J Nutr Sci Vitaminol, **56**, 441–445, 2010

Note

Lactobacillus crispatus KT-11 Enhances Intestinal Immune Functions in C3H/HeN Mice

Keisuke TOBITA[1,2], Hiroyuki YANAKA[2] and Hajime OTANI[1,*]

[1] *Interdisciplinary Graduate School of Science and Technology, Shinshu University, Minamiminowa-mura 8304, Kamiina-gun, Nagano 399–4598, Japan*
[2] *Kitii Co., Ltd., SEI Building, 5 Araki-cho, Shinjuku-ku, Tokyo 160–0007, Japan*

(Received March 30, 2010)

Summary We investigated the effect of *Lactobacillus crispatus* KT-11 (KT-11) on intestinal immune systems in C3H/HeN mice. The level of intestinal total immunoglobulin (Ig) A was significantly higher in mice given KT-11 than in mice not given KT-11. Gene expression relating to antibody production and innate immune response increased more than 2-fold in the former compared with the later. Moreover, the number of IL-6$^+$CD11b$^+$ cells was significantly higher in Peyer's patch cells cultured with KT-11 than in those cultured without KT-11, although the number of CD4$^+$ cells and the cell ratio of CD4$^+$/CD8$^+$ were remarkably lower in the culture with KT-11. These results indicate that KT-11 enhances intestinal IgA production and innate immune response in C3H/HeN mice.

Key Words *Lactobacillus crispatus* KT-11, immunoglobulin A, innate immune system, C3H/HeN mouse, Peyer's patch

Type I allergic diseases are generally characterized by an elevation in the serum immunoglobulin (Ig) E level (*1*). The production of IgE is thought to be due to a skewed type 1 helper T (Th1)/type 2 helper T (Th2) cell balance (*2*–*5*). In a previous paper, we found that *Lactobacillus crispatus* KT-11 (KT-11) reduced some allergic symptoms via a decrease in antigen-specific IgE levels in NC/Nga mice, and the reduction was concluded to be due to an adjustment in the Th1/Th2 balance via Toll-like receptor (TLR) 2, nucleotide-binding oligomerization domain (NOD) 1, and/or NOD2 (*6*). Moreover, we observed that KT-11 suppressed the acquired allergic responses via not only the adjustment in Th1/Th2 balance but also the decrease in spleen mast cell numbers and antigen-presenting cell numbers in ovalbumin-immunized BALB/c mice (*7*). However, the effect of KT-11 on immune systems of normal mice is not known.

Thus, in this paper, we describe the effect of KT-11 on intestinal immune functions in a normal mouse, C3H/HeN strain.

Materials and Methods

Heat-treated KT-11. KT-11 was obtained as a stock culture from Kitii (Tokyo, Japan). KT-11 was inoculated in DeMan-Rogosa-Sharpe medium and cultivated for 24 h at 37°C, collected by centrifugation, washed three times with sterile water, heat-treated at 65°C for 30 min and then lyophilized.

Feeding procedure. Four-week-old male C3H/HeN mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). All animal experiments were conducted in accordance with the Guidelines for Regulation of Animal Experimentation at Shinshu University, and Law No. 105 and Notification No. 6 of Japan. Mice were administered commercial mouse pellets (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) for 1 wk. They were then divided into two groups, in which they were orally administered 0.5 mL of sterile saline solution (control group) or 0.5 mL of sterile saline solution containing 1 mg of heat-treated KT-11 (KT-11 group) once a day. The mice were given the solution from 5 to 10 wk of age. The mice were supplied with commercial mouse pellets from stainless-steel feeders and water ad libitum throughout the course of the experiment. The mice were housed at 23±2°C under a 12-h light-dark cycle. Intestine, spleen and Peyer's patch were collected immediately following a lethal dose of ether at 10 wk of age.

