Oral Intake of *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1 Prevents Collagen-Induced Arthritis in Mice

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ABSTRACT

Oral intake of some lactic acid bacteria can have beneficial effects on the host by activating immune responses and enhancing resistance to infection by pathogens. In this study, effects of *Lactobacillus* sp. on the development of autoimmune disease were examined in mice with collagen-induced arthritis (CIA). CIA, a model of some types of rheumatoid arthritis (RA), can be induced in DBA/1J mice by immunizing them with bovine type II collagen (bCII). Oral intake of skimmed milk (SM) fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1 (SM/OLL1073R-1) was found to markedly inhibit the development of CIA in these mice, compared with a control group fed the control foodstuff. The inhibitory effect of SM fermented with *L. delbrueckii* subsp. *bulgaricus* OLL1102 (SM/OLL1102) or fresh SM was weaker than that of SM/OLL1073R-1. A deMan Rogosa Sharpe (MRS) broth culture of OLL1073R-1 also exhibited the inhibitory effects on both development of CIA and secretion of IFN-γ.

It is well known that some lactic acid bacteria such as *Lactobacillus* sp., *Lactococcus* sp., and *Bifidobacterium* sp. can activate cell-mediated immunity by up-regulating the proliferation of splenocytes (12), secretion of various kinds of cytokines (20, 24), phagocytosis by macrophages (26, 27), and anti-tumor responses (35). Some strains of these bacteria can also increase the production of immunoglobulin A by B cells and stimulate intestinal immunity (30). These activities have been recognized as beneficial effects of these bacteria on the host animals. However, little is known about the effect of each strain of these bacterium species on immune diseases caused by excessive immune responses. Our study was based on the expectation that some strains of *Lactobacillus* sp. have beneficial homeostatic effects, i.e., up-regulation of immune responses relating to host defense and down-regulation of adverse dys-regulated immune responses. Autoimmune diseases, as well as allergy and graft-versus-host diseases, result from excessive immune responses. In this study, we tested whether *Lactobacillus* sp. could inhibit the development of autoimmune disease.

There is growing evidence that the composition of the endogenous intestinal bacterial microflora plays an important role in the development of autoimmune disease in both human and animal models. It has been shown that a DNA component from circulating immune complexes in patients with systemic lupus erythematosus hybridized with the lac gene of *Escherichia coli* (10, 32). The intraluminal bacterial microflora is considered a pivotal factor in the inflammatory process of Crohn’s disease (6, 10). Diversion of the fecal stream after ileal resection can prevent recurrence (10, 25), and lowering the concentrations of luminal bacteria improves active Crohn’s disease (10, 36). In view of these data, it can be supposed that immune responses to intestinal bacteria can cause the development of autoimmune disease. By contrast, other reports have demonstrated that intestinal bacteria can inhibit the development of autoimmune disease. Fisher rats (normally arthritis resistant) are susceptible to an experimental autoimmune disease, adjuvant-induced arthritis, when raised under germ-free conditions (1, 18). However, resistance can be reestablished by infection with *E. coli* (1, 17). On the basis of these findings, we studied whether oral intake of *Lactobacillus* sp. could affect the development of autoimmune disease. *Lactobacillus* sp. are found in the intestines of animals and humans, and some of them are used for processing dairy and fermented foods. Studies on beneficial effects of *Lactobacillus* sp. can reveal the relationship between the host and intestinal bacteria and may also be helpful in developing drugs and foods for preventing the development of autoimmune disease.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology that leads to various degrees of cartilage destruction and joint deformity, and some types of RA have been recognized as autoimmune disease. Collagen-induced arthritis (CIA) in rodents shows the features of an autoimmune disease (37). It is proposed to serve as an experimental model of human autoimmune RA, and it

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is induced in susceptible rodents by injection of the animals with homologous or heterologous native type II collagen (CII) (4, 33). In this study, we tested the effect of oral intake of *Lactobacillus* sp. on CIA induced in susceptible DBA/1 mice.

*Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1 (OLL1073R-1) was fed to DBA/1 mice. A characteristic feature of OLL1073R-1 is the production of phosphopolysaccharides (34). No other *L. bulgaricus* strains are known to secrete phosphate-containing polysaccharides, although nonphosphorylated polysaccharides are produced by various strains. Polysaccharides are a component of bacterial cell walls, and they have been shown to have various immunological effects (14, 23). Phosphopolysaccharides from *Lactococcus lactis* modulate cytokine production (15) and promote the proliferation of B cells (16). In this paper, we compared the effects of oral intake of two *L. bulgaricus* strains, OLL1102 and OLL1073R-1, in prevention of CIA.

**MATERIALS AND METHODS**

**Mice.** Female DBA/1NCrj mice, 3 weeks old, were purchased from Charles River Japan (Yokohama, Japan).

**Microorganisms.** We used *L. delbrueckii* subsp. *bulgaricus* OLL1102 (OLL1102) and *L. bulgaricus* OLL1073R-1 (OLL1073R-1) in this study. These bacteria were grown in 10% (wt/wt) skimmed milk (SM) medium at 37°C for 18 h, followed by lyophilization.

**Foodstuffs containing the OLL1102 and OLL1073R-1 cultures.** The food containing the SM cultures of OLL1102 or OLL1073R-1 was prepared by adding culture lyophilizates at a final concentration of 10% (wt/wt) to normal food for mice (NF; Oriental Yeast, Tokyo, Japan) (designated SMF/OLL1102 and SMF/OLL1073R-1, respectively). NFs were made with wheat, corn, fish meal, soybean, SM, rice bran, and other ingredients. The composition (g/100 g) of NF was as follows: 25.6 g protein, 5.1 g fat, 6.7 g minerals, 3.3 g fiber, 59.1 g carbohydrate, 0.2 g choline chloride, and trace vitamins.

**Preparation of a foodstuff containing polysaccharides from the SM culture of OLL1073R-1.** Crude polysaccharides were isolated as described by Uemura et al. (34) and were mixed with lyophilized SM medium in the same volume as that of the OLL1073R-1 culture medium used for isolation of the polysaccharides. In total, 7.8 mg of crude lyophilized polysaccharides was obtained in this fraction from 1,000 ml of SM culture, and this fraction contained both phosphorylated and nonphosphorylated polysaccharides. The food containing the polysaccharide-SM mixture (PSF) was prepared by adding this mixture to NF at a final concentration of 10% (wt/wt).

**Preparation of a foodstuff containing a deMan Rogosa Sharpe (MRS) broth culture (MRSF/OLL1073R-1) or OLL1073R-1 bacterial cells (BCF).** OLL1073R-1 was grown in MRS broth (Difco Laboratories, Detroit, Mich.) medium at 37°C for 18 h. Half of the culture, including both the bacterial cells and the medium, was lyophilized and used for preparation of MRSF/OLL1073R-1; this lyophilized was added to NF at a final concentration of 10% (wt/wt). The other half was used for preparation of BCF, as follows. The bacterial cells were collected by centrifugation (3,600 × g, 4°C, 10 min) of the culture. After washing with 0.85% NaCl, the cells were lyophilized. This powdered preparation of bacterial cells was added to the same weight of NF as that used in the preparation of MRSF/OLL1073R-1.

**Feeding of various foodstuffs to mice.** Mice were fed various foodstuffs for 2 weeks ad libitum. The concentrations of bacteria in each foodstuff (10⁶ CFU/g) were 5.9 (OLL1102), 16 (OLL1073R-1), and 44 (PSF). However, the numbers of bacteria in MRSF/OLL1073R-1 and BCF could not be verified. In the control groups, the mice were fed NF, 10% SM in NF without *Lactobacillus* sp. (SMF), or 10% MRS broth in NF without OLL1073R-1 (MRSF), depending on the experiment. These NF and SMF preparations were not sterilized. We found no differences in the amounts consumed by the mice among the groups, and there were no apparent differences in the mice among the groups at the start of the study.

