Secreted Factors from Bifidobacterium animalis subsp. lactis Inhibit NF-κB-Mediated Interleukin-8 Gene Expression in Caco-2 Cells

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The objective of the present study was to evaluate the anti-inflammatory effects of Bifidobacterium animalis subsp. lactis strain BB12 in stimulated Caco-2 cells and to characterize the factors responsible for these anti-inflammatory effects. Characterization and purification studies indicate that BB12’s anti-inflammatory factors might include a 50-kDa proteinaceous compound that is stable under a variety of heat and pH conditions.

Human intestinal epithelia and luminal microbiota have a mutualistic relationship in which the resident bacteria aid in nutrient metabolism, development of the intestinal epithelium and lymphoid tissue, and resistance to pathogenic colonization (7, 8, 25). The microbiota also play an important role in the pathogenesis of inflammatory conditions such as systemic inflammatory response syndrome (1), neonatal necrotizing enterocolitis (21, 22), and inflammatory bowel diseases (9, 24).

Probiotics are live organisms which when administered in adequate amounts confer a health benefit on the host (2). Modification of gut microflora by probiotic therapy has therapeutic potential in clinical conditions associated with gut barrier dysfunction and inflamed mucosa (11). The most commonly researched probiotic species belong to the genera Lactobacillus, Bifidobacterium, and Saccharomyces (24). Bifidobacteria are part of the human microflora and dominate the intestinal microbiota of infants, but their abundance decreases over time so that bifidobacteria usually account for approximately 3 to 5% of the adult human colon microbiota (9, 15, 17).

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Bifidobacterium animalis subsp. lactis is a Gram-positive, anaerobic commensal-derived probiotic (26). Interestingly, recent investigations have suggested that B. animalis subsp. lactis has potent anti-inflammatory effects (10, 13). Although a large number of clinical and experimental studies of probiotics have been performed, neither the mechanisms of action nor the true characteristics of probiotic anti-inflammatory molecules are well understood. One mechanistic explanation for probiotic effects is suggested by the work of Neish et al. (18), which was published ahead of print on 16 September 2011.

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FIG. 1. Inhibitory effect of BB12 on TNF-α-induced IL-8 expression is mediated through suppression of NF-κB activation in Caco-2 cells. (A) Total RNA was extracted from BB12-treated Caco-2 cells after TNF-α stimulation, and IL-8 mRNA expression was quantified by real-time PCR. Data are presented as means and standard deviations. ∗, P < 0.01 compared with TNF-α-stimulated cells without the BB12 pretreatment. (B) IL-8 protein secretion in culture supernatants was quantified with an ELISA kit. Data are presented as means and standard deviations. ∗, P < 0.01 compared with TNF-α-stimulated cells without the BB12 pretreatment. (C) Caco-2 cells were pretreated with BB12 (1 × 10^6 CFU/ml) for 12 h and then stimulated with TNF-α (10 ng/ml) for 15 min. Proteins were extracted from the cells. Nuclear extracts were analyzed for NF-κB using anti-human NF-κB antibody. Cytosolic extracts were analyzed with anti-human IκBα and phospho-IκBα (pIκBα) antibodies. (D) Caco-2 cells were transfected with a NF-κB luciferase (firefly) construct together with pRL-TK (Renilla luciferase) as a transfection control using the GeneJammer transfection reagent. After transfection, the cells were stimulated with TNF-α in the presence of BB12. Luciferase activities were determined using a dual-luciferase reporter assay kit and normalized to the control values. Data are means and standard deviations from three independent experiments. ∗, P < 0.01 compared with TNF-α-stimulated cells without the BB12 pretreatment.

(Fig. 1B). However, BB12 significantly suppressed the TNF-α-induced IL-8 expression when added at a concentration of 1 × 10^9 CFU/ml. Because IL-8 expression has been reported to be related to the activation of NF-κB, we also examined whether the inhibitory effect of BB12 on IL-8 expression is mediated through the regulation of NF-κB activation. TNF-α increased NF-κB nuclear translocation and IκBα phosphorylation while decreasing IκBα levels in Caco-2 cells (Fig. 1C). Furthermore, TNF-α stimulation (10 ng/ml) led to a 6-fold increase in NF-κB transcriptional activity in cells transfected with a plasmid bearing genes for NF-κB and luciferase (Fig. 1D). However, treatment of the cells with BB12 (1 × 10^9 CFU/ml) inhibited the TNF-α-induced transcriptional activity of NF-κB. There have been several in vitro investigations that provide some information about the anti-inflammatory mechanisms of probiotics. Kim and colleagues (13) demonstrated that B. animalis subsp. lactis inhibits NF-κB and NF-κB-regulated genes in IECs and prevents acute colitis in mice.

