Oral administration of milk fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 to DBA/1 mice inhibits secretion of proinflammatory cytokines

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Abstract

We have previously shown that oral administration of skimmed milk (SM) fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 (OLL1073R-1/SM) to DBA/1 mice inhibited the development of collagen-induced arthritis (CIA). In this study, our aim was to examine possible mechanisms of inhibiting the development of CIA. We studied the effect of OLL1073R-1/SM on cytokine secretion from cells of popliteal lymph nodes (lymph node cells; LNC) of mice. The results showed that feeding OLL1073R-1/SM inhibited secretion of proinflammatory cytokines such as interferon γ (IFN- γ), interleukin 6 (IL-6), tumor necrosis factor α (TNF- α) and the chemokine, monocyte chemoattractant protein 1 (MCP-1). The most prominent effect was inhibition of TNF- α . Secretion of IL-2 and IL-4 were not influenced. Feeding OLL1073R-1/SM inhibited secretion of proinflammatory cytokines produced by accessory cells, but not T cells. We conclude that CIA may be prevented via downregulation of secretion of proinflammatory cytokines such as IL-6, TNF- α and IFN- γ , and of the chemokine of MCP-1.

Abbreviations: CII – type II collagen; CIA – collagen-induced arthritis; IFN – interferon; IL – interleukin; ELISA – enzyme-linked immunosorbent assay; LNC – lymph node cells; MCP – monocyte chemoattractant protein; OLL1073R-1/SM – skimmed milk fermented with *Lactobacillus bulgaricus* OLL1073R-1; SM – skimmed milk; TNF – tumor necrosis factor.

Introduction

Lactobacillus sp. is used for producing yogurt and other dairy and non-dairy fermented food. It has been shown that feeding these bacteria to animals has beneficial effects on the immune response of host animals. Immunological effects of *Lactobacillus* sp. include modulation of cytokine production by T cells or macrophages (Marin et al., 1997). Cytokines enhance or inhibit production of other cytokines and regulate the immune response (Mosmann and Sad, 1996). Th1 cytokines such as interleukin 2 (IL-2) and interferon γ (IFN- γ), and proinflammatory cytokines such as IL-1 and tumor necrosis factor α (TNF- α) play an important role in eliminating pathogenic bacteria,

virus or tumor cells (Orme and Cooper, 1999; Ramsay et al., 1993; Urban et al., 1986; Onozaki et al., 1985). However, unregulated production of these cytokines may cause inflammation and cell destruction, resulting in the development of autoimmune disease. Conversely, Th2 cytokines such as IL-4 and IL-10 have anti-inflammatory effects and can downregulate the production of Th1 cytokines. Reports have shown that *Lactobacillus* sp. increases production of Th1 cytokines such as IL-2 and IFN- γ in macrophage, T cell or mononuclear cell lines (Haller et al., 2000; Miettinen et al., 1998; Marin et al., 1997; Solis-Pereyra et al., 1997).

Collagen-induced arthritis (CIA) is an animal model for an autoimmune type of rheumatoid arth-

ritis (Wooley et al., 1981). Proinflammatory cytokines such as IL-6 and TNF- α have been shown to be involved in the development of CIA (Joosten et al., 1999; Alonzi et al., 1998), while Th1 cytokines such as IL-2 and IFN- γ are related to both inhibition and development of CIA (Thornton et al., 2000; Boissier et al., 1995; Cooper et al., 1988; Mauritz et al., 1988). The pathogenesis of experimental arthritis has been shown to be prevented by *Lactobacillus* sp. (Kato et al., 1998). We have previously demonstrated that oral intake of milk fermented with a *Lactobacillus* sp. strain, OLL1073R-1 (OLL1073R-1/SM), inhibited the development of CIA (Kano et al., 2002). We have also shown that feeding OLL1073R-1/SM decreased secretion of IFN- γ by lymph node cells (LNC) from type II collagen (CII)-immunized mice. IFN- γ is known to promote production of TNF- α from macrophages and other cells, which is closely related to the pathogenesis of CIA. However, feeding OLL1073R-1/SM did not enhance secretion of IL-10, although this Th2 cytokine can inhibit differentiation into IFN- γ -producing Th1 cells. Therefore, inhibition of CIA development by OLL1073R-1/SM could not be explained by an alteration in Th1/Th2 balance.

The present study was designed to investigate possible mechanisms by which CIA may be inhibited by OLL1073R-1/SM. Thus, we have investigated the effect of OLL1073R-1/SM on the cytokine secretion profile in LNC from CII-immunized mice.

