Institute of Nutrition 93G; mineral mix provided (g/kg diet): calcium, 5.0; phosphorus, 1.6; sodium, 1.0; potassium, 2.3; magnesium, 0.5; iron, 0.03; zinc, 0.03; and copper, 0.01. [c] American Institute of Nutrition 93; vitamin mix provided (g/kg diet): nicotinic acid, 30.0; calcium pantothenate, 16.0; pyridoxine-HCl, 7.0; thiamin-HCl, 6.0; riboflavin, 6.0; folic acid, 2.0; biotin, 2.0; cyanocobalamin, 25.0; α-tocopherol, 150.0; retinyl palmitate, 8.0; cholecalciferol, 2.5; and phylloquinone, 0.75.

for 24 h. The culture was washed twice with saline and suspended in deionized water, followed by autoclaving at 121 °C for 15 min. The heat-killed bacterial suspension was freeze-dried for storage. It was resuspended in saline before use.

Diets. The low-protein diet contained semipurified 4% ovalbumin (OVA), prepared by substituting OVA in the control diet with an equal weight of cornstarch (Japan CLEA Co., Tokyo, Japan). PEM model mice were induced using the low-protein diet.[12] The compositions of the test diets used in the present study are listed in Table 1.

Feeding Procedure. Pathogen-free male C3H/HeN mice (12 weeks of age) were purchased from Japan SLC (Shizuoka, Japan). The mice were housed at 23 ± 2 °C under a standard 12 h light/dark cycle. Mice were given a standard diet, "Labo MR breeder" (Nihon Nosan Co., Kanagawa, Japan) and sterile water ad libitum. After preliminary breeding for 1 week, mice were fed 4 (low-protein diet; PEM model), 20 (standard-protein diet), or 40% (high-protein diet) OVA with or without 0.05% b240 for 4 weeks (Table 1). The schedule appears in the results of Figure 1. Five weeks later, all mice were sacrificed, and organs were extracted for analysis of the immune response. All experimental procedures were carried out in accordance with the Regulations for Animal Experimentation of Shinshu University. The animal protocol was approved by the Committee for Animal Experiments of Shinshu University. On the basis of the national regulations and guidelines, all experimental procedures were reviewed by the Committee for Animal Experiments and finally approved by the president of Shinshu University.

Preparation of Intestinal Extract. The 1 g sample of intestinal tract washes (duodenum to rectum) was ground using a pestle for 20 min at 2 ± 1 °C with sea sand (1 g) in 2.5 mL of 0.01 M sodium phosphate buffer (PBS, pH 7.2) containing 0.15 M sodium chloride. The ground material was then centrifuged at 1200g for 30 min at 4 °C, and the supernatant was collected.

Spleen and Peyer's Patch Cell Suspensions and Cell Cultures. Pathogen-free male C3H/HeN mice (6 weeks of age) were purchased from Japan SLC. After preliminary breeding for 1 week, mouse spleen cells and Peyer's patch cells were prepared using standard methods.[18] The spleen tissue and Peyer's patches were then homogenized in RPMI-1640 medium containing 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The resulting cell suspension was washed three times in this medium and adjusted to 1×10^6 viable cells/mL. The cell suspension (1 mL) was then plated into the wells of a 24-well flat-bottom plate (Sarstedt, Inc., Newton, NC), and the b240 solution was added at a final concentration of 0, 10, or 100 μg/mL. The cells were cultured at 37 °C in a humidified 5% CO_2 incubator for 48 and 120 h for cell functional analysis and Ig ELISA, respectively.

