Stimulation of Cytokine Gene Expression in a Murine Macrophage Cell Line by Lactic Acid Bacteria Isolated from Bhutanese Dried Yak Cheese

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Lactic acid bacteria (LAB) isolated from dried yak cheese from the Bhutan Himalayas were examined for their ability to stimulate the expression of cytokine mRNA on the murine macrophage. The expression of tumor necrosis factor- α , interleukin (IL) 6 or IL-12 p40 gene was stimulated by several strains. The dried yak cheese, *Chugo*, contains LAB that possess immuno-modulating activity.

Key words: lactic acid bacteria; cytokine; macrophage; fermented food

Lactic acid bacteria (LAB) are found in various fermented foods, and their beneficial effects on human health and livestock animals have been stressed (12). Among them, stimulation of the mucosal immune system is one of the most beneficial functions of lactic acid bacteria. A wide range of lactobacilli can stimulate macrophages to secrete pro-inflammatory cytokines which are responsible for the enhancement of the mucosal immune system (4, 11, 14). Many traditional fermented foods that contain LAB are available in Asian countries, and these foods may have beneficial effects on health. Many LAB have been isolated from the cheeses available in Asian countries (2, 3, 9, 20). However, their immunological effects have not been examined in detail. In this study, we isolated LAB from *Chugo*, dried yak cheese, donated or purchased from the local people of northwestern Bhutan, mainly in the Lunana area, and examined their ability to stimulate cytokine production by murine macrophages *in vitro*. Over 100 strains of LAB were isolated from 13 samples of *Chugo* cheese. A portion (0.2 to 0.5 g) of the cheese was suspended in 10 ml of phosphate buffer (50 mM, pH 7.5) and inoculated to LBS (Becton, Dickinson and Company, Cockeysville, MD, USA) and MRS (Becton, Dickinson and Company, Sparks, MD) agar plates. These media were incubated at 37°C under aerobic and anaerobic conditions. Twelve rod-shaped bacteria were selected from 108 isolates and divided into 4 groups according to their morphology and tolerance to oxygen. One strain was randomly selected from each group.

Four selected LAB were incubated in MRS broth at 37°C. After the collection of bacterial cells, a portion was used for DNA extraction (21). The 16S rDNA was amplified by polymerase chain reaction (PCR) with primers 907f (5'-AAACTCAAATGAATTGACGGG-3') and univ-R (5'-ACGGGCGGTGTGTACAAG-3') with *rTaq* polymerase (TOYOBO, Tokyo, JAPAN). The PCR products were sequenced with an LI-COR autosequencer (LIC-4200L; Aloka, Tokyo) using a Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech, Tokyo). The obtained sequences were subjected to a BLAST search with known sequences in the GenBank.

The rest of the bacterial cells were washed and suspended in phosphate buffered saline (PBS) to obtain 10^8 cells/ml. Then they were heat inactivated at 100°C for 10 min. The RAW 264.7 murine macrophage cell line was maintained in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Biological Industries, Kibbutz Beit Haemek, ISRAEL), 50 μ M 2-mercaptoethanol, 1 mM HEPES, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in 5% CO₂. Cultivated macrophages were spread onto a 24-well flat-bottomed plate with 6×10^5 cells/2 ml/well in fresh medium. Then, 100 μ l of the heat-inactivated bacterial suspension was added to each well. For the control, 100 μ l of the sterilized PBS was added to each well instead of the bacterial suspension. After 24 h incubation at 37°C in 5% CO₂, total RNA was extracted from the macrophages with ToTALLY RNA Kit (Ambion, Austin, TX, USA) according to the manufacturer's directions. Total RNA (100 ng) was applied to the

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Table 1. Isolated lactic acid bacteria from Bhutanese dried yak cheese

Strain	Medium	Facultative/obligate	Shape	Closest relative
28-2	LBS	Facultative	Rod	
28-2-2*	LBS	Facultative	Rod	<i>L.parakefiri</i>
12115-15	LBS	Facultative	Rod	
1219-03*	LBS	Obligate	Rod	<i>L.plantarum</i>
1219-09	LBS	Obligate	Rod	
1219-12	MRS	Obligate	Rod	
1219-21	LBS	Obligate	Rod	
0108-1*	LBS	Obligate	Long rod	<i>L.paracasei</i>
0108-2-2	LBS	Obligate	Long rod	
0108-4	LBS	Obligate	Long rod	
0108-4-2	LBS	Obligate	Long rod	
1211-06*	MRS	Facultative	Short rod	<i>Leuconostoc</i> sp.

