Functional modulation of enterocytes by gram-positive and gram-negative microorganisms

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Otte, Jan-Michel, and Daniel K. Podolsky. Functional modulation of enterocytes by gram-positive and gram-negative microorganisms. Am J Physiol Gastrointest Liver Physiol 286: G613–G626, 2004; 10.1152/ajpgi.00341.2003.—Clinical studies have suggested that so-called probiotic bacteria may be effective as therapy in inflammatory bowel disease. However, the molecular mechanisms of their interaction with the intestinal surface remain undefined. The influence of whole probiotic bacteria [Escherichia coli Nissle 1917 (EcN); probiotic mixture VSL#3 (PM)], bacterial cell lysates, and conditioned media on transepithelial resistance (TER), IL-8 secretion, mucin gene expression, and tight junction proteins were determined in T84 and HT-29 intestinal epithelial cells (IEC). In addition, effects on pathogen (Salmonella dublin)-induced alterations were analyzed. EcN as well as debris and cell extracts induced IL-8 secretion from IEC, whereas no such effect was observed following incubation with the PM. The PM and soluble protein(s) released from the PM increased TER, prevented pathogen-induced decrease in TER, and were shown to stabilize tight junctions. The PM induced expression of mucins in IEC, and these organisms as well as EcN diminished S. dublin-induced cell death. Inhibition of MAPKs with PD-98059 or SB-203580 significantly decreased alterations in IL-8 synthesis and mucin expression and affected the regulation of TER. Probiotics and protein(s) released by these organisms may functionally modulate the intestinal epithelium of the host by different mechanisms, including the competition of whole organisms for contact with the epithelial surface as well as stabilization of the cytoskeleton and barrier function and the induction of mucin expression. Gram-negative and gram-positive organisms differ in the mechanisms activated, and a combination of organisms might be more effective than the application of a single strain.

probiotics: Escherichia coli Nissle 1917; probiotic mixture VSL#3; intestinal epithelial cell lines; interleukin-8; transepithelial resistance; zonula occludens-1; mucins

THE HUMAN GASTROINTESTINAL tract is colonized by a vast community of symbionts and commensals with essential roles in nutrient processing, innate and adaptive immunity (5, 48), and a broad range of other host functions including regulation of intestinal epithelial development and activity (3, 19). There is continuous epithelial microbial cross-talk, presumably mediated in part by Toll-like receptors (TLR) (36, 37), putative internal NOD receptors (20, 21), and other mechanisms. However, persistent, uncontrolled activation of the mucosal immune system may be linked to a loss of “tolerance” to luminal commensals (12, 29, 39). Unregulated, ongoing mucosal inflammation is characteristic of inflammatory bowel disease (IBD) (12, 34) which proceeds through stages of initiation, amplification, and healing. There is accumulating evidence that the luminal flora plays a critical role in the initiation and perpetuation of colitis (8, 10, 43). The break or lack of tolerance might be due to an imbalance between protective and aggressive luminal bacterial species, a decreased barrier function, and an impaired mucosal clearance allowing access of bacteria to the mucosal immune system. Whereas no specific pathogen has been identified as causal, reduced numbers of Lactobacillus and Bifidobacteria have been found in colonic biopsies of patients with IBD (13, 14), and the presence of increased numbers of mucosal adherent and perhaps even intraepithelial bacteria in the mucosa of IBD patients (45) has been noted.

Despite increasing recognition of the importance of “commensal” intestinal bacteria in clinical and experimental colitis, therapeutic approaches that modulate the composition of the bacterial load are hampered by the limited knowledge of the intestinal flora and its interaction with the epithelial barrier. The use of antibiotics in a number of randomized control trials have been disappointing, and it has not been determined yet how antibiotic combinations would be most effectively used. However, a limited number of studies using so-called probiotics to manage IBD has, however, shown surprisingly consistent results. Probiotic microorganisms have been defined as live microbes that, when administered in adequate amounts, confer a health benefit to the host (15, 16). Both laboratory and clinical studies have shown that certain probiotics may be useful in treatment and prevention of a number of intestinal disorders including inflammatory diseases. There is great variation in the number and combination of probiotic organisms provided in various supplements; some contain a single organism, and others contain multiple microbes. Successful treatment has been reported with Escherichia coli Nissle 1917 (EcN) in patients with ulcerative colitis (UC) (24, 41) and with a multiple probiotic organisms mixture (VSL#3; PM) in UC (47), Crohn’s disease, and pouchitis (17, 18). Improvement of symptoms has been achieved in patients with irritable bowel syndrome (IBS) by treatment with EcN and, more recently, with the PM.