**Induction and assessment of CIA.** On day 15 after the start of feeding the various foodstuffs, CIA was induced by immunization with bovine type II collagen (bCII), and, after induction, the mice were fed NF. The mice were administered bCII (200 µg) in complete Freund’s adjuvant (CFA) containing *Mycobacterium tuberculosis* strain H37Ra (Difco) by injection intradermally at the base of the tail. Twenty-one days later, the mice were boosted with bCII (200 µg) in incomplete Freund’s adjuvant (Difco) by intradermal immunization at the base of the tail. Arthritis development was graded from 0 to 3: grade 0, no swelling; grade 1, one-digit swelling or mild swelling; grade 2, two-digit swelling or swelling of ankle or wrist joints; and grade 3, severe inflammation or bony deformity.

**Cytokine assays.** Mice were sacrificed 10 days after immunization with bCII/CFA in the hind footpads. Draining lymph nodes were removed, and the cells (2.5 × 10⁶ cells/ml) were cultured with 100 µg/ml bCII, 10 µg/ml purified protein derivative (PPD; BCG Japan, Tokyo, Japan), or 5 µg/ml concanavalin A (Sigma Chemical Co., St. Louis, Mo.) in X-vivo 20 (Bio-Whittaker, Walkersville, Md.). Supernatants were collected after 72 h for measurement of levels of cytokines IFN-γ and IL-10 by enzyme-linked immunosorbent assay as described by Matsumoto et al. (21). The antibody pairs used were as follows (capture and biotinylated detection): IFN-γ, R4-6A2/XG1.2; and IL-10, 2A5/SXC-1. Standard curves were generated using recombinant cytokines (IFN-γ, Peprotech, Rocky Hill, N.J.; and IL-10, Stratham Biotech, Hannover, Germany).

**Statistical analyses.** The statistical analyses were performed with Student’s *t* test for arthritis index, and the concentration of cytokines, with the χ² test for the incidence of arthritis. *P* < 0.05 was accepted as the level of significance.

**RESULTS**

Oral intake of SMF/OLL1073R-1 strongly suppressed the development of CIA. The DBA/1 mice were fed various foodstuffs for 2 weeks and then immunized with bCII to induce CIA. After immunization, all mice were fed NF. Oral intake of SMF/OLL1073R-1 decreased the severity of arthritis in comparison with the mice in the control groups fed NF or SMF (Fig. 1A). SMF/OLL1073R-1 also suppressed the development of CIA as assessed on the basis of the number of arthritic paws. However, only a slight difference was found in the number of arthritic mice (Fig. 1C), and the difference was not statistically significant. The development of CIA was also inhibited in the groups treated with SMF/OLL1102 (Fig. 1B) or SMF SMF/OLL1102
FIGURE 1. Oral intake of SMF/OLL1073R-1 inhibited the development of CIA. Mice were fed SMF/OLL1102, SMF/OLL1073R-1, SMF, or NF for 2 weeks and then immunized with bCII twice. The first immunization was performed on day 0. The mice were then routinely monitored for the development of clinical symptoms of CIA by inspection of distal joint inflammation. The arthritis index was determined by summation of the total score for each joint in each group of mice and dividing by the total number of animals in each group. n = 10. (a) and (b) Significantly different from the SMF or NF group, respectively (P < 0.05). (c) Significantly different from both SMF and NF groups (P < 0.05).

