Anti-Inflammatory Effect of *Bifidobacterium longum* on Macrophage-Like THP-1 Cells via Epithelial Cell Caco-2

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We examined the immunomodulation capability of *Bifidobacterium longum* strains via a Transwell co-culture system using human colonic epithelial cells, Caco-2, in the upper chamber, and human macrophage-like cells, THP1, in the lower chamber of the culture. Heat-treated cells of three *B. longum* strains, JCM1217^T, KT237 or H7-115, were added to the upper chamber to allow direct contact with Caco-2 cells and the culture was incubated for 24 hr. After incubation, THP-1 cells in the lower chamber were placed in a separate well containing fresh medium with LPS and incubated for 6 hr. After incubation, we found that TNF- α secretion from THP-1 cells, that had been co-cultured with Caco-2 directly contacting heat-treated cells of *B. longum* strains, especially H7-115, was suppressed. This was, however, not the case in an the almost identical experiment using *B. longum* cells killed under ultra-violet light (not heat-treated). We then blocked Caco-2 TLR2 with anti-TLR 2 antibodies in another co-culture experiment and found that blocking TLR2 canceled the indirect anti-inflammatory effect of *B. longum* H7-115. The evidence suggests that some heat-resistant somatic structures of *B. longum* can modulate a host's immune response at least via TLR2 expressed on intestinal epithelial cells.

Key words: *Bifidobacterium longum*; immunomodulation; co-culture system; TLR2

The genus *Bifidobacterium* is the most predominant members of the human intestinal microflora. It has long been suggested that *Bifidobacterium* species have important roles in maintaining or promoting general health, preventing infection by enteric pathogens, suppressing the growth of other members of intestinal microflora that might produce cancer-causing substances, and modulating the host immune system in humans (2, 13). Bifidobacterial cultures have thus been increasingly used as probiotics in pharmaceuticals and foods (6, 10), and strains belonging to *B. longum*, *B. breve*, and *B. bifidum* have been intensively employed as probiotic bifidobacteria to date (1, 3).

The immunomodulatory properties of bifidobacterial strains have been described in a number of studies. For example, He et al. (4) reported that a cultured murine macrophage-like cell line produced a range of cytokines in the presence of different species of heat-inactivated bifidobacteria. More recently, Medina et al. (7)

demonstrated that different strains of *Bifidobacterium longum* had different abilities to induce cytokine production in human peripheral blood mononuclear cells, suggesting that immunomodulation by bifidobacteria present in the human host intestine varies not only at the species level but also at the strain level. It should be noted, however, that most of these findings were based on *in vitro* experiments, in which some antigen-presenting cells (i.e., macrophages, dendritic cells) were in direct contact with bifidobacterial cells. Such direct contact of commensal bacteria and antigen-presenting cells (APCs) may be less likely *in vivo* except for specialized epithelial cells (M cells), since they are usually separated by host intestinal epithelial cells (ECs). Using an *in vitro* co-culture system, however, Rimoldi et al. (9) reported that dendritic cells underneath ECs could be activated by inflammatory mediators released from ECs stimulated by direct contact with commensal bacteria. This points to the possibility of EC-mediated activation of the host immune system by bifidobacteria. In this context, we here describe indirect and strain-specific immunomodulation of *Bifidobacterium longum* in a novel *in vitro* co-culture system using human ECs (Caco-2) and APCs (THP-1).

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In the present study, we used 3 *B. longum* strains, JCM1217^T purchased from the Japanese Collection of Microorganisms, KT237 isolated from yogurt, and H7-115 isolated from adult feces. The strains were incubated anaerobically in an Anaero pack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 37°C for 24 hr in MRS broth (Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with 0.5% L-cysteine (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The bacteria were harvested by centrifugation, washed three times with sterile phosphate-buffered saline (PBS; pH 7.4) and resuspended in PBS. Bacterial cells were then either killed under ultraviolet light (UV-killed) or heat-treated at 100°C for 30 min, washed three times with sterile PBS, and re-suspended in PBS at a final cell concentration of ca. 10⁸/ml as measured by direct microscopic counting. The bacterial suspension thus prepared was used for subsequent experiments.

The monocytic cell line THP-1 was purchased from the American Type Culture Collection. THP-1 cells were maintained in RPMI1640 (Invitrogen, Tokyo, Japan) supplemented with 10% FBS (Biological Industries, Beit, Israel), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C under a humidified 5% CO₂ atmosphere. THP-1 cells for co-culture experiments were differentiated to macrophage-like cells at a density of 1.0×10⁵ cells/ml by treatment with 162 nM phorbol myristate acetate (Sigma, St. Louis, MO, USA) for 72 hr and quiescence in complete RPMI1640 medium for 24 hr before the co-culture experiments.

