Inhibitory Activity of *Bifidobacterium longum* HY8001 against Vero Cytotoxin of *Escherichia coli* O157:H7

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ABSTRACT

Vero cytotoxin (VT)-producing *Escherichia coli* (VTEC), such as *E. coli* O157:H7, are emerging foodborne pathogens worldwide. VTs are associated with hemorrhagic colitis and hemolytic uremic syndrome in humans. Attachment of the B subunit of VTs to its receptor, globotriaosylceramide (Gb₃), at gut epithelium is the primary step and, consequently, the A subunit of VTs inhibits protein synthesis in the target cell. Proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, up-regulate Gb₃ expression, increase sensitivity to VTs, and enhance VT action in developing disease. Currently, there is a growing interest in probiotics, given the increasing occurrence of antibiotic-resistant bacteria. In particular, much work on bifidobacteria among probiotics, regarded as microorganisms targeted for technological and therapeutic applications, has been performed. In Korea, the neutralizing effect of the culture supernatant of *Bifidobacterium longum* HY8001, Korean isolate, against the VTs from *E. coli* O157:H7 was found. Therefore, this study focused on the raveling of the inhibitory effect of *B. longum* HY8001 against VTs, through the interference B subunit of VTs and Gb₃ interaction. Mice were inoculated intragastrically with *B. longum* HY8001 culture supernatant before and after challenge with *E. coli* O157:H7. Control mice were inoculated intragastrically only with *E. coli* O157:H7. Cytokine, TNF-α, and IL-1β levels in sera and expression of their mRNA were decreased, and expression of Gb₃ in renal tubular epithelial cells was reduced in mice treated with *B. longum* HY8001 culture supernatant. In competitive enzyme-linked immunosorbent assays (ELISAs), the culture supernatant of *B. longum* HY8001 primarily binds VTs to interfere the VTs with Gb₃ interaction. These results suggest that soluble substance(s) in *B. longum* HY8001 culture supernatant may have inhibitory activity on the expression of Gb₃, VT-Gb₃ interaction, or both. Further study should be done to elucidate the property of soluble substances in *B. longum* HY8001 culture supernatant.

Enterohemorrhagic *Escherichia coli* (EHEC), such as *E. coli* O157:H7, is one of the emerging foodborne pathogens worldwide (1, 16, 17) and produces one or more Vero cytotoxins (VTs). VTs are the major virulence factor of EHEC and are believed to be associated with hemorrhagic colitis and life-threatening hemolytic uremic syndrome in humans (18, 28). VT is a 69-kDa complex of proteins comprised of five receptor-binding B subunits (7 kDa each) and one A subunit with enzymatic activity (30 kDa) (29). The B subunit recognizes glycosphingolipids on the target eukaryotic cell surface, and the A subunit cleaves an adenine residue from the 28S rRNA within the 60S ribosome and, consequently, inhibits protein synthesis in the target cell (7, 22, 26).

Globotriaosylceramide (Gb₃; Galα1-4-Galβ1-4 glucosyl ceramide) is the functional receptor for VTs (5, 24, 29), and attachment of the B subunit of VTs to Gb₃ at gut epithelium is the primary step to develop disease (27). The importance of the B subunit-Gb₃ interaction is clearly illustrated by the fact that all cells susceptible to VTs express Gb₃ on their cell surfaces, whereas cells that do not express Gb₃ are resistant to the toxins (22, 23). Furthermore, reconstitution of Gb₃-deficient, toxin-resistant mutant cell lines with exogenous Gb₃ restores sensitivity (34).

Proinflammatory cytokines also have an important role, as they enhance VT action in developing hemolytic uremic syndrome. In vitro cultures of vascular endothelial cells, it has been found that the cytotoxic activity of VTs is markedly potentiated by proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1, which interact with vascular endothelial cells, then up-regulate the synthesis of the Gb₃ receptors (13, 31).