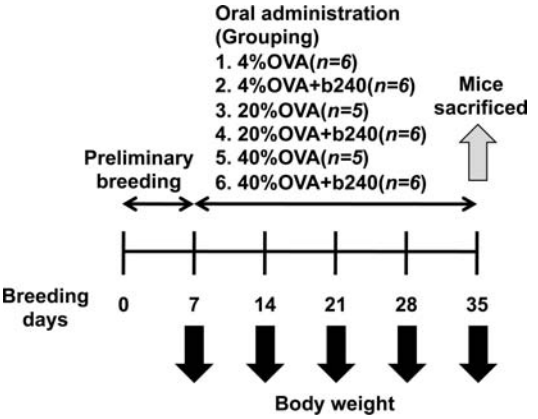

Figure 1. Schedule for the oral administration of the test diets. After preliminary breeding for 1 week, mice were fed 4, 20, or 40% OVA with or without 0.05% b240 in the diet. The body weight of mice was monitored during the experiment. Five weeks later, all mice were sacrificed, and organs were extracted for analysis of the immune response.

Analysis of Igs A and G. Total and OVA-specific IgA and IgG levels in the intestinal extract were measured using a mouse enzyme-linked immunosorbent assay (ELISA) Quantitation kit (Bethyl Laboratories) as described previously.[18] The antibody level was calculated using the following formula: antibody level = ELISA value (A450) × dilution-fold of the test sample.

Cell Functional Analysis. The suspensions of mouse spleen cells were prepared as described previously.[19,20] Cell surface marker antigens of spleen cells were reacted with biotin-labeled antimouse mAbs specific to CD4, CD8, CD11b, or CD19 or PE-labeled antimouse mAb specific to CD49b for 15 min at 4 °C and visualized by incubation with PE/Cy5-labeled streptavidin for 15 min at 4 °C. The visualized cells were analyzed by means of a Guava Personal Cell Function Analyzer (Guava Technologies, Hayward, CA). An assay of intracellular cytokines was performed by permeabilization of PE-labeled antimouse cytokine mAbs specific to IL-4, IL-6, and IFN-γ. Briefly, the cells were incubated with 40 μg/mL BFA, 4 μg/mL ionomycin, and 40 ng/mL PMA for 4 h. The incubated cells were washed and fixed in IntraPrep reagent 1. After 15 min, the cells were washed again and permeabilized by incubation with IntraPrep reagent 2. The cells having cytokines were visualized with incubation of PE-labeled antimouse mAbs specific to IL-4, IL-6, or IFN-γ, and the cell number was determined using a Guava personal cell functional analyzer (Guava PCA; Guava Technologies).

Statistical Analysis. Statistical analyses were performed using MedCalc, version 9.3.7.0 (MedCalc Softwere, Mariakerke, Belgium) and S-Plus (Version 7, Insightful Corp.). Differences between groups were assessed using a one-way analysis of variance (ANOVA) with Bonferroni posthoc test control for type I error. Differences in survival were determined using the log rank test of Kaplan−Meier. Analysis of weight loss was performed using mixed effects longitudinal regression models. All tests were two-sided; probability values less than 0.05 were

