Journal of Bioscience and Bioengineering
VOL. 112 No. 4, 363 – 368, 2011

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TLR ligands of *Lactobacillus sakei* LK-117 isolated from seed mash for brewing sake are potent inducers of IL-12

Yasuyuki Masuda,[1,*] Toshinari Takahashi,[1] Kazutoshi Yoshida,[2] Yosuke Nishitani,[3] Masashi Mizuno,[4] and Haruhiko Mizoguchi[1]

General Research Laboratory, Kiku-Masamune Sake Brewing Co. Ltd., 1-8-6 Uozaki-nishimachi, Higashinada-ku, Kobe, Hyogo 658-0026, Japan,[1] Hyogo Prefectural Institute of Technology, 3-1-12 Yukihira-cho, Kobe, Hyogo 654-0037, Japan,[2] Organization of Advanced Science and Technology, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe, Hyogo 657-8501, Japan,[3] and Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe, Hyogo 657-8501, Japan[4]

Received 24 November 2010; accepted 18 June 2011
Available online 8 July 2011

Many studies have investigated the immunostimulatory effects of bacteria, such as the anti-allergic effects of lactic acid bacteria (LAB) and LAB-fermented milk. Importantly, these anti-allergic effects have been observed for both viable and nonviable bacteria. However, there are no reported immunological effects of LAB isolated from *kimoto*, the traditional yeast starter culture used for brewing sake, which also involves spontaneous lactate fermentation. In this study, we determined whether the *Leuconostoc mesenteroides* and *Lactobacillus sakei* bacterial strains obtained from *kimoto* affected the production of interleukin-12 (IL-12), an inducer of the T-helper type-1 immune response. By incubating autoclaved bacteria with J774.1 macrophage-like cells, we found that *L. sakei* LK-117 induced a sustained increase in IL-12p40 production. The IL-12-inducing ability of LK-117 was unaffected by anti-TLR2 neutralization and was entirely inhibited when the LK-117 cells were treated with RNase. When LK-117 cells were treated with M-1, an *N*-acetylmuramidase, at varying concentrations and for different periods of time, the ability of the bacteria to induce IL-12 decreased quickly. Although an active fraction could be prepared by chromatography from the soluble products of enzymolysis, the fraction's induction ability was <2% of that of intact organisms, and induction ability disappeared completely upon anti-TLR2 neutralization after treating the active fraction with RNase. These results suggest that single-stranded RNA released from cells that were disrupted by autoclaving might act as a TLR ligand and provide a novel mechanism in which heat-killed LAB could be used to regulate immune activity.

[**Key words:** Macrophage; *Lactobacillus sakei*; Sake brewing; IL-12; Toll-like receptor; TLR ligand; Th1 immune response]

Recently, allergy-related diseases have become more common in urban areas compared with rural areas. In particular, the number of cases of atopic diseases, such as pollinosis, atopic eczema, allergic rhinitis, and asthma, has increased in urban areas. Many studies have focused on developing anti-allergy agents, and a number of such agents are commercially available. One representative agent, a histamine antagonist, inhibits the action of histamines by blocking histamine receptors, but this anti-histamine offers only temporary symptomatic relief. Furthermore, curative drugs, such as steroids that are prescribed to patients with severe allergies, may have adverse effects. Therefore, a need remains for more extensive and novel allergy cures.

According to the hygiene hypothesis, when the level of an infection decreases, suppressive factors against the infection are lost from a host's immune repertoire, and the likelihood of becoming allergic to an allergen is increased accordingly (1). This hypothesis is, in part, the reason that the immunomodulating effects of lactobacilli, which are present in many foods and are frequently used as probiotics, have been studied extensively (2,3).

Common allergic reactions include a wide range of various atopic diseases. During an allergic response, exogenous antigens, such as pollen and mites, are taken into antigen-presenting cells (APCs) and are processed into fragments. APCs then present these fragments to naïve $CD4^+$ T cells, and the $CD4^+$ T cells are subsequently induced to differentiate into antigen-specific T helper (Th) cells (i.e., Th1 and Th2 cells). The major cytokine secreted by Th2 cells, interleukin (IL)-4, stimulates immunoglobulin class switching in B cells and promotes the synthesis of IgE antibodies. Furthermore, IgE is secreted and binds to high-affinity receptors on the surface of mast cells. When sensitized populations of cells subsequently encounter the same allergen, IgE-coated mast cells release substances, such as histamine, that cause swelling and/or inflammation in the surrounding tissues.

The interaction of APCs with lactobacilli stimulates the production of cytokines, such as IL-12 and IL-18 (4,5). Furthermore, IL-12 secreted by APCs induces differentiation of the Th1 cells that subsequently impair Th2 cell activity by secreting both IFN-γ and IL-2. Thus, lactobacilli appear to reduce the Th2-mediated allergic

* Corresponding author. Tel.: +81 50 2403 6136; fax: +81 78 854 1058.
E-mail address: y-masuda@kikumasamune.co.jp (Y. Masuda).