*Strains which were used for identification and *in vitro* experiments with the murine macrophage cell line RAW 264.7.

reverse transcription-PCR (RT-PCR) with TaKaRa RNA PCR Kit Ver. 3.0 (Takara Bio, Kyoto, JAPAN). Reverse transcription was performed with Oligo dT primer at 60°C for 30 min. The primers for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (5'-TGAAGGTCGGTGTGAACGGATTTGGC-3' and 5'-CATGTAGGCCATGAGGTCCACCAC-3'), IL-6 (5'-ATGAAGTTCCTCTCTGCAAGAGACT-3' and 5'-CACTAGGTTTGCCGAGTAGATAT-3'), TNF- α (5'-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3' and 5'-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3'), and IL-12 p40 (5'-CCA CTCACATCTGCTGCTCCACAAG-3' and 5'-ACTTCTCATAGTCCCTTTGGTCCAG-3') used for the PCR, were purchased from Clontech (Palo Alto, CA, USA). The PCR was performed with a temperature program of 3 min at 94°C; 30 cycles of 20 s at 94°C, 45 s at 60°C, and 1 min at 72°C; followed by extension for 5 min at 72°C. After the PCR products were electrophoresed, the bands were quantified with NIH Image (National Institute of Health, Bethesda, MD). The density of each mRNA band was normalized to that of G3PDH. The results were expressed as means with standard deviations of three independent experiments. The differences between the values obtained with and without cell suspension were statistically analyzed by a Bonferroni/Dunn analysis after one-way ANOVA.

Twelve strains of rod-shaped LAB were selected (Table 1). The partial sequences of 16S rDNA (containing V6, V7, and V8 regions) retrieved from strains 28-2-2, 0108-1, 1211-06, and 1219-03 were similar to those of *L.parakefiri* [GenBank accession number AY026750, 98% similarity], *L.paracasei* [AJ271854, 97% similarity], *Leuconostoc* sp.

[AF349931, 98% similarity], and *L.plantarum* [AJ272031, 95% similarity], respectively. The strain 1211-06 significantly stimulated the expression of TNF- α , and strain 0108-1 stimulated the expression of IL-6 and IL-12 p40 genes in murine macrophages (Fig. 1). The level of mRNA was determined to investigate the cytokine production, since cytokine production at the protein level reflects the expression of the cytokine gene (6–8). Consequently the strain 1211-06 stimulated TNF- α production, and the strain 0108-1 stimulated IL-6 and IL-12 p40 production. TNF- α production by macrophages is one of the anti-tumor characteristics of lactobacilli (11). Therefore, strain 1211-06 may have the potential to inhibit tumor growth.

Strain 0180-1 stimulated expression the of IL-12 p40 and IL-6 genes. Many probiotic lactobacilli have been reported to enhance IL-12 and IL-6 production (4, 14, 19). IL-12 induces the proliferation of Th1 cells (10, 18). Induction of a Th1-type immune response is critical for the prevention and treatment of type-1 allergic diseases in which Th2-type immune responses exceed those of the Th1 type (1). IL-6 promotes differentiation of B cells into plasma cells and enhances the secretion of IgA (3, 13, 17). In addition, IL-6 polarizes naïve CD4⁺ T cells transforming them to effector Th2 type cells (16). Furthermore, IL-6 activates pathogen specific activity with block of the suppressive activity of CD4⁺ CD25⁺ T_R cells (15). Therefore, the strain 0180-1 may modulate the host immune system through the stimulation of cytokine production of antigen present cells, such as macrophages and dendritic cells.

Although the identification of LAB in this study was incomplete, the dried yak cheese, *Chugo*, contains LAB that possess immuno-modulating activities.

Fig. 1. The mRNA expression of cytokines in the murine macrophage cell line RAW 264.7 cultured with lactic acid bacteria isolated from Bhutaneese dried yak cheese. Total RNA was extracted from cells, and RT-PCR was performed. The quantification of each PCR product was performed by the method described in the text. The results were expressed by means and standard deviations of three independent experiments. *Values significantly different ($p < 0.05$) from control.

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