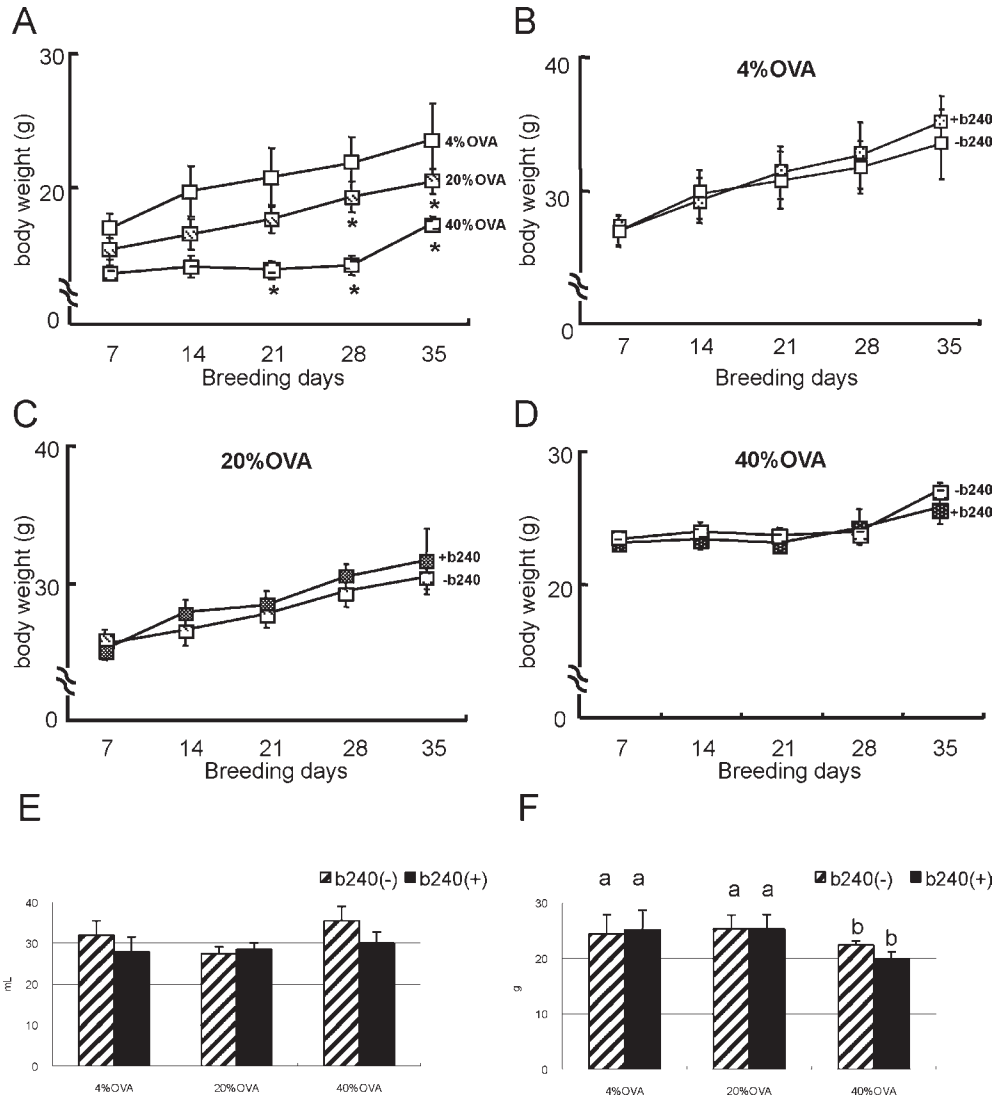

Figure 2. Changes in body weight (A−D), food intake (E), and water consumption (F) of mice fed the 4, 20, and 40% OVA diet with or without b240 intake. Mean body weight of the mice fed diet without b240 (A) or with b240 (B−D) during the experimental period. Values significantly differ from the 4% OVA diet group at $^*p < 0.05$ (A). Items indicated with different letters (i.e., a, b) were significantly different ($p < 0.05$) (F).

considered significant. All values are expressed as means ± SEs unless otherwise noted.

RESULTS AND DISCUSSION

Changes in Body Weight, Food Intake, and Water Consumption. To investigate the effect of the test diets on the body weight of the mice, body weight was monitored during the experiment (Figure 1). There were no significant differences in body weight in mice fed the same level of protein, whether or not they received b240 (Figure 2B−D). The addition of b240 had a minor influence on mean weight when administered with diets of identical protein levels. The body weight of mice fed 20 and 40% OVA diets without b240 was significantly decreased from day 21 to 35 of the study as compared with the 4% OVA diet without b240 (Figure 2A). This is due to the significant decrease in food intake in the 40% OVA diet with or without b240 (Figure 2F). There were also no significant effects on water consumption induced by the intake of test diets during the experiment (Figure 2E). These results suggest that with identical protein levels, the presence of b240 had a minor effect on the body weight of mice. The b240 shows no influence on nutritive function. The body weight decreased significantly in mice consuming the 20 and 40% OVA diets as compared with those consuming the 4% OVA diet. Batterhem et al. reported that a high-protein intake induced the greatest release of the anorectic hormone peptide YY (PYY) and resulted in the most pronounced satiety.[21] Therefore, the high level of dietary protein induces the decreasing food intake and increasing plasma PYY levels in mice.