Materials and methods

Preparation and administration of food containing the Lactobacillus bulgaricus culture

We used *Lactobacillus delbrueckii* subsp. *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 and the whole culture was lyophilized. The food containing the SM culture of OLL1073R-1 (OLL1073R-1/SM) was prepared by adding the culture lyophilizate at a final concentration of 10% (wt/wt) to the standard food for mice (CRF-1 from Oriental Yeast, Tokyo, Japan). This foodstuff contained 4.4×10^8 OLL1073R-1 CFU g⁻¹. The composition of CRF-1 (g 100 g⁻¹) was as follows: 22.6 g protein, 5.6 g fat, 6.6 g minerals, 3.3 g fiber, 53.8 g carbohydrate, 0.3 g choline, and trace vitamins.

Female, 4 week old, DBA/1J Sea mice (supplied by Seac Yoshitomi, Fukuoka, Japan) were fed various foodstuffs *ad libitum* throughout the experimental

period. In the two control groups, mice were fed CRF-1 or CRF-1 containing 10% (wt/wt) skimmed milk lyophilizate.

Cell culture

On day 15 after the start of feeding, mice were immunized with 10 μ g of CII plus adjuvant complete H37Ra (Difco Laboratories, Detroit, MI) in the hind footpads. Mice were sacrificed in an approved manner 10 days after immunization. Draining lymph nodes were removed. LNC were prepared and 2.5×10^6 cells ml⁻¹ were cultured with 100 μ g ml⁻¹ CII in RPMI 1640 medium (Sigma, St. Louis, MO) containing 10% fetal calf serum (Intergen, Purchase, NY), 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin (Gibco BRL, Gaithersburg, MD). Supernatants were collected after 24 h incubation for measurement of IL-2, IL-4, IL-6, IL-12 (p40 plus p70), TNF- α and monocyte chemoattractant protein 1 (MCP-1) and after 72 h incubation for IFN- γ and IL-10. The enzyme-linked immunosorbent assay (ELISA) kit from R&D systems (Minneapolis, MN) was used for measuring TNF- α levels and the kits from Endogen (Woburn, CA) were used for other cytokines.

Statistical analyses

Statistical analyses for ELISA data were performed using ANOVA-Scheffé test. $P < 0.05$ was accepted as the level of significance.

Results

Feeding OLL1073R-1/SM inhibited secretion of IFN- γ but not IL-2 by LNC

DBA/1 mice were fed with OLL1073R-1/SM, SM, or CRF-1 and immunized with bovine CII plus adjuvant complete H37Ra. LNC were pooled from each group of the mice and tested for secreting Th1 type cytokines of IL-2 and IFN- γ in response to CII (Figure 1). IFN- γ secretion was higher in cultures of LNC with CII than those without CII for each group of mice. The data showed a decrease in secretion of IFN- γ by LNC from mice fed with OLL1073R-1/SM, compared with those with the SM-fed and CRF-1-fed groups, as previously reported by us (Kano et al., 2002). This decrease was found in cultures with and without CII. Feeding SM also decreased IFN- γ secretion, compared with the control diet of CRF-1. However, the

Figure 1. Oral intake of OLL1073R-1/SM did not inhibit IL-2 production by LNC. Mice were fed OLL1073R-1/SM, SM or CRF-1 for 14 days and then immunized with bovine CII plus adjuvant complete H37Ra. Ten days after immunization of the mice, LNC were collected and cultured with or without bovine CII ($100 \mu\text{g ml}^{-1}$) for 24 or 72 h. Supernatants were collected and the amounts of IL-2 and IFN- γ were measured by ELISA. Data represent the mean $\pm$ SD of triplicate cultures. a = Significantly different from both SM and CRF-1 group ($P < 0.05$). b = Significantly different from the CRF-1 group ($P < 0.05$).

amount of secreted IL-2 for the OLL1073R-1/SM-treated group was similar to that in other groups, when LNC were cultured with CII.

Feeding OLL1073R-1/SM inhibited secretion of IL-10 but not IL-4 by LNC

We tested the effect of feeding OLL1073R-1/SM on secretion of Th2 cytokines of IL-4 and IL-10. The ELISA results (Figure 2) indicated that secretion of IL-10 by LNC cultured with CII in the group fed OLL1073R-1/SM was lower than that in the CRF-1-fed group, but similar to that in the SM-fed group as previously reported by us (Kano et al., 2002). However, secretion of IL-4 by LNC cultured either with or without CII was not affected by feeding OLL1073R-1/SM.