Lymph node cells (LNCs) from mice fed SMF/OLL1073R-1 showed decreased secretion of IFN-γ in response to bCII. To investigate the mechanisms contributing to the inhibition of CIA development by SMF/OLL1073R-1, we analyzed the cytokine response of LNCs to bCII. When stimulated with bCII, LNCs from mice in the SMF/OLL1073R-1–treated group showed an obvious decrease in production of IFN-γ (Fig. 2A) in comparison with the SMF-treated or NF-fed group. By contrast, the level of IFN-γ production by LNCs from mice treated with SMF/OLL1102 was not diminished. A reduced level of production of IFN-γ in response to bCII was also found in the case of the SMF group, but the level displayed was higher than that in the SMF/OLL1073R-1–treated group. Upon PPD recall, LNCs from SMF/OLL1073R-1–treated mice displayed a level of production of IFN-γ similar to that of all other groups.

Little significant difference in bCII-induced production of IL-10 was found comparing the SMF/OLL1073R-1 and SMF groups. LNCs from both groups produced a lower amount of IL-10 than those from the SMF/OLL1102 or NF group (Fig. 2B and 2C).

Oral intake of polysaccharides from SM/OLL1073R-1 strongly inhibited the development of CIA. For comparison with the effects of the fermented SM, OLL1073R-1 was cultured in a different medium, MRS. This medium shares no major components of SM, such as casein, β-lactoglobulin, and α-lactalbumin, and therefore can be considered quite different from SM. Two foodstuffs were prepared using part of the whole culture (MRSF/OLL1073R-1) and bacterial cells isolated from another part of the same culture of OLL1073R-1 in MRS medium (BCF). The development of CIA was inhibited in the group treated with MRSF/OLL1073R-1 when evaluated on the basis of the number of arthritic feet (Fig. 3). By contrast, both the arthritis index and number of arthritic mice suggested that arthritis was exacerbated in the group treated with BCF, although the differences were not statistically significant. These data indicate that the supernatant of the culture of OLL1073R-1 contained the component(s) able to inhibit the development of CIA.

In an effort to identify soluble active component(s) of SM/OLL1073R-1 capable of suppressing the development of CIA, we investigated inhibition of CIA by a polysaccharide fraction from OLL1073R-1 by preparing a foodstuff containing this fraction (PSF). In PSF-treated mice, both the severity (Fig. 4A) and the incidence of arthritis (Fig. 4B and 4C) were very strongly reduced compared with the NF or SMF group.
FIGURE 2. Oral intake of SMF/OLL1073R-1 inhibited IFN-γ production by LNCs. Mice were fed SMF/OLL1102, SMF/OLL1073R-1, SMF, or NF and immunized with bCII/H37Ra. Ten days after immunization of the mice, LNCs were collected and cultured in the presence of bCII (100 μg/ml), PPD (10 μg/ml), or concanavalin A (5 μg/ml) for 72 h. Supernatants were collected, and the amounts of IFN-γ and IL-10 were measured by enzyme-linked immunosorbent assay. Data represent the mean ± standard deviation of triplicate cultures. (a) and (b) Significantly different from the SMF or NF group, respectively \((P < 0.05)\). (c) Significantly different from both SMF and NF groups \((P < 0.05)\).

FIGURE 3. Oral intake of MRSF/OLL1073R-1 but not BCF inhibited the development of CIA. Mice fed MRSF/OLL1073R-1, BCF, or NF were immunized with bCII twice. Other experimental designs are the same as described in Figure 1. \(n = 10\) or 11. (b) Significantly different from the NF group \((P < 0.05)\).

LNCs from mice fed PSF showed a reduced level of secretion of IFN-γ in response to bCII. Oral intake of PSF resulted in a marked decrease in bCII-induced production of IFN-γ by LNCs from these mice (Fig. 5A) com-
FIGURE 4. Oral intake of PSF strongly inhibited the development of CIA. Mice fed PSF, SMF, or NF were immunized with bCII twice. Other experimental designs are the same as described in Figure 1. \( n = 10 \) or 11. (a) and (b) Significantly different from the SMF or NF group respectively \( (P < 0.05) \). (c) Significantly different from both SMF and NF groups \( (P < 0.05) \).