Intestinal IgG and IgA Production in Vivo. The mice were fed 4, 20, or 40% OVA with or without 0.05% b240 in the diet for 5 weeks. OVA-specific IgA and IgG levels in the intestinal extract are shown in Figure 3. The OVA-specific IgA was significantly increased by 20% OVA diets with b240 intake (Figure 3A). The total IgA was also significantly increased by all test diets with b240 intake (Figure 3B). The OVA-specific IgG was significantly increased in the 4% OVA diet with b240 group ($P < 0.05$), whereas no differences were found in the 20 and 40% OVA diet with or without b240 groups (Figure 3C). The total IgG was also significantly increased by 20% OVA diet with b240 intake,

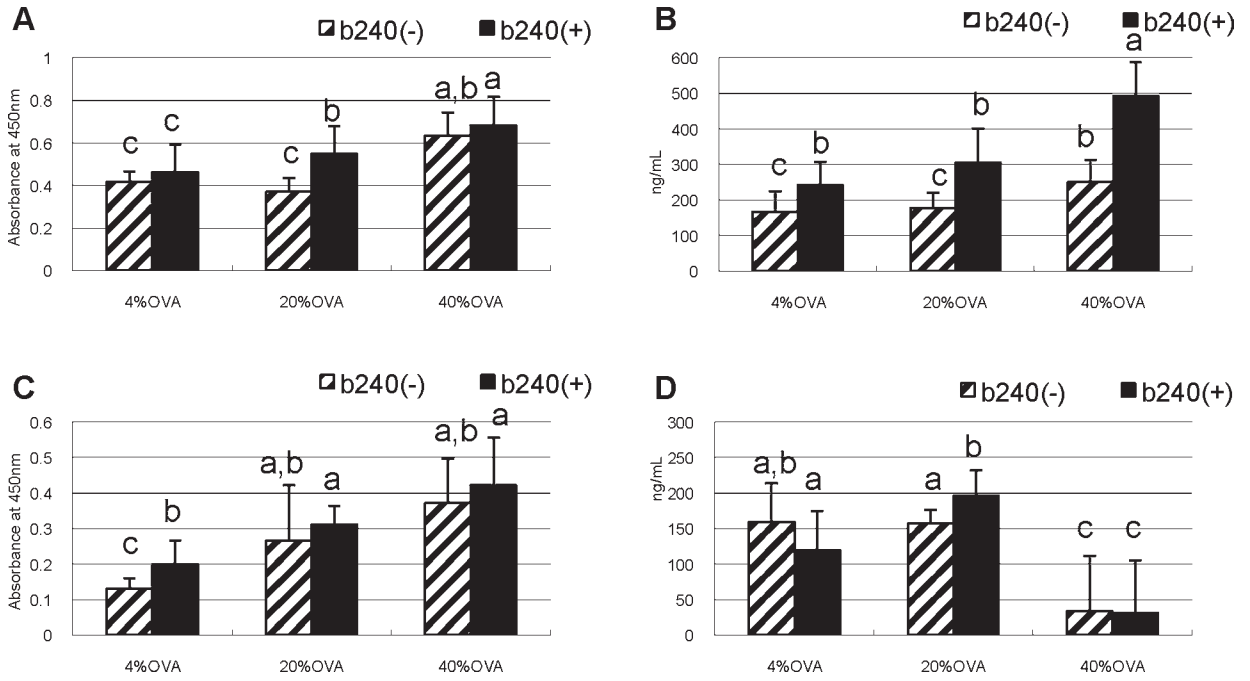

Figure 3. Intestinal anti OVA IgA (A), total IgA (B), anti OVA IgG (C), and total IgG (D) levels of mice fed diets containing 4, 20, and 40% OVA with or without 0.05% b240 for 35 days. Items indicated with different letters (i.e., a, b, c) were significantly different ($p < 0.05$).

whereas no differences were found in the 4 and 40% OVA diet with or without b240 groups (Figure 3D). These results suggest that the high-protein diet induces antigen-specific IgA and total IgA production in the intestine more than the low- and standard-protein diets. Moreover, b240 intake significantly enhanced the intestinal total IgA level in the low-protein diet group as well as the standard group.