Preparation of intestinal extract and antibody analysis. One gram of intestinal tract tissue (duodenum to rectum), including contents, was ground using a pestle for 15 min at 2±1°C with 1.5 g sea sand in 2.5 mL of 0.01 M sodium phosphate buffer containing 0.15 M NaCl (PBS, pH 7.2). The ground material was then centrifuged at 1,200 ×*g* for 30 min at 4°C. The supernatant was collected and stored at −30°C until use. The level of intestinal total IgA was measured using a mouse IgA enzyme-linked immunosorbent assay (ELISA) quantitation kit (Bethyl Laboratories, Montgomery, TX, USA) according to the manufacturer's pro-

*To whom correspondence should be addressed at present address: Graduate School of Agriculture, Shinshu University, Minamiminowa-mura 8304, Kamiina-gun, Nagano 399–4598, Japan.
E-mail: otani84@shinshu-u.ac.jp

tocol.

Cell suspensions. Seven Peyer's patches were collected from a mouse. The Peyer's patches collected from 6 mice in the control group and 5 mice in the KT-11 group were pooled, respectively. Spleens were individually collected from 6 mice in the control group and 5 mice in the KT-11 group. The Peyer's patches and spleens were homogenized in RPMI-1640 medium containing 5% defined fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. The cell suspensions were then washed three times in this medium, and adjusted to 1×10^6 viable cells/mL. Peyer's patch cell suspensions were used for DNA microarray analysis. Spleen cell suspensions were used for cell functional analysis.

Peyer's patch cell cultures. Peyer's patches used in vitro were collected from 6-wk-old male C3H/HeN mice. Peyer's patch cell suspensions were prepared as described above, and were plated into the wells of a 24-well flat bottom plate (Sarstedt, Inc., Newton, NC, USA). PBS containing heat-treated KT-11 was added to the wells at a final whole cell concentration of 0 μg/mL (control culture) or 100 μg/mL (KT-11 culture). The cells were cultured at 37˚C in a humidified 5% CO_2 incubator for 48 h.

Cell functional analysis. The cell surface markers for CD4 (clone RM4-5) and CD8α (clone 53-6.7) were then observed using a specific biotin-conjugated anti-mouse monoclonal antibody (mAb) for 15 min at 4˚C, followed by incubation with phycoerythrin/cyanine 5 (PE/Cy5)-labeled streptavidin for 15 min at 4˚C. Cell numbers were then determined using a personal cell functional analyzer (Guava PCA: Guava Technologies, Hayward, CA, USA). When observing intracellular cytokines in $CD4^+$ cells or $CD11b^+$ cells, the cells were incubated at 37˚C in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 20 μg/mL brefeldin A, 2 μg/mL ionomycin and 20 ng/mL phorbol 12-myristate 13-acetate for 4 h. The cell surface marker antigens for CD4 or CD11b (clone M1/70) were then labeled using a specific biotin-conjugated anti-mouse mAb for 15 min at 4˚C, followed by incubation with PE/Cy5-labeled streptavidin for 15 min at 4˚C. Intracellular cytokines were measured following permeabilization and labeling with a PE-labeled anti-mouse cytokine mAb specific for interferon (IFN)-γ (clone XMG1.2), interleukin (IL)-4 (clone 11B11) or IL-6 (clone MP5–20F3). In order to achieve this, cells were fixed with IntraPrep (Beckman Coulter, Marseille, France) reagent 1 for 15 min, washed and then permeabilized with IntraPrep reagent 2. The cells were then incubated with the appropriate antibodies and intracellular cytokine levels determined using Guava PCA.

Microarray analysis. The genome-wide gene expression of the pooled Peyer's patch cells was examined using the Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA) that contains 45101 probe sets for approximately 34,000 mouse genes. Briefly, total RNA was extracted from Peyer's patch cell suspensions using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). GeneChip analysis was performed according to the GeneChip Eukaryotic Target Preparation and Hybridization Manual (Affymetrix). Data analysis was performed with GeneChip Operating software 1.4 (Affymetrix). Expression data were selected when there was more than a 2-fold difference or less than a 0.5-fold difference between the control group and the KT-11 group.