FIGURE 5. Oral intake of PSF inhibited IFN-\( \gamma \) secretion but not IL-10 secretion by LNC. LNCs from mice fed PSF, SMF, or NF for 2 weeks before immunization with bCII/H37Ra were cultured as described in Figure 2. (a) and (b) Significantly different from the SMF or NF group, respectively \( (P < 0.05) \). (c) Significantly different from both SMF and NF groups \( (P < 0.05) \).

In this study, we tested whether intake of \textit{Lactobacillus} sp. could inhibit the development of autoimmune disease. We found that oral intake of \textit{L. bulgaricus} OLL1073R-1 inhibited the onset and development of CIA (Fig. 1). It seems that the inhibitory effect of \textit{L. bulgaricus} on CIA...
depends on the bacterial strain used, because the OLL1102 strain of *L. bulgaricus* was not as effective in suppressing CIA as the OLL1073R-1 strain (Fig. 1).

We also examined the cytokine profile of LNCs from mice fed SMF/OLL1073R-1. It is now well established that activated CD4 T lymphocytes can differentiate into subsets of memory-effector T lymphocytes that show distinct patterns of cytokine secretion. The best defined subsets are Th1 cells, which typically produce IL-2 and IFN-γ, and Th2 cells, which typically produce IL-4, IL-5, and IL-10 (22). These two subsets produce cytokines that cross-regulate each other’s development and activity (8, 9). In the case of CIA, an anti-CII Th1 response is involved in the induction of experimental autoimmune arthritis, although the IFN-γ response is related to both inhibition and development of experimental arthritis (2, 3).

SMF/OLL1073R-1 attenuated the production of IFN-γ by LNCs in response to bCII (Fig. 2). It is supposed that suppression of the development of CIA is attributable to reduced levels of production of IFN-γ, although other mechanisms may also play a role in the inhibition observed. By contrast, bCII-induced production of IL-10 was not enhanced by SMF/OLL1073R-1 (Fig. 2). Therefore, the effect of SMF/OLL1073R-1 did not result from a preferential shift to a Th2-type response.

We found that SMF also suppressed the secretion of IFN-γ that occurred in response to bCII (Fig. 2). This result may imply that constituents of SM may contribute in part to the inhibition of IFN-γ secretion by SMF/OLL1073R-1. However, SMF/OLL1102 was not effective to inhibit bCII-induced IFN-γ production (Fig. 2). MRSF/OLL1073R-1, which has no major components of SM, inhibited bCII-induced IFN-γ production (Fig. 6). The level of IFN-γ secretion was more greatly reduced in the SMF/OLL1073R-1–treated group than in the SMF-treated group (Fig. 2). From these results, it is evident that OLL1073R-1 is involved in the suppressed IFN-γ response to bCII. OLL1073R-1 inhibited both the development of CIA and IFN-γ secretion. However, SMF/OLL1102 suppressed the development of CIA more efficiently than SMF (Fig. 1), although LNCs from mice in the SMF/OLL1102 group produced about twice as much IFN-γ as those from mice in the SMF group (Fig. 2). These results suggest that mechanisms other than interference with the production of IFN-γ by bCII-specific LNCs are predominant in inhibition of CIA.