Intestinal IgA plays a principal role in the intestinal immune system; it prevents infection at the early stages by excluding bacteria and viruses from the gastrointestinal tract.[22] To date, the respective effects of PEM and aging have been shown to decrease IgA levels and the antigen-specific response.[23] Therefore, our findings suggest that aging might modify the influence of PEM on IgA production by b240 intake.

Immune Effects of b240 in Vitro. Surface expression of CD4 (Th cells), CD8 (cytotoxic T cells), CD19 (B cells), CD49 (NK cells), and CD11b (macrophages) was examined in mouse spleen cells after treatment with b240 for 48 h (Figure 4). The expression of $CD19^+$ cells was significantly higher following treatment with b240 (Figure 4C) than in the control medium, whereas $CD4^+$ (Figure 4A), $CD49^+$ (Figure 4D), and $CD11b^+$ (Figure 4E) cells were significantly lower than in the control medium. The b240 treatment had a minor effect on $CD8^+$ cells (Figure 4B). We also used flow cytometric analysis to investigate the effect of b240 on IL-6^+CD4^+ [type 2 helper T (Th2) cells], IL-4^+CD4^+ (Th2 cells), and IFNγ^+CD4^+ [type 1 T helper (Th1) cells] cell populations in vitro. Treatment of mouse spleen cells with 10 μg/mL of b240 significantly increased the ratio of IL-6^+CD4^+ and IL-4^+CD4^+ cells (Figure 5A,B), whereas b240 treatment had a minor effect on IFNγ^+CD4^+ cells (Figure 5C). The intestinal IgA and IgG production in mouse Peyer's patch cells cultured with or without b240 for 120 h is shown in Figure 6. b240 significantly up-regulated IgA (Figure 6A) and IgG (Figure 6B) production. Mature Th cells are believed to always express the surface protein CD4.[24] CD19 is a member of the Ig superfamily and has two Ig-like domains. The CD19 molecule is expressed on 100% of the peripheral B cells as defined by the expression of κ- or λ-light chains.[25] In the present study, we also observed a significant up-regulation in Th2 immune responses by treatment with b240 in vitro (Figures 4 and 5). Th2 cells can help B cells with IgE synthesis, as well as with IgG, IgM, and IgA production. Particularly, they are essential in determining B cell antibody class switching. IFN-γ^+, IL-4, and IL-6^+CD4^+ cells are Th1 and Th2 cells, respectively, and Th2 cells stimulate antibody responses.[26] IgA and IgG production is regulated by various cytokines, such as IL-6 and IL-4. IL-6 is a cytokine produced by several cell types, including antigen presenting cells (APC) such as macrophages, dendritic cells, B cells, and T cells. IL-6 is involved in the acute phase response, B cell maturation, and macrophage differentiation. IL-6 activates NFAT- (nuclear factor of activated T cells) mediated transcription, leading to the production of IL-4 by naive $CD4^+$ T cells and subsequent differentiation into effector Th2 cells. While the induction of Th2 differentiation by IL-6 is dependent upon endogenous IL-4, inhibition of Th1 differentiation by IL-6 is IL-4- and NFAT-independent.[27] In this study, we found a significant up-regulation in IL-4 and IL-6^+CD4^+ cells in mouse spleen cells by b240 coculture. We speculate that b240 has the ability to induce intestinal IgA production by augmentation of Th2 immune responses.