The molecular basis of the effects evoked by probiotics have not been well characterized. Lactic acid bacteria are known to have a wide range of effects on the immune system including both enhancing phagocytosis as well as more specific humoral and cellular immune responses. Evidence from animal studies using E. coli is contradictory. It has been speculated that the beneficial effects of various probiotics may reflect species-
specific properties (25) with mechanisms of action including competition for nutrients and adhesion sites, effects on mucin, immunoglobulin, and antimicrobial peptide secretion (19, 38).

The current study was designed to compare and further characterize effects of two widely used probiotic preparations, a single strain gram-negative E. coli and a multistrain gram-positive probiotic mixture (PM) on naïve intestinal epithelial cells (IEC). The capacities of pre- and coinoculation with these preparations on bacterial invasion were also studied by characterizing signaling pathways likely to be involved in cell protection.

MATERIALS AND METHODS

Cell lines, media, and bacterial strains. T84 and HT-29 cells were obtained from American Type Culture Collection (Manassas, VA). All cells were cultured at 37°C in a 5% CO₂ atmosphere in DMEM/F-12 (1:1) and DMEM, respectively (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (GIBCO). Culture media were supplemented with 50 μM penicillin and 50 μg/ml streptomycin (GIBCO). E. coli Nissle 1917 (Mutafull) was from Ardeypharm (Herdecke, Germany), the Salmonella dublin strain was purchased from ATCC, and the PM, also designated as VSL#3, comprised of four strains of Lactobacillus, three species of Bifidobacterium, and one strain of Streptococcus was a generous gift of Dr. C. De Simone (University of L’Aquila, Italy). Batch-to-batch consistency was verified before experiments. Bacterial strains were stored in Luria-Bertani (LB) medium plus 15% glycerol at −80°C and grown in LB broth overnight at 37°C without shaking. For conditioned media, the PM was incubated in cell culture medium for 12 h. Afterward, the bacteria were removed by filtration, and the efficiency of removal was determined by plating serial dilutions.

Reagents. TNF-α and IL-1β were obtained from Sigma-Aldrich (Munich, Germany) and used in concentrations ranging from 1 to 100 ng/ml. Monochloramine, also from Sigma-Aldrich, was added to cultured cells (Munich, Germany) and used in concentrations ranging from 1 to 100 μM. Phosphospecific antibodies for detection of activated MAPK as well as antibodies for the quantification of total p42/44, p38, and p65/50 were obtained from Cell Signaling (Beverly, MA). Inhibitors of p42/44 mitogen-activated protein kinases (MAPKs; PD-98059) and an inhibitor of p38 MAPK [SB-203580 (9)] were purchased from Cell Signaling Technology (Beverly, MA). Before infection, the cells were incubated for 90 min with conditioned media containing 10% fetal bovine serum (GIBCO). Culture media were obtained from American Type Culture Collection (Manassas, VA). All cells were cultured at 37°C ± 1°C in a 10% CO₂ atmosphere in DMEM/Ham’s F12 medium plus 15% glycerol at −80°C and grown in LB broth overnight at 37°C without shaking. For conditioned media, the PM was incubated in cell culture medium for 12 h. Afterward, the bacteria were removed by filtration, and the efficiency of removal was determined by plating serial dilutions.

Infection procedure. Cultured cells were washed and incubated for at least 2 h in antibiotic-free medium. Bacteria grown overnight in LB broth were pelleted by centrifugation, resuspended in sterile buffered saline (PBS), and added to the cells in a concentration of 100/cell.