The mechanism(s) responsible for the suppression of CIA observed in the present study are yet to be identified. Considering the involvement of bacteria in modulating the immune response in cases of RA and its experimental models, we can suggest two possible mechanisms. First, milk fermented with *Lactobacillus* sp. may influence the intestinal microflora of the mice, causing a decrease in abundance of certain types of bacteria such as those provoking the production of proinflammatory cytokines by intestinal epithelial cells, which is related to the induction of CIA. HT-29 cells, a human colonic adenocarcinoma cell line, express IL-8, MCP-1, and tumor necrosis factor-α mRNA upon stimulation with *E. coli*, whereas *Lactobacillus* sp. do not enhance expression of these mRNA (5). It has also been demonstrated that tumor necrosis factor-α is associated with the pathogenesis of RA (7). Second, improvement of the intestinal flora may be associated with another nonimmunological mechanism involved in prevention of CIA. Serum biotin levels are lower than normal in RA patients and remain almost unchanged, even after administration of biotin. However, supplementary administration of a probiotic agent together with biotin has been shown to significantly increase serum biotin levels and to maintain levels of the vitamin high enough to improve the clinical manifestations. Low serum biotin levels may also be attributable to the proliferation of harmful intestinal bacteria that degrade or take up biotin (19). Moreover, some reports suggest that induction of RA is associated
with *Clostridium perfringens* infection (28, 29). Improvement in RA disease symptoms following treatment with sulphasalazine has been shown to be correlated with decreased counts of intestinal anaerobic bacteria, including *C. perfringens* and *E. coli* (13). Thus, oral intake of milk fermented with OLL1073R-1 at the time of induction of CIA may alter the intestinal bacterial flora and decrease the percentage of harmful bacteria, such as *C. perfringens*, and other bacteria that take up or degrade biotin. It is unlikely that these effects are due to viable bacteria. In this experimental design, the OLL1073R-1 strain does not become predominant in the intestinal microflora, because the cells scarcely survive in the gastrointestinal tract. It seems that before oral intake, the number of viable bacteria decreases due to exposure to oxygen. By contrast, fermented milk can reduce the pH in the intestinal tract and thereby diminish the percentage of *C. perfringens* (31). It is not clear whether polysaccharides secreted by OLL1073R-1 affect the growth of some lactic acid bacteria. However, transgalactosylated disaccharides are known to promote the growth of bifidobacteria (11). The species composition of the intestinal microflora can be modified by bacterial metabolites and components of ingested. These changes may be related to the observed effects on the development of autoimmune disease.

We investigated the effect of polysaccharides isolated from OLL1073R-1 cells. PSF exerted extremely strong activity in inhibiting the development of CIA (Fig. 4) and the IFN-γ response of LNCs to bCII (Fig. 5), similar to SMF/OLL1073R-1. MRSF/OLL1073R-1 also inhibited the development of CIA (Fig. 3). MRSF/OLL1073R-1 inhibited the bCII-induced production of IFN-γ more strongly than MRSF (Fig. 6). OLL1073R-1 bacterial cells from the OLL1073R-1 culture in MRS were not effective in preventing the development of CIA in the mice nor in regulating the LNC response to bCII (Figs. 3 and 6). These results indicate that cellular products such as polysaccharides, but not OLL1073R-1 bacterial cells, are responsible for the observed inhibition of CIA development. OLL1073R-1 cells secrete phosphorylated polysaccharides (34). No other *L. bulgaricus* strains have been reported to secrete phosphate-containing polysaccharides, whereas nonphosphorylated polysaccharides are produced by various strains. Therefore, the production of phosphorylated polysaccharides is distinctive of this bacterial strain, OLL1073R-1. Because OLL1073R-1 but not OLL1102 showed the inhibitory effect, the phosphorylated polysaccharides characterized produced may be responsible for this effect.

Our results show that OLL1073R-1 prevents CIA in mice serving as a model of autoimmune disease, although SM also played a significant role. It is also suggested that a reduced Th1 response to CII, as shown by down-regulation of IFN-γ secretion, may be partly involved in suppression of CIA. However, we could not demonstrate up-regulation of Th2-type cytokine responses or any significant change in the immunoglobulin G subclass pattern of bCII-specific antibodies (data not shown). Thus, mechanism(s) other than a shift from a Th1-type response to a Th2-type response may account for the inhibition of CIA. This inhibitory activity was dependent on the bacterial strain used. We also found that a polysaccharide fraction, but not the isolated bacterial cells themselves, strongly inhibited the development of CIA. Although the mechanisms and components involved in inhibition of CIA remain to be fully elucidated, OLL1073R-1 may be an attractive agent for use in preventing autoimmune diseases, such as some types of RA, in the future.

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REFERENCES