Feeding OLL1073R-1/SM inhibited secretion of IL-6 and TNF- α by LNC

The effects of feeding OLL1073R-1/SM on secretion of the proinflammatory cytokines IL-6, IL-12 and TNF- α were also tested (Figure 3). The secretion of IL-6 by LNC cultured with CII was decreased in the OLL1073R-1/SM-treated group, compared with those in both the SM and CRF-1-treated groups. Feeding OLL1073R-1/SM and SM inhibited secretion of TNF- α by LNC cultured with or without CII, compared with the CRF-1-fed group. However, the inhibitory effect of OLL1073R-1/SM was greater than that of SM ($P < 0.05$). Secretion of IL-12 by LNC cultured with CII was similar to that without CII in each

group. Only a small, but statistically significant difference was found in the level of IL-12 between the OLL1073R-1/SM-treated and two control groups.

Feeding OLL1073R-1/SM inhibited secretion of MCP-1 by LNC

The effect of OLL1073R-1/SM on LNC secretion of MCP-1 was tested (Figure 3). The ELISA result indicated that the amount of MCP-1 secreted by LNC of mice in the OLL1073R-1/SM-fed group was lower than those of the SM and CRF-1 groups, when LNC were cultured with or without CII. Feeding SM also inhibited secretion of MCP-1, although the inhibitory effect was weaker than that of OLL1073R-1/SM ($P < 0.05$).

Discussion

In the present study we have investigated possible mechanisms by which feeding OLL1073R-1/SM may inhibit the development of CIA. We studied the effect of feeding OLL1073R-1/SM on cytokine secretion by LNC from bovine CII-immunized mice. As previously reported (Kano et al., 2002), feeding OLL1073R-1/SM inhibited the production of both Th1 and Th2 cytokines, IFN- γ and IL-10, respectively, by LNC (Figures 1 and 2). However, oral intake of OLL1073R-1/SM did not inhibit the production of the Th1 and Th2 cytokines, IL-2 and IL-4, respectively (Figures 1 and 2). The change in cytokine secretion by LNC induced

Figure 2. Oral intake of OLL1073R-1/SM had no effect on secretion of IL-4 by LNC. The amounts of IL-4 and IL-10 in culture supernatants were measured by ELISA. Data represent the mean $\pm$ SD of triplicate cultures. a = Significantly different from both SM and CRF-1 group ($P < 0.05$). b = Significantly different from the CRF-1 group ($P < 0.05$).

Figure 3. Oral intake of OLL1073R-1/SM reduced secretion of proinflammatory cytokines and a chemokine by LNC. The amounts of IL-6, TNF- α , IL-12 and MCP-1 in culture supernatants were measured by ELISA. Data represent the mean $\pm$ SD of triplicate cultures. a = Significantly different from both SM and CRF-1 group ($P < 0.05$). b = Significantly different from the CRF-1 group ($P < 0.05$).

by OLL1073R-1/SM does not appear to be explained by modulation of the Th1/Th2 balance.

IL-2 has been shown to enhance IFN- γ synthesis by Th1 cells (Mosmann and Sad, 1996). The present study suggested that the reduced production of IFN- γ by feeding OLL1073R-1/SM was mediated by mechanisms other than suppression of the IL-2 response. Feeding OLL1073R-1/SM inhibited secretion of IL-12, although the inhibitory effect of OLL1073R-1/SM on secretion of IL-12 was smaller than that of IFN- γ . IL-12 can upregulate Th1 responses including IFN- γ secretion (Trinchieri et al., 1992). These results indicate that the decrease in IFN- γ secretion was only partly dependent on the IL-12 response.

Proinflammatory cytokines have been shown to be related to progression of CIA. In the present study feeding OLL1073R-1/SM inhibited secretion of the proinflammatory cytokines IL-6 and TNF- α by LNC (Figure 3). Inhibition of TNF- α secretion was the most marked response. Thus TNF- α would seem to be a good target for prevention of CIA. It has been shown by others that neutralizing TNF- α ameliorated disease activity, when anti-TNF- α antibody or soluble TNF- α receptor was administered before or shortly after the onset of CIA (Joosten et al., 1996; Wooley et al., 1993; Williams et al., 1992). Our data suggest that feeding OLL1073R-1/SM may prevent the development of CIA via inhibition of proinflammatory cytokine secretion.

