The influence of the immunostimulation by bacterial cell components derived from altered large intestinal microbiota on probiotic anti-inflammatory benefits

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Abstract
Using murine macrophage-like J774.1 cells and fecal precipitates prepared from the feces of elderly volunteers whose acute inflammation had been inhibited by LKM512 yogurt consumption, we investigated the likelihood that immunostimulation by altered intestinal bacterial cell components contribute to the anti-inflammatory benefits of this yogurt. Tumor necrosis factor-α production due to stimulation by fecal precipitates obtained during LKM512 yogurt consumption tended to be higher than due to stimulation by precipitates obtained from preconsumption (P = 0.0827), although acute phase response was suppressed by LKM512 yogurt consumption. We suggest that the anti-inflammatory benefits of LKM512 yogurt on elderly volunteers are independent of direct immunostimulation by the bacterial cell components derived from altered intestinal microbiota.

Introduction
Some reports have demonstrated that the in vivo host cytokine pattern is improved by the consumption of probiotics or yogurt (Isolauri et al., 2000; Shimada et al., 2004). This benefit probably depends on two factors, namely, the intestinal bacterial cell components and the metabolites produced by the probiotics or altered intestinal microbiota. Many researchers demonstrated that the former stimulate lymphocytes (Hesse et al., 2000; Karlsson et al., 2004; Kimoto et al., 2004) and are recognized by Toll-like receptors (TLR) (Takeuchi et al., 1999). Therefore, it is generally believed that intestinal bacterial cell components play a more important role than bacterial metabolites in the control of the host cytokine pattern. However, the complexity of the intestinal microbiota, which number some 400–500 bacterial species with c. 1011 bacteria per gram of wet feces in the gut (Moore & Holdman, 1974; Finegold et al., 1983), was completely ignored in these experiments with a single culture. The essential probiotic benefits through the complex intestinal microbiota were not addressed by these experiments.

In our previous study, we administered Bifidobacterium animalis ssp. lactis LKM512 (Matsumoto et al., 2002, 2004)-containing yogurt (LKM512 yogurt) to hospitalized elderly volunteers and observed that the fecal haptoglobin content, which is used as a marker of acute inflammation, decreased due to LKM512 yogurt consumption (Matsumoto et al., 2001). Furthermore, using murine macrophage-like J774.1 cells and fecal extracts prepared from the feces of these volunteers, we concluded that the intestinal bacterial metabolites produced by LKM512 yogurt consumption contribute to suppressing the production of inflammatory cytokines by macrophages (Matsumoto & Benno, 2006).

In this study, using fecal precipitates prepared from the same feces samples as those used in the previous study, we investigated the possibility that direct immunostimulation by altered intestinal bacterial cell components induce the anti-inflammatory benefits of LKM512 yogurt.

Materials and methods
Preparation of the fecal precipitate
This study used the feces of six volunteers (three males and three females with an average age of 78.0 years) from whom feces could be collected in all of the designed test periods (preconsumption, 2 weeks of LKM512 yogurt consumption,
after consumption 2 weeks, and 2 weeks of placebo consumption) from the study on elderly volunteers (Matsumoto et al., 2000, 2001). Frozen feces were diluted 10-fold with 10 mM phosphate-buffered saline (PBS; pH 7.2) and washed intensely mixing for 1 min. After washing, the supernatant was removed after centrifugation (10 000 g for 20 min at 4 °C). After washing three times the precipitate was lyophilized. Bacteria in dried fecal precipitate was killed by Ultraviolet radiation for 16 h and used as the fecal precipitate. This precipitate was stored – 20 °C until use.

**Stimulation of J774.1 with the fecal precipitate**

The J774.1 cells were maintained in the same manner as that in previous study (Matsumoto & Benno, 2006). The J774.1 cells were suspended in culture medium at a concentration of 5 x 10⁶ cells mL⁻¹, and 1 mL of the cell suspension was plated in a Falcon 24-well culture plate (Becton Dickinson Labware, Oxnard, CA). The cells were incubated with 10 mg fecal precipitate at 37 °C for 48 h in a humidified atmosphere containing 5% CO₂. After incubation, we harvested the supernatants and stored at – 80 °C until needed for use.

**Measurement of the cytokine content in the culture medium**

We measured the concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-1α, and IL-10 using commercially available sandwich ELISA kits, namely, the Mouse TNF-α ELISA MAX Set Standard kit (BioLegend, Inc., San Diego, CA), Murine IL-1α ELISA Development Kit (PeproTech EC, London, UK), and Mouse IL-10 ELISA Development Kit (GT, Minneapolis, MN), respectively.