Electrical resistance measurements. The human colon cancer cell line T84 (passage 54–60) was grown on filters (0.4 μM; Becton Dickinson, Franklin Lakes, NJ). Cell monolayers reached confluence within 5–7 days and were polarized and differentiated state within 30–45 days, respectively (6). Transmembrane electrical resistance (TER) was measured to quantify changes in epithelial cell culture integrity and confluence with a Millicell-ERS apparatus (Millipore, Danvers, MA) as described elsewhere. Experiments were performed using differentiated T84 cells with a TER of >1,500 Ω/cm (a nondifferentiated state was defined as <500 Ω/cm) (12).

Distribution of zonula occludens-1. Zonula occludens (ZO)-1 distribution was analyzed in unstimulated controls, after infection, and in coculture models using immunohistochemistry and confocal microscopy. After 60 min, the monolayers were washed extensively with PBS and fixed with 2% paraformaldehyde for 30 min. The cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and then washed with PBS. Polyclonal rabbit anti-ZO-1 antibody (Zymed, South San Francisco, CA) was incubated with the permeabilized cells for 45 min at 37°C. The monolayers were washed and then treated with fluorescein-conjugated anti-rabbit immunoglobulin G (DAKO, Hamburg, Germany) for 45 min at 37°C. After washes with PBS, the filters were excised from the supports, mounted, and observed under a Zeiss confocal laser-scanning microscope.

ELISA. For IL-8 ELISA, 1 × 10⁶ cells were plated per well in six-well plates. Cells were cultured with live bacteria for 12 h or incubated with MAPK inhibitors and then infected with microorganisms. Subsequently, supernatants were harvested and centrifuged for 10 min at 1,500 rpm to pellet residual bacteria. IL-8 ELISA (BD Pharmingen, San Diego, CA) was performed as per the manufacturer’s instructions. Samples were read at 450 nm using a 7520 Microplate reader (Cambridge Technology, Cambridge, MA). To exclude deviation of cell viability, trypan blue exclusion assays were performed as indicated below.

RNA extraction and expression analysis by real time analysis. Total RNA was isolated from cells using the RNaseasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. All samples were treated twice with RNase-free DNase I. Samples were stored at −80°C before the assay. Reverse transcription of RNA was performed in a final volume of 20 μl containing 200 μM of each nucleotide triphosphate, 6.7 mM MgCl₂, 5 U of RNase inhibitor (Promega, La Jolla, CA), 5 μM random hexamer (Pharmacia, Uppsala, Sweden), and 200 U of Moloney murine leukemia virus RT (Life Technologies, Rockville, MD), and 14 μl of the extracted RNA or H₂O were added. The samples were incubated at 65°C for 5 min, then at 42°C for 30 min. Negative controls included the amplification of samples without prior RT reaction. Colon tissue was used as positive control. Primers and probes were chosen using the Primer Express computer program (PE Applied Biosystems, Forster City, CA). Primer sequences for muc1-1, -2, -3, SAGC, and GAPDH are given in Table 1. All PCR fragments were purified, subcloned into pCR 2.1 vector (Invitrogen, Carlsbad, CA), and sequenced.

PCR amplification was performed with FAST DNA SYBR Green I (Roche, Mannheim, Germany), which allowed an automated quantification of the amplified products in real time with the Light Cycler (Roche). Of the total reverse transcription volume of 20 μl, 2 μl were used for each PCR. The polymerase amplification was performed in a total volume of 20 μl containing 1× PCR buffer [3 mM MgCl₂; 10 mM Tris-HCl (pH, 8.3); 50 mM KCl; 5% glycerol; 200 μM dATP, dCTP, and dGTP; 400 μM dUTP; 500 nM of each primer], H₂O, and Fast Start DNA SYBR Green I according to the manufacturer’s instructions. Samples were incubated at 95°C for 10 min and then subjected to 40 cycles of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C. PCR efficiencies were determined for each primer pair using a 10-fold dilution series of cDNA.

Table 1. Sequences and product size of PCR primers

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<th>Gene</th>
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instructions. The thermal cycling conditions comprised 10 min at 95°C and then 45 cycles at 95°C for 15 s, 56°C for 5 s, and 72°C 2 min followed by a standard melting curve analysis. Annealing temperatures were optimized for the primer pairs used. Each experiment was performed in two independent runs (RNA extraction and reverse transcription), each in duplicate. The assessment of quality control was performed in standardized PCR conditions, including two negative controls (1 with no template and 1 negative reverse transcription control). Calculations and statistical analysis were performed using GAPDH as endogenous standard, run in duplex with the candidate gene primers, and with 18S and β-actin, run separately.