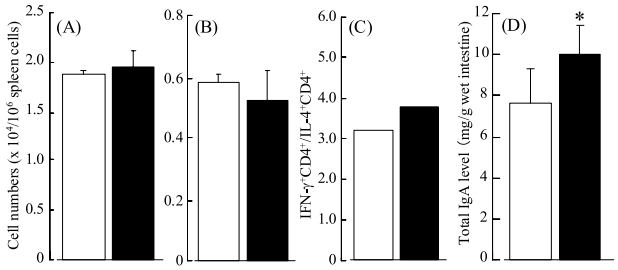

Fig. 1. The number of spleen IFN-γ^+CD4$^+$ and IL-4$^+$CD4$^+$ cells, the ratio of IFN-γ^+CD4$^+$/IL-4$^+$CD4$^+$, and the level of intestinal total IgA in C3H/HeN mice given KT-11. The mice were orally administered KT-11 once a day from 5 to 10 wk of age. The spleen was collected at 10 wk of age. The number of spleen IFN-γ^+CD4$^+$ cells (A) and IL-4$^+$CD4$^+$ cells (B) was determined using Guava PCA. The ratio of IFN-γ^+CD4$^+$/IL-4$^+$CD4$^+$ (C) was presented as the mean IFN-γ^+CD4$^+$ cell number against the mean IL-4$^+$CD4$^+$ cell number. The level of intestine total IgA (D) was determined using ELISA. Control and KT-11 groups are presented as open and filled bars, respectively. Data are presented as mean±SD (control group, $n=6$; KT-11 group, $n=5$). $^*p<0.05$ (compared to control group using Student's t-test).

Statistical analysis. Data are presented as the mean±standard deviation (SD). Statistical analyses were performed using Student's t-test. Differences were considered significant when p values were less than 0.05.

Results and Discussion

There were no significant differences in body weight between the control group and the KT-11 group (data not shown). This result indicates that there are no differences in the stress level or nutritive value between the groups.

It is recognized that IFN-γ^+CD4$^+$ and IL-4$^+$CD4$^+$ cells are categorized as Th1 and Th2, respectively (*6*). As shown in Fig. 1A–C, the number of spleen IFN-γ^+CD4$^+$ and IL-4$^+$CD4$^+$ cells, and the ratio of IFN-γ^+CD4$^+$/IL-4$^+$CD4$^+$ did not remarkably differ between the control and KT-11 groups. Hence, this result indicates that KT-11 does not influence the Th1/Th2 balance. As described already, type I allergic diseases are thought to be due to a skewed Th1/Th2 balance. We previously reported that KT-11 reduced some allergic symptoms by shifting the Th1/Th2 balance from a Th2-dominant state to a Th1-dominant state in type I allergic model mice (*6*, *7*). However, KT-11 does not influence the Th1/Th2 balance in the normal C3H/HeN mouse.

Intestinal IgA, which is a major mucosal antibody and plays an important role as one of the intestinal barrier components, prevents the adhesion and/or entry of antigens into the epithelium, and neutralizes proinflammatory antigen (*8*). As shown in Fig. 1D, the level of intestinal total IgA was significantly higher in the KT-11 group than in the control group. It is generally accepted that the development of IgA-producing B cells requires cytokines, such as IL-5 and IL-6, produced by Th2 cells (*9–11*). However, Sato et al. (*12*) reported that IL-6 produced by Peyer's patch $CD11b^+$ dendritic cells enhanced IgA production from B cells. In addition, Kuraoka et al. (*13*) reported that a novel subset of Peyer's patch $CD4^-$c-$kit^-CD3\varepsilon^-$IL-$2R\alpha^+$ cells, but not T and B cells, contributed markedly to IL-5 production, and promoted IgA production by B cells via a helper T cell-independent pathway. $CD4^+$ was a typical cell-surface antigen of helper T cells (*14*). As shown in Table 1, the number of IL-6^+CD11b^+ cells was significantly higher in the KT-11 culture than in the control culture. In contrast, the number of $CD4^+$ cells was significantly lower in the KT-11 culture than in the control culture. These findings suggest that KT-11 induces IgA production via a helper T cell-independent pathway.