A co-culture system was established essentially following the methodology described by Tanoue et al. (14). Briefly, a human intestinal epithelial cell line Caco-2 cells was maintained in Dulbecco's modified Eagle's minimal essential medium (glutamine, high glucose, Sigma) supplemented with 1% non-essential amino acids (Sigma), 10% FBS (Biological Industries), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C under a humidified of 5% CO₂ atmosphere. The cells were incubated at 37°C under a humidified 5% CO₂ atmosphere. Caco-2 cells used for the co-culture experiments were seeded in the upper chamber of a Transwell filter (Corning CoStar Corp., Cambridge, USA) for 3 weeks (48–60 passages) until transepithelial resistance (TER) of 100 ohm/cm² was observed. The upper chamber Caco-2 cells were then placed in the lower chamber of the Transwell preloaded with THP-1 cells and incubated for 24 hr in complete RPMI1640 (Fig. 1). After incubation, all media were replaced with RPMI1640 and 1.0 × 10⁸ cells of heat-treated or UV-killed *B. longum* strains were applied to the apical side of

Fig. 1. Schematic drawing of a Transwell co-culture system. Caco-2 cells form a continuous monolayer epithelium on a microporous filter, which constitutes the bottom of the Transwell insert. The monolayer of Caco-2 cells is continuous and thus separates the chamber into an upper chamber (A) and a lower chamber (B) containing THP-1 cells at the bottom of well.

the Caco-2 cell layer in the upper chamber and further incubated at 37°C under a humidified 5% CO₂ atmosphere for 24 hr. During incubation, the TER value of the upper chamber filter was periodically measured by a Millicell-ERS instrument (Millipore, Eschborn, Germany) and there was no marked decrease in the TER value, indicating that the Caco-2 cell layers were intact throughout the experiments.

After incubation, TNF-α concentrations in the culture supernatants of the lower chamber were quantified with a cytotoxicity assay with L929 cells (actinomycin D-treated murine fibroblast cell line) using human rTNF-α (Wako) as the standard, as described by Takada et al. (11). Concentrations of IL-10, TGF-β1 and PGE2 in culture supernatants of the lower chamber were also measured by commercially available ELISA kits (Invitrogen for IL-10, and R&D Systems, Minneapolis, USA for TGF-β1 and PGE2). The assay results showed no significant production of either Caco-2 cells or THP-1 cells in the lower chamber regardless of the presence of bifidobacterial cells in the upper chamber (data not shown).

Subsequently, THP-1 cells were then placed in wells containing complete RPMI1640 with LPS (LPS from *Escherichia coli* O26:B6, Sigma) at a concentration of 100 ng/ml and incubated at 37°C in a humidified 5% CO₂ atmosphere for 6 hr. After incubation, culture supernatants were measured for TNF-α concentrations using the cytotoxicity assay described above. The assay results showed that there was no significant LPS-induced TNF-α secretion by the THP-1 cells that had been co-cultured with Caco2 cells in direct contact with the UV-killed bifidobacteria (data not shown), while the TNF-α secretion was significantly suppressed in the THP-1 cells that had been co-cultured with Caco2 cells in direct contact with the heat-treated cells of *B. longum* H7-115

Fig. 2. TNF- α production by THP-1 after LPS (100 ng/ml) stimulation after indirect co-culturing with *B. longum* strains. * $p < 0.05$ compared with control.

(Fig. 2). This result suggests that some heat-resistant somatic structures of *B. longum* triggered Caco-2 cells to produce some substances immunosuppressive for THP-1 cells that have yet to be identified. Kumar et al. (5) reported epithelial cells in contact with enteric commensal bacteria generate reactive oxygen species (ROS) accounting for blockade of the NF- κ B pathway. ROS may be involved in the immunosuppressive effect observed in this study.

The above results encouraged us to determine how Caco-2 cells recognized *B. longum* H7-115. Since TLR2 is known to play an important role for host cells, including ECs, in recognizing Gram-positive bacterial lipopeptide and peptidoglycan (12), we prepared another set of the Transwell co-culture, in which Caco-2 cells in the upper chamber were incubated in complete RPMI1640 medium containing 10 μ g/ml TLR2 antibody (Cosmo Bio Co., Ltd., Tokyo, Japan) or a control antibody (Sigma) for 1 hr at 37°C in a humidified 5% CO₂ atmosphere before the heat-treated cell preparation of *B. longum* H7-115 was applied to the chamber. Co-cultures were then incubated for 24 hr at 37°C in a humidified 5% CO₂ atmosphere. After incubation, THP-1 cells in the lower chambers were placed in wells containing complete RPMI1640 with LPS (Sigma) at a concentration of 100 ng/ml and incubated at 37°C in a humidified 5% CO₂ atmosphere for 6 hr. After incubation, the culture supernatants were measured for TNF- α concentrations using the cytotoxicity assay as described above and we found that such blocking TLR2 of Caco-2 cells before direct contact with *B. longum* H7-115 cells abolished the suppression of TNF- α production by THP-1 cells upon LPS stimulation (Fig. 3). This

Fig. 3. Effect of TLR2 neutralization of Caco-2 on TNF- α production by THP-1 after LPS (100 ng/ml) stimulation after indirect co-culturing with *B. longum* H7-115. * $p < 0.001$ compared with control. # $p < 0.05$ compared with antiTLR2.

suggests that *B. longum* H7-115 cells can induce an immunosuppressive effect via TLR2 of EC and TLR2 of EC might be involved in the immunotolerance. Moreillon and Majcherczy (8) described that innate immunity via TLR2 can be used to detect very subtle differences in Gram-positive walls; thus, the present results suggest that *B. longum* has strain-specific cell-surface structures which are differentially recognized by TLR2 of host ECs. This in turn suggests that the immunomodulatory effects of probiotic *B. longum* on human hosts vary from strain to strain. Further studies are in progress to evaluate these possibilities.

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