Currently, there is a growing interest in probiotics, microbial cell preparations, or components of microbial cells that have a beneficial effect on the health and well-being of the host (17), with the increasing occurrence of antibiotic-resistant bacteria by overuse of antibiotics (4, 6, 11, 25, 30, 37). Bifidobacteria, among probiotics, are important microorganisms in the active and complex ecosystem of the intestinal tract of humans and other warm-blooded animals (10). Much work on bifidobacteria, regarded as microorganisms targeted for technological and therapeutic applications, has been performed (10), and several studies have shown the possible mechanisms responsible for therapeutic effects of bifidobacteria (3, 8, 9, 19). Bifidobacteria may...
promote the immunologic and nonimmunologic defense barriers in the gut (3) and prevent pathogen adherence or pathogen activation via production of inhibitory metabolites, such as organic acids, hydrogen peroxide, bacteriocins, and deconjugated bile salts (8, 9), or via ferrous iron uptake, thus making it unavailable for pathogens (19). In Korea, Yang et al. (35) showed that the cytopathic effect of the VT of E. coli O157:H7 was neutralized by the culture supernatant of Bifidobacterium longum HY8001, Korean isolate. This represents the presence of soluble VT-neutralizing substance(s) in the B. longum HY8001 culture supernatant and the possibility of using B. longum HY8001 for treatment of VT-producing E. coli (VTEC) infection (35). Furthermore, antibiotic use is controversial for treatment of VTEC infection, because antibiotics can induce the bacterial SOS response, with resulting VT prophage induction, which is associated with increased VT production (36). In this respect, the use of probiotics, such as bifidobacteria, may have beneficial effects on VTEC infection and is possibly a treatment for VTEC infection.

This study focused on the raveling of the inhibitory effect of B. longum HY8001 against VTs, through the interference in the interaction of VTs with Gb3, to assess the possibility of using B. longum HY8001 for treatment of VTEC infection.

**MATERIALS AND METHODS**

**Bacterial strains and culture.** The bacterial strains used in this study were E. coli O157:H7 (ATCC 43894), which produces both VT1 and VT2, and B. longum HY8001, Korean isolate, from Korea Yakult Co., Ltd. For inoculation into mice, E. coli O157:H7 was grown in tryptic soy broth (Difco Laboratories, Sparks, Md.) at 37°C overnight in the shaking incubator (90 rpm), harvested by centrifugation (30,000 × g, 20 min; Union 32R, Hanil Science, Korea), and resuspended in phosphate-buffered saline (PBS), pH 7.2, at a concentration of 4 × 10^8 CFU/ml. B. longum HY8001 was grown in blood-glucose-liver broth at 37°C for 16 h and harvested by centrifugation (30,000 × g, 20 min; Union 32R), and the supernatant was inoculated into mice. For enzyme-linked immunosorbent assays (ELISAs), overnight cultures of E. coli O157:H7 in tryptic soy broth were incubated for an additional 6 h with ciprofloxacin (2 mg/ml in 5% dextrose) for increased production of VTs (36). The culture supernatant was used for the ELISA after centrifugation (30,000 × g, 20 min; Union 32R).

**Experimental animals.** Female specific pathogen-free BALB/c mice at 5 weeks of age from the Laboratory Animal Center at Seoul National University were used. Mice were randomly assigned to treatment cages (three per cage for the negative control group and five per cage for the other groups) and were kept in the temperature- and humidity-controlled housing facility (MJ-72ICS, Myung-Jin, Korea) with a 12-h light:dark cycle. Mice were used experimentally after acclimating for 1 week in the housing facility. Food, water, and wood-chip bedding for mice were autoclaved before use.

**Experimental design.** Mice were divided into four groups (1 through IV) and fasted (except for water supply) for 24 h before challenge. An E. coli O157:H7 suspension (0.5 ml of 4 × 10^8 CFU/ml per mouse) was challenged intragastrically with a stainless steel tube (Zonde, 18G) into mice of group II (32). The supernatant of B. longum HY8001 (0.5 ml per mouse) was inoculated with Zonde into mice of groups III and IV for 5 days before and was inoculated into mice of group IV for 7 days after challenge. Mice in the control group (I) received 0.5 ml of PBS.

Mice were examined for gastrointestinal (changes in fecal color and consistency) and systemic (lethargy, anorexia, ruffled fur, and restlessness) symptoms every 8 h after challenge. After bacterial inoculation, fecal samples were collected from each mouse. They were suspended in brain heart infusion broth and plated on O157:H7 ID medium, a selective chromogenic medium for the detection of E. coli O157:H7 (BioMerieux, France), to assess colonizing ability. Mice were sacrificed 7 days after challenge and subjected to full necropsy. Intestines and kidneys were obtained for reverse-transcription polymerase chain reaction (RT-PCR) and immunohistochemistry, and blood samples from each mouse were also obtained for cytokine assay.