In the study of Dahan et al., nonpathogenic Salmonella did not inhibit the phosphorylation of IκBα, whereas Saccharomyces boulardii (4) inhibited phosphorylation of IκBα that was induced by enterohemorrhagic Escherichia coli infections. In addition, Jijon et al. (12) reported that probiotic DNA inhibited IL-8 secretion and delayed NF-κB activation in the presence of proinflammatory stimuli. These different results could be caused by differences in the probiotic strains, the number of probiotic treatments, or the proinflammatory stimuli used in the studies. Our results demonstrate that BB12 attenuated TNF-α-induced NF-κB pathway activation by interfering with IκBα degradation in Caco-2 cells. Further studies will be required to determine the precise step associated with the effect of BB12 in the NF-κB pathway.

BB12 produces soluble factors that inhibit TNF-α-induced IL-8 elevation. To elucidate the mechanism of IL-8 inhibition, potential components of the bacterium-cell interaction were separated and studied individually. The inability of heat-killed BB12 to generate a response allowed us to exclude bacterial wall components as stimuli. Interestingly, conditioned medium (CM) from cocultured bacteria and Caco-2 cells, even when heat inactivated, was sufficient to inhibit IL-8 expression (Fig. 2A). To find out whether bacterium-cell coculture was required for the production of stimulatory molecules, culture supernatant (CS) from BB12 grown without Caco-2 cells was used. This CS was virtually as effective as CM in which bacte-
rium-cell contact had occurred (Fig. 2B). These results indicate that soluble factors generated from bacteria alone, independent of contact between bacteria and cells, inhibit IL-8 production. Other studies have reported similar observations, demonstrating that soluble factors secreted by probiotics are sufficient to beneficially modulate host cell functions (14, 27).

Physical and chemical characteristics of soluble factors from the CS. Attempts were made to determine the properties of the active IL-8-inhibitory fraction by extracting and precipitating with ethanol, ethyl acetate, or 60% ammonium sulfate (AS). The majority of activity was found in the 60% AS precipitate, whereas little or no activity was extracted by ethanol or ethyl acetate, indicating that the active component may have a proteinaceous rather than carbohydrate or lipid nature ($P < 0.05$) (Fig. 2C). To further investigate the nature of the active components, various treatments were tested. The IL-8-inhibitory activity of the CS was abrogated after treatment with trichloroacetic acid (TCA) and SDS at final concentrations of 12% (vol/vol) and 0.5%, respectively (data not shown). The CS was also found to be relatively stable in the neutral and acidic pH ranges, whereas the activity disappeared rapidly at pH values over 8.0 (Fig. 2D). The activity of CS also remained unaffected by 15 min of heating up to 90°C, and 50% of the original activity persisted after 15 min of heat treatment at 121°C (data not shown). These results may indicate that the active components of BB12 CS are active in a low pH range and heat stable over a fairly wide temperature range. The compounds may also be protein or peptides, as suggested by the results of TCA precipitation and SDS denaturation in conjunction with AS precipitation. The heat and pH stability of this activity may prove to be useful in applications where CS components are used as food adjuncts.

Purification of the active IL-8 inhibitory components in CS. To purify proteins from CS, filtered CS was loaded onto UNOsphere S ion exchange medium (with negatively charged functional groups), and bound proteins were eluted using 40 mM Tris (pH 6.9) containing an increasing gradient of NaCl from 100 mM to 800 mM. Eluted proteins were analyzed by SDS-PAGE to identify the proteins contained in each fraction (data not shown). A 50-kilodalton protein (p50) was eluted from the cation exchange resin at NaCl concentrations of 350 mM (Fig. 3A). SDS-PAGE analysis indicated that the chromatography procedure successfully separated p50.

To determine the biological roles of purified proteins from CS, the effects of p50 on the TNF-α-induced IL-8 production in Caco-2 cells was evaluated. Cells were treated with various concentrations of p50, and IL-8 production was determined by ELISA. As controls, we tested the flowthrough fraction from the ion exchange column and the fraction eluted from the column with 200 mM NaCl: neither of these preparations contained p50, and neither of these preparations inhibited IL-8 production (data not shown).
To summarize, a soluble factor secreted by BB12 inhibited the TNF-α-induced production of IL-8 in Caco-2 cells. We showed that the inhibitory effect of BB12 on TNF-α-induced IL-8 production was at the transcriptional level and operated at least in part through inhibition of the NF-κB signaling pathway. The active component in CS might include a 50-kDa compound with a proteinaceous nature that is stable under a variety of temperature and pH conditions. These findings support the potential application of probiotics or probiotic components in the prevention of cytokine-mediated injury in inflammatory intestinal diseases.

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REFERENCES

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