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doi:10.1016/j.jbiosc.2011.06.007

response by shifting the Th1/Th2 balance from a Th2-dominant state toward a Th1-dominant state.

However, the ability of lactobacilli to induce IL-12 production is strain-dependent but not dependent on species (6). Specifically, it has been reported that lactic acid bacteria (LAB) strains isolated from fermented foods, including soy sauce, miso, and traditional Japanese pickles, in addition to strains from dairy products strongly induce IL-12 production (7,8). Many of the probiotic effects obtained from viable cells were also obtained from populations of dead cells (9). This paradox is explained by the fact that live probiotic cells influence both the gastrointestinal microflora and the immune response, whereas the components of dead cells primarily affect the intestinal immune system. Although, compared with live cells, less attention has been paid to the immunomodulatory effect of killed LAB, the effect of dead cells appears to be bacterial strain-dependent (10). The use of killed LAB as a potent immunomodulator presents certain advantages, such as a long shelf life.

Although it has been reported that immunomodulatory LAB can be isolated from traditional plant-derived fermented foods, to our knowledge, there have been no reports of such activity associated with the LAB used in traditional seed mash making for brewing sake, which are limited to *Lactobacillus sakei* and *Leuconostoc mesenteroides* (11). Unlike the LAB species that spoil sake, such as *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus hilgardii*, *L. sakei* and *L. mesenteroides* are capable of growing at relatively low temperatures, and they are completely killed during the late stage of the seed mash because of their extreme sensitivity to alcohol (12). Accordingly, it is of considerable interest to determine how bacteria with such different properties exhibit immune-stimulating activity.

In this study, we screened a collection of LAB that were isolated from seed mash used for brewing sake to identify strains that potently induce IL-12 production, investigating which component (s) may be strong IL-12 inducers that acts as Toll-like receptor (TLR) ligands. Our ultimate goal was to determine the mechanism of efficient bacterial killing that could these products to be used as immune-stimulators.

MATERIALS AND METHODS

Bacteria The LAB strains used in this study were five strains of *L. sakei* (LT-13, LK-117, LK-127, LK-133, and LK-134), two strains of *L. mesenteroides* (LK-16 and LK-103), three strains of *Lactobacillus plantarum* (LK-25, LK-64, and LK-132), two strains of *L. casei* (LK-32 and LK-53), two strains of *Lactobacillus curvatus* (LK-141 and LK-142), and *Lactobacillus citreum* LK-95. All of these strains, except LT-13, were isolated from seed mash during the first half of the *kimoto*-making process, and the strains were identified by 16S rRNA sequence analysis using BLAST homology. LT-13 was purchased from the Japan Collection of Microorganisms (Wako, Japan). LAB cultures were grown in 10 mL of deMan Rogosa and Sharpe (MRS) broth (BD Biosciences, Franklin Lakes, NJ, USA) at 30°C for 2 days, harvested by centrifugation at 16,000 × *g* for 15 min, and washed twice in 10 mL of distilled water. The cells were sterilized by autoclaving at 121°C for 10 min and were subsequently lyophilized.

Stimulation of macrophage-like cells with LAB The murine macrophage cell line J744.1 was purchased from the Human Science Research Resources Bank (Osaka, Japan). The cells were grown in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Tissue Culture Biological, Los Alamitos, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2 atmosphere. Cells (1.25×10^5/100 μL) were first pre-cultured in 96-well plates at 37°C for 90 min, and 100 ng of lyophilized bacteria was that subsequently suspended in 10 μL of sterile water was transferred into each well. This mixture was subsequently incubated at 37°C for an additional 18 h. To examine the effects of M-1 enzymatic decomposition products, 10 μL of the M-1 reaction mixture supernatant or its concentrated form was heated at 100°C for 5 min and incubated with the J744.1 cells.

For the TLR2 blocking assays using anti-TLR2 neutralization, 3 μg of anti-TLR2 antibody (clone T2.5, Cosmo Bio, Tokyo, Japan) or control antibody (Bay Bioscience, San Diego, CA, USA) was added to each well of the pre-culture plates, followed by the addition of lyophilized bacteria or their decomposition products. Each antibody was added in combination with lyophilized bacteria at the same time for subsequent culture.

After 18 h incubation, the supernatants were subjected to IL-12p40 and IL-10 quantification by enzyme-linked immunosorbent assay (ELISA) using murine IL-12p40 and IL-10 ELISA sets (BD Biosciences, Franklin Lakes, NJ, USA), respectively, as per the manufacturer's instructions.