Protein isolation and Western blot analysis. Cells were exposed to EcN or the PM in the indicated periods of time. Cells were then washed with PBS and harvested by scraping into ice-cold lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and phosphatase inhibitors (400 mM sodium orthovanadate and 4 mM NaF). Lysates were homogenized, and protein contents were determined using the Bradford method. Aliquots of equal homogenate protein were resolved by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto BioBlot-NC nitrocellulose membranes (Costar, Cambridge, MA). Membranes were quenched with 5% nonfat dry milk in 1% blocking solution (Boehringer-Mannheim, Mannheim, Germany) overnight at 4°C with 5% dry milk in PBS containing 0.1% Tween 20 (PBS-T), followed by incubation for 2 h at room temperature with primary antibodies diluted in blocking buffer at 1 μg/ml for the mucins and the signaling molecules of the MAPK pathway. After the blots were washed in PBS-T for 30 min, they were incubated with secondary antibodies diluted 1:1,500 in blocking buffer for 45 min at room temperature. Hybridized bands were detected using an enhanced chemiluminescence Western blotting system (Amersham, Braun- schweig, Germany). Images of Western blots were acquired in a standardized way (800 dots/in.) using an Epson perfection 1640SU photo scanner. To confirm equal loading, blots were stripped in 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate containing 100 mM 2-ME at 50°C for 30 min, and reprobed with anti-GAPDH as well as anti-total p42/p44, total p38, or total SAPK/JNK, respectively.

Trypan blue exclusion assay. Intestinal epithelial cells were incubated with the PM, E. coli Nissle 1917, or S. dublin. After removal of microbes, the cells were exposed to Trypan blue dye, and both viable (cells excluding trypan blue) and nonviable cells (cells not excluding trypan blue) were counted.

Statistical analysis. Data are expressed as means ± SE. Significance of differences was determined using the two-tailed Student’s t-test and analysis of variance. P values <0.05 were considered to be statistically significant.

RESULTS

Effect of the PM, E. coli Nissle, and S. dublin on IL-8 secretion. Microbial organisms and their products are known to induce expression of cytokines by IEC. Model IEC lines were stimulated with 10^2, 10^4, and 10^6 colony-forming units (CFU/ml) of the indicated organisms for 2–12 h. As shown in Fig. 1, A and B, 10^4 CFU/ml E. coli and S. dublin time-dependently induced IL-8 secretion by T84 and HT-29 human intestinal epithelial cell lines. A significant (P < 0.01) increase was observed after 6–8 h, which plateaued at the end of the observation period (Fig. 1, A and B). The induced secretion of IL-8 was concentration dependent (10^2 to 10^6 CFU/ml; data not shown). When cells were infected with E. coli and S. dublin, no additive effect on IL-8 secretion was observed. The effect of coinfection was lower compared with the one detected following an infection with an equal amount of S. dublin but more pronounced compared with an infection with an equal amount of E. coli (data not shown). In contrast to the results obtained with gram-negative organisms, no induction of IL-8 secretion was observed when the cells were incubated with the PM (Fig. 1, A and B). IEC incubated with the PM did not release significant amounts of IL-8. Comparable results were obtained when the experiments were performed with microbial cell lysates (50 μg/ml; 200 μg/ml) instead of living microorganisms (data not shown).

To further investigate the effect of various bacterial loads on cytokine release from IEC, cells were costimulated with increasing concentrations (10^2 to 10^6 CFU/ml) of the PM and a constant (10^4 CFU/ml) concentration of E. coli (Fig. 1C) or S. dublin (Fig. 1D). Significant progressive inhibition of E. coli- or S. dublin-induced IL-8 secretion by IEC was observed when increasing concentrations of the PM were added simultaneously to each well. A smaller inoculum (10^4 instead of 10^6 CFU/ml) of the PM was needed to effectively decrease E. coli- or S. dublin-induced IL-8 secretion in cells preincubated for 3–6 h with the PM (data not shown).