It is known that regulatory T cells control immune responses to bacteria and play crucial roles in immunohomeostasis of Th1/Th2 balance (*15*). The regulatory T cells are identified by expression of forkhead box P3 (Foxp3) on $CD4^+$ and $CD8^+$ T cells (*16*). $Foxp3^+CD8^+$ T cells are activated by IL-6, and suppress $CD4^+$ T cell proliferation (*17*, *18*). Latvala et al. (*19*) reported that some lactic acid bacteria induced IL-6-production from predation cells such as macrophages and dendritic cells. In this study, the numbers of $CD8^+$ and IL-6^+CD11b^+ cells were higher in the KT-11 culture than in the control culture. In contrast, the number of $CD4^+$ cells and the cell ratio of $CD4^+/CD8^+$ were remarkably lower in the KT-11 culture than in the control culture. Therefore, KT-11 would induce IL-6-production from macrophages and/or dendritic cells via TLR2, NOD1 and/or NOD2 and control the Th1/Th2 balance via the activation of regulatory $CD8^+$ T cells in the normal mice, although KT-11 adjusted the Th1/Th2 balance via TLR2, NOD1 and/or NOD2 in the type I allergic model mice reported previously (*6*).

Peyer's patch, a major secondary lymphoid tissue in the intestine, plays an important role in the development of not only the local immune system but also the systemic immune system (*20*, *21*). The relative mRNA expression of 71 genes in Peyer's patch cells increased more than 2-fold in the KT-11 group when compared with the control group. In contrast, the relative mRNA expression of 79 genes in Peyer's patch cells decreased less than 0.5-fold in the KT-11 group. Almost all of these influenced genes were related to biological processes for cellular transport, cell adhesion, protein binding and protein folding. Out of these 150 influenced genes, as shown in Table 2, 8 genes related to immunity increased more than 2-fold while 2 genes decreased less than 0.5-fold. The proteins produced by transcription of *Ccl21*, *Defa5*, *Defa-rs7*, *Defa-rs2*, *Igkv12-46* and *Muc13* are chemokine (C-C motif) ligand 21 (CCL21), α-defensin 5, α-defensin related sequence 7, α-defensin related sequence 2, immunoglobulin κ chain variable 12-46 and mucin 13, respectively. The immunoglobulin κ variable chains are related to the composition of antibodies such as IgA (*22*). This fact supports the assump-

Table 1. The number and ratio of immunocompetent cells in C3H/HeN mouse Peyer's patch cells cultured in the presence of KT-11.

Immunocompetent cell	Control culture	KT-11 culture
	Cell number ($\times 10^4/10^6$ cells)	
IL-6^+CD11b^+	0.8±0.1	2.9±0.1***
$CD4^+$	36.3±0.9	29.9±0.5***
$CD8^+$	13.5±0.7	14.2±0.3
	Cell ratio	
$CD4^+/CD8^+$	2.69	2.10

Values are represented as mean±SD (n=3). ***$p<0.001$ (compared to control culture using the Student's t-test).

Table 2. The gene expression transcriptome relating to immunity of Peyer's patch cells in C3H/HeN mice given KT-11.

Gene symbol	Gene title	Relative mRNA expression (KT-11 group/control group)
Increased genes		
Ccl21	chemokine (C-C motif) ligand 21	3.7
Defa5	defensin, alpha, 5	2.6
Defa-rs7	defensin, alpha, related sequence 7	2.6
Defa-rs2	defensin, alpha, related sequence 2	2.5
Igkv12-46	immunoglobulin kappa chain variable 12-46	2.3
Rnf128	ring finger protein 128	2.0
Muc13	mucin 13, epithelial transmembrane	2.0
Leap2	liver-expressed antimicrobial peptide 2	2.0
Decreased genes		
Ccr9	chemokine (C-C motif) receptor 9	0.5
Lck	lymphocyte protein tyrosine kinase	0.5