**Cytokine assay.** The Quantikine M murine TNF-α kit and the Quantikine M murine IL-1β kit from R&D systems Inc. (Minneapolis, Minn.) were used for serum TNF-α and IL-1β. Procedure of the ELISA followed the manufacturer's directions. The concentration of TNF-α and IL-1β in serum was expressed as picograms per milliliter.

RT-PCR was applied to analyze the expression of mRNA for TNF-α, IL-1β, and β-actin (internal control) in the intestines and kidneys from each mouse. Total RNA was isolated from intestines and kidneys using TRIzol reagent (Gibco BRL, Grand Island, N.Y.) according to manufacturer's directions. RT was performed with the SuperScript First-Strand Synthesis System for RT-PCR (Gibco BRL) according to manufacturer's directions. The primers were designed based on the previous study (15), and the forward and reverse primers used were 5′CTCTTTCAAGGGA-CAAGGTCTG3′ and 5′CGGACTCCCGAAAATGTCAG3′ for TNF-α, 5′GCAACTGTTCCTGAACTC3′ and 5′CTCGGAG- CTTGATCGAC3′ for IL-1β, and 5′ATGGATGAGCGA- TAGCCTG3′ and 5′ATGAGGTAGTCTGAGTGTTG3′ for β-actin, respectively. The 25 μl of PCR reaction mixture contained 1.5 μl of 25 mM MgCl2, 1 μl of 10 mM dNTPs, 20 pmol of each primer, 1 unit of Taq DNA polymerase (Promega, Madison, Wis.), and 2 μl of cDNA in diethylpyrocarbonate–treated water. The thermal cycles consisted of denaturation at 94°C for 30 s, annealing (TNF-α, 54°C; IL-1β, 56°C; and β-actin, 58°C) for 30 s, and extension at 72°C for 5 min. Amplification was performed for 35 cycles in a GeneAmp PCR system (Model 2400, Perkin-Elmer Co., Norwalk, Conn.). The PCR-amplified products were fractionated on 2.0% agarose gel and analyzed using a model GS-710 imaging densitometer (BIO-RAD Laboratories, Inc., Hercules, Calif.) scanner and PDQuest 6.1 2-D analysis software (BIO-RAD). The scanned images were digitized and quantified using PDQuest 6.1 2-D analysis software, and the values for each cytokine were normalized to those of β-actin.

**Analysis of Gb3 expression.** Intestines and kidneys were surgically removed immediately after sacrifice for immunohistochemical analysis of Gb3. Tissues were then fixed in 10% buffered neutral formalin, embedded in paraffin, and sectioned (4 μm thick). The distributions of Gb3 in paraffin-embedding sections of mouse intestines and kidneys were determined by means of immunohistochemistry, as previously described (20, 24). In brief, sections were prepared as above and blocked in PBS containing 1% bovine serum albumin (BSA, fraction V; Sigma Chemical Co., St. Louis, Mo.) for 1 h. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol. After washing with PBS, the sections were reacted with monoclonal anti-Gb3, IgM antibody (Biodiag, Kennesaw, Maine), diluted in PBS 1:100, and in-
FIGURE 1. Standardization of IL-1β using the ELISA method. The straight line indicates the linear fit (P < 0.0001) (A). Serum IL-1β concentrations were measured at 7 days after E. coli O157:H7 challenge with the ELISA kit. Data are presented as means ± SEM (P < 0.05 for group III and P < 0.1 for group IV versus group II).

cubated overnight at 4°C. After washing with PBS, horseradish peroxidase-conjugated monoclonal mouse anti-rat IgM (Zymed, San Francisco, Calif.) diluted 1:100 in PBS was added and then incubated for 1 h. Binding of the secondary antibody was detected by using a 3,3′-diaminobenzidine substrate kit for peroxidase (Vector Laboratories Inc., Burlingame, Calif.). Sections were counterstained with Mayer’s hematoxylin and examined under light microscopy. Paraffin-embedding sections of tissues were incubated with the primary antibody (monoclonal anti-Gb3 IgM antibody) for negative control. The intensity and distribution of Gb3 in renal glomeruli, cortex, and medulla were estimated in five different microscopic fields (×100) in each renal section from mice in each group, as described in a previous study (24).