Feeding OLL1073R-1/SM also inhibited secretion of the chemokine MCP-1 by LNC (Figure 3). The level of MCP-1 in the hind paws of arthritic mice was shown to increase as the severity of the disease increased (Parks et al., 1998). Our data suggest that reduced secretion of MCP-1 is part of the mechanism by which feeding OLL1073R-1/SM prevents progression of CIA.

Feeding SM also inhibited secretion of IFN- γ , IL-10, TNF- α and MCP-1, but not IL-2, IL-4, IL-6 and IL-12. However, inhibition of IFN- γ , TNF- α and MCP-1 secretion by OLL1073R-1/SM was greater than that by SM alone. OLL1073R-1/SM but not SM alone inhibited secretion of IL-6 and IL-12. Our previous report indicated that feeding SM inhibited CIA, although the inhibition was less marked than that by OLL1073R-1/SM (Kano et al., 2002). These data suggest that although SM contains components capable of inhibiting CIA and responses of proinflammatory cytokines, OLL1073R-1 bacteria modify those inhibitory molecules and/or produce additional inhibitory molecules.

We have previously reported that CIA and secretion of IFN- γ by LNC was inhibited by feeding polysaccharides of OLL1073R-1/SM (Kano et al., 2002). These polysaccharides may reduce the production of TNF- α by LNC. However, Kitazawa et al. (1996) have shown that secretion of IL-1 α and IFN- γ are upregulated in macrophages incubated with *Lactococcus* sp.-derived polysaccharide. This difference in cytokine responses may result from distinct molecules produced by each strain of *Lactobacillus* sp. and *Lactococcus* sp. Further investigation is necessary to examine this.

This study has shown that feeding OLL1073R-1/SM inhibits secretion of IFN- γ , IL-6, IL-10, IL-12, TNF- α and MCP-1, but not IL-2 and IL-4 by LNC from bovine CII-immunized mice. These data indicate that feeding OLL1073R-1/SM mainly inhibits secretion of proinflammatory cytokines. The most abundant cells in the synovial membrane of rheumatoid arthritis patients are macrophages and T cells (Feldmann et al., 1996). Macrophages can produce IFN- γ , IL-6, IL-10, TNF- α , IL-12 and MCP-1. Secretion of these cytokines was inhibited by feeding OLL1073R-1/SM. Secretion of IL-2 and IL-4 which macrophages do not produce was not inhibited by the same treatment. The results of downregulation by this feeding can be reasonably understood by postulating that macrophages were affected. However, it is still possible that the activity of other cells can be modulated. Secretion of IFN- γ from purified NK cells was increased in the presence of macrophages primed with *Lactobacillus johnsonii* La1 (Haller et al., 2000). Cytokine secretion of other cells may be modified through change in responses of macrophages. It is also possible that cytokines secreted from other types of cells changed in response to OLL1073R-1/SM. However, most reports note that *Lactobacillus* sp. can activate macrophages and enhance secretion of those cytokines *in vitro* and *in vivo* (Matsuzaki et al., 1998; Marin et al., 1997; Rangavajhyala et al., 1997; Solis-Pereyra and Lemonnier, 1993; Sato et al., 1988). This difference may result from different experimental conditions such as target cells, antigens and tested bacterial strains. In this study, we tested responses of popliteal lymph node cells to OLL1073R-1, while in other studies, peripheral blood mononuclear cells or spleen cells from normal animals were tested for responses to other bacterial species or strains. We need to further compare different lymphoid organs, antigens, mouse strains or bacterial strains for differential effects on macrophages. Feeding *Lactobacillus casei* strain Shirota

inhibited secretion of IFN- γ by spleen cells from CII-immunized mice (Kato et al., 1998), whereas the same strain enhanced the production of Th1 cytokines such as IFN- γ and IL-2 by spleen cells from mice immunized with foreign antigen of ovalbumin (Matsuzaki et al., 1998). These inhibiting and enhancing effects may depend on T cells rather than directly on macrophages. The inhibitory effect of OLL1073R-1/SM on secretion of proinflammatory cytokines may be dependent on the strain of *Lactobacillus* sp. employed. Our previous report demonstrated that feeding another strain of *Lactobacillus* sp., *Lactobacillus bulgaricus* OLL1102, did not suppress the production of IFN- γ (Kano et al., 2002). Although further inhibitory mechanisms need to be investigated, our study indicates that some *Lactobacillus* sp. may be effective in prevention of rheumatoid arthritis and other inflammatory diseases via their effects on proinflammatory cytokines.

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