Enzymatic treatment with M-1 *N*-acetylmuramidase Bacteria were suspended at a density of 2 mg dry weight (DW)/mL in 50 mM Tris-maleate buffer (pH 7.0) containing 4 mM $MgCl_2$ and M-1 enzyme (Seikagaku Corp., Tokyo, Japan) in a concentration range of 1–10 μg/mL, and the mixture was incubated at room temperature for up to 120 min. The reaction at each time point was terminated by heating in a boiling water bath for 5 min, unless otherwise noted. To estimate the degree of protoplast formation, we added a 10% sodium dodecyl sulfate solution to the reaction mixtures to achieve a final concentration of 2%, stirring vigorously to dissolve spheroplasts resulting from digestion of the cell wall (13). Finally, we measured the optical density of the mixtures at 600 nm (OD_{600}) as an approximate indicator of protoplast formation.

Molecular weight distribution of LAB enzymatic decomposition products - Size-exclusion chromatography experiments were performed using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Briefly, 20 μL of sample was loaded onto a TSK-gel G2000SWXL (Tosoh, Tokyo, Japan) and eluted at 0.5 mL/min with a mobile phase containing 20 mM phosphate-buffered saline (PBS; pH 6.8) and 0.3 M NaCl. Elution profiles were monitored by ultraviolet light absorbance at 280 nm. Estimation of peak molecular weights was performed using the following five molecular weight standards purchased from Merck (Darmstadt, Germany): glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), myokinase (32 kDa), and cytochrome *c* monomer (12.4 kDa).

Fractionated concentrate of decomposition products The reaction mixture supernatants, which contained 50 mM Tris-maleate buffer (pH 7.0), 4 mM $MgCl_2$, M-1 enzyme, and 2 mg DW LAB cells per mL, were applied to diethylaminoethyl (DEAE)-cellulose columns (2.0 × 30 cm) that were equilibrated with 10 mM Tris–HCl buffer (pH 7.4), and the columns were washed with the same buffer at a flow rate of 3.2 mL/min. After collection of the unadsorbed fractions, the adsorbed fractions were eluted stepwise with increasing concentrations of NaCl from 0.2 to 0.5 M in the same buffer. Elution was monitored by absorbance at 280 nm, and the peak fractions were concentrated by ultrafiltration with an Amicon Ultra 10 K device (Millipore, Billerica, MA, USA). For desalting, we applied the concentrates to PD-10 desalting columns (GE Healthcare, Uppsala, Sweden). Finally, ultrafiltration was used to re-concentrate each fraction 5-fold relative to the volumes of the original supernatants.

Scanning electron microscopy (SEM) Cells treated with M-1 enzyme were washed twice with 0.1 M phosphate buffer (pH 7.4) and were subsequently fixed in 2% glutaraldehyde overnight at 4°C. The fixed cells were washed with phosphate buffer and sterile water and were dehydrated using a graded ethanol series and *t*-butyl alcohol. The cells were subsequently freeze-dried, mounted on carbon tape, and sputter-coated with platinum and palladium *in vacuo*. SEM images were obtained using an XL30-SFEG FEI scanning electron microscope (FEI Company, Eindhoven, Netherlands).

Composition analyses We determined the composition of amino acids by hydrolyzing the cell decomposition products in 6 N HCl and analyzing the hydrolysates with an amino acid analyzer (Hitachi L-7000, Tokyo, Japan). Amino acid analyses of proteins and peptides were performed separately, according to a previously reported method (14). Protein concentrations were determined by the Bradford assay, with bovine serum albumin used as the standard (15).

RESULTS AND DISCUSSION

IL-12-inducing ability of LAB isolated from *kimoto* There is evidence that nonpathogenic gram-positive bacteria may be strong inducers of IL-12 in mononuclear adherent cells (16). In the present study, we tested 14 strains of gram-positive *kimoto* LAB for their ability to induce the secretion of both IL-12 and IL-10 after 18 h of culture with J744.1 macrophage-like cells. All of the strains induced IL-12p40 secretion, although at widely varying levels (Fig. 1A). Furthermore, species identification by 16S rRNA sequence analysis demonstrated that most of the potential IL-12 inducers belonged to *L. sakei*, a characteristic LAB commonly present in *kimoto*. Importantly, we observed that *L. sakei* LK-117 was the strongest IL-12p40 inducer (>140 ng/mL). In addition, we found that almost all LAB strains also induced IL-10 synthesis, although the induced IL-10 levels were much lower than the induced levels of IL-12p40 (Fig. 1B).

It has been suggested that improved public hygiene and exposure to fewer infections in urban life may reduce the Th1 response present in individuals (1), thereby increasing the risk of developing allergies because of IgE production and eosinophilia (17,18). Based on these findings, augmentation of the Th1 response may be beneficial in combating allergies, and lactobacilli may be effective for affecting the Th1 response through the induction of IL-12, which