tion that KT-11 induces IgA production in mucosal immune systems via Peyer's patch. On the other hand, the innate immune system plays an important role as the first line of protection against infective pathogens. CCL21, one of the secondary lymphoid tissue chemokines, induces rapid endocytosis of mature dendritic cells (*23*). Mucins, such as mucin 13, protect the epithelium against the constant attack of digestive fluids, microorganisms, pollutants, toxins, etc. (*24*). α-Defensin, released by epithelial cells onto the mucosal surface, functions as an antimicrobial peptide (*25*). TLRs and NODs that are expressed mainly in predation cells, such as macrophages and dendritic cells, are known as pathogen pattern recognition receptors. The cellular signal via TLRs and NODs activates these cells, and leads to innate immune responses with subsequent initiation of adaptive immune responses (*26*). As described already, we observed that KT-11 modulated Th1/Th2 balance via TLR2, NOD1 and/or NOD2 (*6*). Therefore, it is suggested that KT-11 enhances innate immune responses via TLR2, NOD1 and/or NOD2 in C3H/HeN mice.

On the other hand, Matsuguchi et al. (*27*) and Shimosato et al. (*28*) reported that cell components of lactic acid bacteria such as lipoteichoic acids and specific DNA motifs modulated immune responses via TLRs. Moreover, Bessa and Bachmann (*29*) demonstrated that TLR signals played an important role in IgA responses. Thus, it is suggested that some cell components of KT-11 might be related to the modulation of intestinal immune functions via TLRs.

In conclusion, we propose that *L. crispatus* KT-11 modulates intestinal immune functions via enhancement of IgA production and the innate immune response in normal mice, although the bacterium reduces allergic symptoms via adjustment of the Th1/Th2 balance in type I allergic model mice. Thus, KT-11 may be an excellent immunoregulatory supplement for animals.