VT-B. longum HY8001 culture supernatant-binding assay. B. longum HY8001 culture supernatants were plated in triplicate on flat-bottomed Immunopure Polysorp 96-well plates (Nunc, Naperville, Ill.). The plates were incubated at 37°C for 1 h and then overnight at 4°C, washed once with 400 μl of PBST (0.05% Tween-20; Sigma), and then blocked with 400 μl of 3% BSA in PBS at 37°C for 1 h. Plates were washed once with 400 μl of PBST, incubated with 400 μl of crude VT at 37°C for 3 h, and washed three times as described above, followed by incubation with the monoclonal anti-VT IgG antibody (Biodesign) diluted 1:100 in BSA (0.1% in PBS) at 37°C for 1 h. Plates were washed three times and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) (Zymed) diluted 1:5,000 in BSA (0.1% in PBS) at 37°C for 1 h. Plates were washed five times and developed with O-phenylene diamine at room temperature for 15 to 30 min. Reactions were terminated by the addition of 100 μl of 1.25 M H2SO4 with gentle shaking. The optical density was read at 490 nm on an ELISA reader.

Statistical analysis. Results are presented as mean ± standard error of the mean (SEM). Data were analyzed by the Student’s t test or by analysis of variance as appropriate. The analyses were performed with Microcal Origin 6.0 (Microcal Software, Inc., Boston, Mass.).

RESULTS

Clinical symptoms of inoculated mice. Mice were inoculated intragastrically with E. coli O157:H7 as described previously and monitored for gastrointestinal and systemic symptoms. Gastrointestinal symptoms developed within 5 to 95 h after inoculation and consisted of loose, watery stools in mice of group II (100%), group III (100%), and group IV (60%). Systemic symptoms developed within 4 to 96 h and included lethargy and anorexia in all groups except for the PBS-treated group. After inoculation of the E. coli O157:H7 suspension, colonization of E. coli O157:H7 from fecal samples was detected in all groups except for the PBS-treated group.

Cytokine assay. TNF-α and IL-1β concentrations in serum were measured by a commercial ELISA kit at 7 days after challenge (14). The standard curves of IL-1β are shown in Figure 1. Levels of serum IL-1β significantly decreased in mice of group III (P < 0.05) and group IV (P < 0.1) compared to mice of group II (Fig. 1). Concentration of TNF-α in serum was not detected in mice of all groups by the ELISA kit used in this study. The expression levels of mRNA for TNF-α and IL-1β were examined in the intestines and kidneys by RT-PCR (Figs. 2 and 3). Expressions of TNF-α mRNA in intestines and kidneys were decreased in mice of group III (P < 0.15) and group IV (P
FIGURE 2. The expression of TNF-α mRNA in intestines (A) and kidneys (B) was examined by RT-PCR at 7 days after E. coli O157: H7 challenge. The values for TNF-α were normalized to that of β-actin. Data are presented as means ± SEM (P < 0.15 for group III and P < 0.15 for group IV versus group II).

< 0.15) (Fig. 2), and IL-1β mRNA in intestines and kidneys were also decreased in mice of group III (P < 0.01) and group IV (P < 0.01) compared to mice of group II (Fig. 3).

Analysis of Gb3 expression. The expressions of Gb3 in intestines and kidneys were examined by immunohistochemistry with a monoclonal anti-Gb3 antibody. The intensity and distribution of Gb3 in the intestines were estimated in five different microscopic fields (×100) in each section from mice in each group. Expressions of Gb3 were not different from group II to group III and group IV in intestines. No staining was detected in renal tubular epithelial cells and glomeruli from mice in the control group without the primary antibody (monoclonal anti-Gb3 antibody). Gb3 expressions decreased in kidneys of groups III and IV, whereas the Gb3 expression was remarkably high in group II compared to group I (Fig. 4). The Gb3 was localized on renal tubular epithelial cells in the renal cortex and medulla.

VT-B. longum HY8001 culture supernatant-binding assay. The interaction between VT and B. longum HY8001 culture supernatant was examined by ELISA. The optical density of VT (0.32 ± 0.02; mean ± SEM, n = 3) was higher than those of controls, PBS (0.11 ± 0.0005), and tryptic soy broth (0.16 ± 0.002) (P < 0.01). And the optical density value was decreased as VT was diluted (Y = A + B × X; A = −0.01 ± 0.34, and B = 1.51 ± 0.09).

Competitive ELISAs. The inhibitory activity of B. longum HY8001 culture supernatant on the VT-Gb3 interaction was examined by competitive ELISAs. The optical density value of VT with B. longum HY8001 culture supernatant (0.14 ± 0.02; mean ± SEM, n = 3) was significantly lower than that of VT with blood-glucose-liver broth (0.37 ± 0.02) (P < 0.01). And the optical density value of control was 0.10 ± 0.01.