PEM has been shown to exert diverse influences on host immune systems, including systemic and mucosal immune responses.[28] The immune system's effects on PEM are related and might have a cumulative effect. At present, the number and proliferative response of lymphocytes, cytokine production, cytotoxic T-lymphocyte activity, and antigen-specific response to vaccinations are known to be damaged by PEM in aging.[29] However, intestinal immune function and its mechanisms have not been sufficiently clarified. PEM is a common and serious problem that results in an immunocompromised condition and aggravates infection-related mortality in elderly subjects.[30,31]

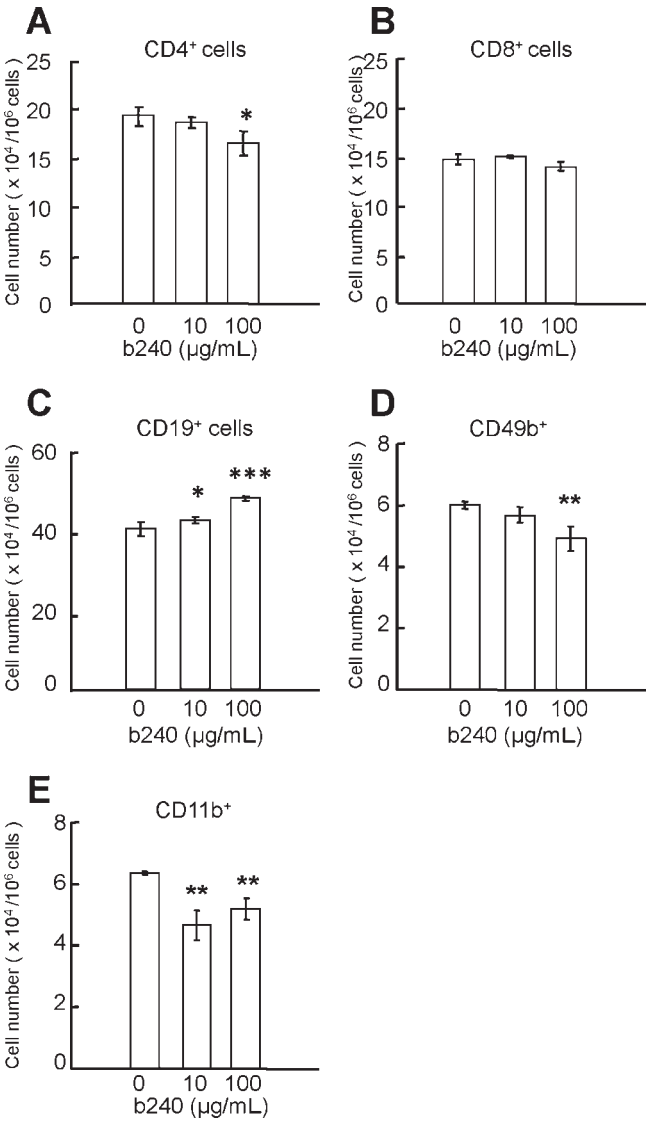

Figure 4. Determination of $CD4^+$ (A), $CD8^+$ (B), $CD19^+$ (C), $CD49b^+$ (D), and $CD11b^+$ (E) cell numbers in mouse spleen cells using Guava PCA. Spleen cells from mice were cultured with b240 (0, 10, and 100 μg/mL) for 48 h. Data are presented as the mean ± SD (n = 3). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (as compared to the control, 0 μg/mL b240). All assays were carried out through at least three separate experiments using three separate culture wells. Similar results were obtained from at least three different mice.

In the present study, the oral ingestion of b240 improved intestinal IgA production when mice were fed the low-protein diet. Moreover, we found that b240 had the ability to augment Th2 type immune responses via IL-4 and IL-6 induction. The b240 strains may modulate host immunity through incorporation of Peyer's patches cells and stimulation of the immune cells in the intestinal tract. In any event, in terms of nutritional status, our results suggest that b240 may improve nutritional status without changing the quantity of food consumption. This improvement in nutritional status might contribute indirectly to restoring an immunocompromised condition, which is cumulatively controlled by direct intestinal stimulation with b240.