REFERENCES

1) Dreborg S. 2002. The implications of nomenclature. *Ann Allergy Asthma Immunol* **89**: 83–85.
2) Shirakawa T, Enomoto T, Shimazu S, Hopkin JM. 1997. The inverse association between tuberculin responses and atopic disorder. *Science* **275**: 77–79.
3) Prescott SL, Macaubas C, Smallacombe T, Holt BJ, Sly PD, Holt PG. 1999. Development of allergen-specific T-cell memory in atopic and normal children. *Lancet* **353**: 196–200.
4) Hopkin JM. 2002. The rise of atopy and links to infection. *Allergy* **57**: 5–9.
5) Shirai T, Suzuki K, Inui N, Suda T, Chida K, Nakamura H. 2003. Th1/Th2 profile in peripheral blood in atopic cough and atopic asthma. *Clin Exp Allergy* **33**: 84–89.
6) Tobita K, Yanaka H, Otani H. 2009. Heat-treated *Lactobacillus crispatus* KT strains reduce allergic symptoms in mice. *J Agric Food Chem* **57**: 5586–5590.
7) Tobita K, Yanaka H, Otani H. 2010. Anti-allergic effects of *Lactobacillus crispatus* KT-11 strain on ovalbumin-sensitized BALB/c mice. *Anim Sci J* **81**: 699–705.
8) Corthésy B. 2007. Roundtrip ticket for secretory IgA: role in mucosal homeostasis? *J Immunol* **178**: 27–32.
9) Schoenbeck S, McKenzie DT, Kagnoff MF. 1989. Interleukin 5 is a differentiation factor for IgA B cells. *Eur J Immunol* **19**: 965–969.
10) Kunimoto DY, Nordan RP, Strober W. 1989. IL-6 is a potent cofactor of IL-1 in IgM synthesis and of IL-5 in IgA synthesis. *J Immunol* **143**: 2230–2235.
11) Platts-Mills TA. 2001. The role of immunoglobulin E in allergy and asthma. *Am J Respir Crit Care Med* **164**: 1–5.
12) Sato A, Hashiguchi M, Toda E, Iwasaki A, Hachimura S, Kaminogawa S. 2003. CD11b$^+$ Peyer's patch dendritic cells secrete IL-6 and induce IgA secretion from naive B cells. *J Immunol* **171**: 3684–3690.
13) Kuraoka M, Hashiguchi M, Hachimura S, Kaminogawa S. 2004. CD4$^-$c-kit$^-$CD3ε^-IL-2Rα^+ Peyer's patch cells are a novel cell subset which secrete IL-5 in response to IL-2: implications for their role in IgA production. *Eur J Immunol* **34**: 1920–1929.
14) Sallusto F, Lanzavecchia A. 2009. Heterogeneity of CD4$^+$ memory T cells: functional modules for tailored immunity. *Eur J Immunol* **39**: 2076–2082.
15) Corthay A. 2009. How do regulatory T cells work? *Scand J Immunol* **70**: 326–336.
16) Pillai V, Karandikar NJ. 2007. Human regulatory T cells: a unique, stable thymic subset or a reversible peripheral state of differentiation? *Immunol Lett* **114**: 9–15.
17) Konya C, Goronzy JJ, Weyand CM. 2009. Treating autoimmune disease by targeting CD8$^+$ T suppressor cells. *Expert Opin Biol Ther* **9**: 51–65.
18) Nakagawa T, Tsuruoka M, Ogura H, Okuyama Y, Arima Y, Hirano T, Murakami M. 2010. IL-6 positively regulates Foxp3$^+$CD8$^+$ T cells in vivo. *Int Immunol* **22**: 129–139.
19) Latvala S, Pietila TE, Veckman V, Kekkonen RA, Tynkkynen S, Korpela R, Julkunen I. 2008. Potentially probiotic bacteria induce efficient maturation but differential cytokine production in human monocyte-derived dendritic cells. *World J Gastroenterol* **14**: 5570–5583.
20) Sato A, Iwasaki A. 2005. Peyer's patch dendritic cells as regulators of mucosal adaptive immunity. *Cell Mol Life Sci* **62**: 1333–1338.
21) Tsuji M, Suzuki K, Kinoshita K, Fagarasan S. 2008. Dynamic interactions between bacteria and immune cells leading to intestinal IgA synthesis. *Semin Immunol* **20**: 59–66.
22) Taussig MJ. 1988. Molecular genetics of immunoglobulins. *Immunol Suppl* **1**: 7–15.
23) Yanagawa Y, Onoé K. 2003. CCR7 ligands induce rapid endocytosis in mature dendritic cells with concomitant up-regulation of Cdc42 and Rac activities. *Blood* **101**: 4923–4929.
24) Porchet N, Aubert JP. 2004. MUC genes: mucin or not mucin? That is the question. *Med Sci* (*Paris*) **20**: 569–574.
25) Mastroianni JR, Ouellette AJ. 2009. Alpha-defensins in enteric innate immunity: functional Paneth cell alpha-defensins in mouse colonic lumen. *J Biol Chem* **284**: 27848–27856.
26) Monie TP, Bryant CE, Gay NJ. 2003. Activating immunity: lessons from the TLRs and NLRs. *Trends Biochem Sci* **34**: 553–561.
27) Matsuguchi T, Takagi A, Matsuzaki T, Nagaoka M, Ish-

ikawa K, Yokokura T, Yoshikai Y. 2003. Lipoteichoic acids from *Lactobacillus* strains elicit strong tumor necrosis factor alpha-inducing activities in macrophages through Toll-like receptor 2. *Clin Diagn Lab Immunol* **10**: 259–266.

28) Shimosato T, Kitazawa H, Katoh S, Tohno M, Iliev ID, Nagasawa C, Kimura T, Kawai Y, Saito T. 2005. Augmentation of T(H)-1 type response by immunoactive AT oligonucleotide from lactic acid bacteria via Toll-like receptor 9 signaling. *Biochem Biophys Res Commun* **326**: 782–787.

29) Bessa J, Bachmann MF. 2010. T cell-dependent and -independent IgA responses: role of TLR signalling. *Immunol Invest* **39**: 407–428.