**The ratios of Gram-positive bacteria to Gram-negative bacteria in fecal precipitates**

Fecal precipitates were suspended in PBS and smeared on slide glasses. We performed Gram-staining and measured the number of Gram-positive bacteria and Gram-negative bacteria using microscope. The number of bacteria was counted in five random microscopic areas.

**Statistical analysis**

Changes in each cytokine caused by LKM512 yogurt and placebo consumption were analyzed using the paired t test. Calculations were performed using the computer software STATISTICA (Design Technologies Inc., Tokyo, Japan).

**Results and discussion**

Using the cell culture systems, it has been demonstrated that the cell components of probiotics and some intestinal commensal bacteria stimulate lymphocytes to alter the systemic cytokine production pattern (Hessle et al., 2000; Karlsson et al., 2004). However, the complexity of the intestinal microbiota has not been addressed in these experiments that employ single bacterial cultures. The present study was performed using fecal precipitates, and thus, all the findings of the present study reflect the complexity of the intestinal microbiota. In addition, fecal precipitates also contain unabsorbed diet ingredients which have antigenic. Therefore, in this study, we could also investigate the immunostimulation by diet ingredients. In adults, the colon contains 0.1% of fecal solids, which approximately half consists of bacterial cells (Cummings et al., 1990). Microorganisms are therefore a major component of feces, comprising c. 50% of fecal solids in a person consuming a British-style diet (Stephen & Cummings, 1980).

The effects of fecal precipitates on TNF-α, IL-1α, and IL-10 production by J774.1 cells are shown in Fig. 1. TNF-α concentrations preconsumption, during LKM512 yogurt consumption, after consumption, and during placebo consumption were 21.7 ± 11.3, 31.3 ± 9.7, 28.4 ± 11.2, and 27.2 ± 12.0 ng mL⁻¹, respectively (mean ± SD). In four of the six volunteers, TNF-α production due to stimulation by the fecal precipitates collected during LKM512 yogurt consumption tend to be higher than that due to stimulation by precipitates collected before consumption (P = 0.0827). There was no change in IL-1α and IL-10 concentrations throughout the test periods and some amount of variation was observed between individuals.

The ratios of Gram-positive bacteria to Gram-negative bacteria preconsumption, during LKM512 yogurt consumption, and during placebo consumption are shown in Fig. 2. The ratios of Gram-positive bacteria to Gram-negative bacteria during preconsumption, LKM512 yogurt consumption tend to be higher than the preconsumption baseline level by the fecal precipitates taken during the period of LKM512 yogurt consumption. There was no change in IL-1α and IL-10 concentrations throughout the test periods.

![Fig. 1. The effects of fecal precipitates on TNF-α, IL-1α, and IL-10 production by J774.1 cells. TNF-α production in J774.1 cells stimulated by the fecal precipitates taken during the period of LKM512 yogurt consumption tend to be higher than the preconsumption baseline level on the basis of paired t-test (P = 0.0827). There was no change in IL-1α and IL-10 concentrations throughout the test periods.](image-url)
are influenced by intestinal bacterial metabolites than preconsumption (Matsumoto & Benno, 2006). These data than that due to stimulation by precipitates obtained during LKM512 yogurt consumption was lower than that due to stimulation by fecal precipitates obtained from all the volunteers would be identical if diet ingredients contribute to these results, because all the volunteers were hospitalized and were on almost identical diets (Matsumoto et al., 2000). The TNF-α and IL-10 production patterns of the individuals were not identical (data not shown). Thus, we believe that diet ingredients influence these results to a very slight extent.

This study demonstrated one of the mechanisms of the anti-inflammatory benefits of LKM512 yogurt on elderly volunteers who have developed immune tolerance and complex large intestinal microbiota for a long time. Therefore, their host cytokine pattern may not be influenced by the temporary changing of immunostimulation due to large intestinal bacterial cell components. Anti-inflammatory benefits of probiotics on adults and elderly volunteers are
probably induced by anti-inflammatory metabolites produced by altered large intestinal microbiota rather than direct immunostimulation of bacterial cell components derived from altered large intestinal microbiota. However, we agree that cell components from probiotics and the altered large intestinal microbiota can directly stimulate the immature immune system in infants who have simple large intestinal microbiota.

Based on these findings, we suggest that the anti-inflammatory benefits of LKM512 yogurt on elderly volunteers are independent of direct immunostimulation by the bacterial cell components derived from altered large intestinal microbiota.

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References