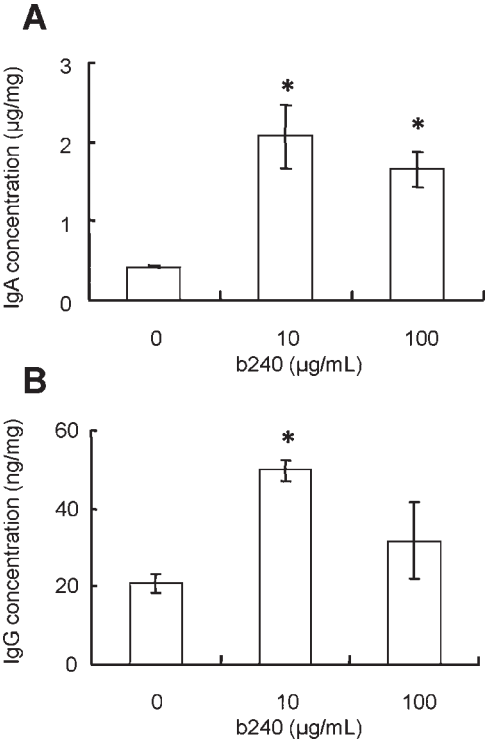

Figure 6. Effect of b240 (0, 10, and 100 μg/mL) on the production of IgA (A) and IgG (B) in mouse Peyer's patch cell cultures. The IgA and IgG levels were determined by ELISA. The data are presented as the mean ± SD (n = 3). $^*P < 0.05$ (as compared to the control, 0 μg/mL b240). All assays were carried out through at least three separate experiments using three separate culture wells. Similar results were obtained from at least three different mice.

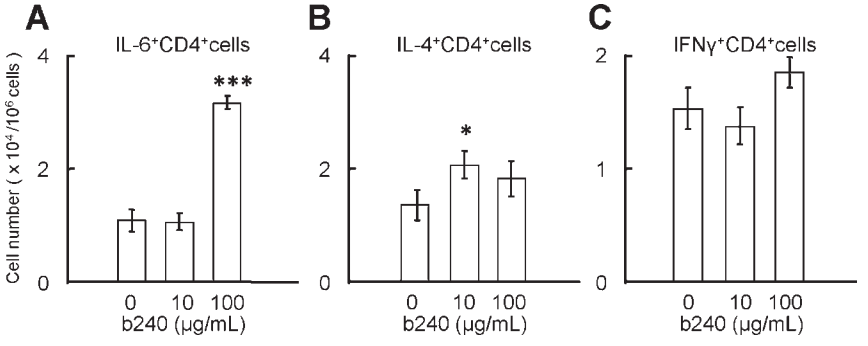

Figure 5. Determination of IL-6^+CD4^+ (A), IL-4^+CD4^+ (B), and IFN-γ^+CD4^+ (C) cell numbers in mouse spleen cells cultured with b240 (0, 10, and 100 μg/mL) for 48 h using Guava PCA. Data are presented as the mean ± SD (n = 3). $^*P < 0.05$, and $^{***}P < 0.001$ (as compared to the control, 0 μg/mL b240). All assays were carried out through at least three separate experiments using three separate culture wells. Similar results were obtained from at least three different mice.

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Therefore, we propose that the oral ingestion of b240 stimulates certain natural cellular immune systems, while enhancing humoral adaptive immune responses in PEM model mice. Exploiting this property may also prove useful in the design and production of new physiologically functional foods using probiotics.

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ABBREVIATIONS USED

b240, *Lactobacillus pentosus* ONRIC b0240; PEM, protein-energy malnutrition; CD, cluster of differentiation; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; Guava PCA, Guava personal cell functional analyzer; IFN, interferon; Ig, immunoglobulin; IL, interleukin; OVA, ovalbumin; PE, phycoerythrin; ANOVA, analysis of variance; SD, standard deviation; Th1, type 1 T helper; Th2, type 2 T helper; NK cells, natural killer cells; NFAT, nuclear factor of activated T cells

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