DISCUSSION

Currently, there is no specific treatment for EHEC disease, and antibiotic treatment is also controversial (27,36). New clinical trials are now evaluating including Synsorb-Pk, which consists of a chemically synthesized analog of Gb3 and could absorb toxin from the intestine and prevent the development of hemolytic uremic syndrome (2). The neutralizing effect of B. longum HY8001 culture supernatant against VT of E. coli O157:H7 was also reported (35). This finding suggests the possibility of using probiotics for treatment of EHEC infection. In this respect, this study was focused on the raveling of the inhibitory effect of B. longum HY8001 against VTs, through the interference in the interaction of the B subunit of VTs with Gb3 interaction.

FIGURE 3. The expression of IL-1β mRNA in intestines (A) and kidneys (B) was examined by RT-PCR at 7 days after E. coli O157: H7 challenge. The values for IL-1β were normalized to that of β-actin. Data are presented as means ± SEM (P < 0.01 for group III and P < 0.01 for group IV versus group II).
The concentration of serum IL-1β significantly decreased in mice of group III, treated with *B. longum* HY8001 culture supernatant before challenge, and group IV, treated with *B. longum* HY8001 culture supernatant before and after challenge, compared to mice of group II, challenged with *E. coli* O157:H7. Expressions of TNF-α mRNA and IL-1β mRNA in intestines and kidneys were also decreased in mice of groups III and IV compared to mice of group II. These results first suggest the possibility that *B. longum* HY8001 culture supernatant may inhibit the production of TNF-α and IL-1β. The concentration of TNF-α in serum was not detected by ELISA in any group, whereas TNF-α mRNA expression in intestines and kidneys was measured. This discrepancy could be attributable to the difference in methods used for cytokine estimation (measurement of protein by ELISA versus measurement of mRNA by RT-PCR). Second, it is also possible that the TNF-α was released and degraded so rapidly that the concentrations were not detected at 7 days after challenge with *E. coli* O157:H7. Isogai et al. (14) showed the same results—i.e., that no proinflammatory cytokines were detectable in the serum at days 1 and 7 after inoculation of...
EHEC. Third, TNF-α production could occur within specific tissues, such as the kidneys and intestines, in an organ-specific manner after challenge. Harel et al. (12) and Isogai et al. (14) showed that no circulating TNF-α in serum was detected by ELISA, whereas TNF-α production occurred within specific tissues such as the kidney and brain. This result suggested that VT induces TNF-α synthesis in the kidney and, at the same time, increases renal sensitivity to the toxic effects of TNF-α. The concentrations of serum IL-1β in mice of group IV, treated with B. longum HY8001 culture supernatant before and after challenge with E. coli O157:H7, were lower than in mice of group III, treated with B. longum HY8001 culture supernatant only before challenge. As mentioned earlier, VT induces cytokine within specific tissues rather than circulating cytokine (14), so this discrepancy may not be significant. It is also possible that mice of group IV were under stress by intragastric inoculation with Zonde for 7 more days compared with mice of group III. Laugero and Moberg (21) showed that circulating IL-1β was significantly higher in mice under repeated stress, compared with mice under no stress.

In immunohistochemical study, renal tubular epithelial cells from mice of groups III and IV show decreased Gb3 expression, whereas those from mice of group II show significantly increased Gb3 expression. Recent studies have suggested that renal tubular epithelial cells may be more sensitive to the effect of VTs than endothelial cells (24, 33). Thus, it is feasible that B. longum HY8001 culture supernatant may inhibit renal damage by VT. These findings correlate with the results of other findings—i.e., that tubules in the renal medulla are more significantly injured when mice are fed bacteria-producing VTs (22, 24).

Fujiwara et al. (8) demonstrated that B. longum culture supernatant inhibits the binding of some strains of E. coli to the gangliotetraosylceramide, one of the glycosphingolipids. VTs produced from EHEC bind the Gb3 of mouse erythrocytes, which is also a glycosphingolipid, so it is plausible that the B. longum HY8001 culture supernatant also inhibits the binding of VTs to Gb3. The competitive ELISA showed that the culture supernatant of B. longum HY8001 primarily binds VTs to interfere the VTs with Gb3 interaction.

These results suggest that soluble substances in B. longum HY8001 culture supernatant have inhibitory activity on the expression of Gb3, VT-Gb3 interaction, or both. If this is the case, further study should be performed to elucidate the property of soluble substances in B. longum HY8001 culture supernatant.

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REFERENCES