Effect of the PM, E. coli Nissle, and S. dublin on T84 monolayer resistance. To study the effects of probiotics and pathogens on epithelial barrier function, we assessed the TER of differentiated T84 monolayers exposed to 10^3 CFU/ml of the PM, E. coli, or S. dublin. As shown in Fig. 2A, the PM significantly (P < 0.05) increased TER in a time-dependent manner, with alterations first observed after 4–6 h of incubation. Effects on TER leveled off toward the end of the observation period (10–12 h). In contrast, incubation with E. coli induced minimal changes in TER, and infection with S. dublin resulted in a significant (P < 0.01) decrease in TER (Fig. 2A).

Smaller impairments in barrier function were observed in cultures coincubated with E. coli and S. dublin than in those incubated with S. dublin alone (data not shown). Coincubation of various concentrations of the PM (10^2 to 10^6 CFU/ml) and a constant amount of S. dublin (10^4 CFU/ml) significantly attenuated S. dublin-induced alterations in TER (P < 0.01; Fig. 2B).

Immunohistochemical localization of the tight-junction component ZO-1. Immunohistochemical staining revealed uniform circumferential distribution of ZO-1 in noninfected T84 cells (Fig. 3A). On infection with S. dublin, a distinct accumulation of ZO-1 was observed in parts of the monolayer with a contraction at the apical circumferential junctional ring of some cells and subsequent enlargement of neighboring cells (Fig. 3B). Degradation or disappearance of the tight junction protein was not observed. Coincubation with the PM concentration-dependently resulted in a significant decrease of S. dublin-induced alterations in the distribution of ZO-1 (Fig. 3C). Incubation with PM-conditioned medium was less effective in this assay compared with incubation with live organisms of the PM (data not shown).

Effect of conditioned media on IEC monolayer resistance and IL-8 secretion. To determine whether the alterations in T84 monolayer resistance and IL-8 secretion in IEC observed in the present study required the direct contact between the bacteria and the IEC or could be mediated by a secreted product, conditioned media were generated and added to the T84 monolayer. Serial dilutions (undiluted, 1:10, or 1:100 diluted) of conditioned media induced an increase in T84 TER comparable with the one detected when the cells were incubated with various concentrations of the living organism (Fig. 4).

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PROBIOTICS IN INTESTINAL HOMEOSTASIS

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Culture of T84 cells in various concentrations of PM-conditioned media and 10^4 CFU/ml of *S. dublin* indicated a stabilizing effect of the conditioned media that was comparable with the one observed when coculturing the cells with living PM and *S. dublin* (Fig. 4B). Preconditioned media from *E. coli* had no effect on the *S. dublin*-induced alterations in TER (data not shown). These results suggest that a soluble factor is responsible for the modulation of barrier integrity.

In addition, the application of medium that was conditioned by organisms of the PM or *E. coli* to intestinal epithelial cell cultures had no effect on IL-8 secretion induced by a subsequent challenge to *S. dublin* or *E. coli* (data not shown).

Effect of pretreatment of IEC with live bacteria on monolayer resistance and IL-8 secretion. To determine whether prior exposure to “benign” bacteria had any effect on response to subsequent challenge with *S. dublin*, IEC were preincubated
that incubation with this organism had some stabilizing effect on the cells. The effect of the PM was slightly but not significantly decreased when the cells were also preincubated with E. coli (data not shown).

Preincubation with EcN resulted in a reduction of S. dublin-induced IL-8 secretion. This effect was significant when S. dublin was applied in low concentration, whereas after stimulation with a high concentration, no significant reduction was detected. In contrast to the effect on TER, preincubation with the PM had no significant effect on subsequent S. dublin-induced IL-8 secretion. (Fig. 5B).

Effect of the PM, E. coli Nissle, and S. dublin on cell survival. Incubation of IEC with E. coli or the PM alone (10⁴ CFU/ml) had only a minimal effect on cell survival as measured by trypan blue exclusion (3–5% dead cells/field; control: 2–4% dead cells/field). During the 8-h observation period, probably due to alterations in culture medium pH, damage to the cells was also observed in cultures incubated with the PM (Fig. 6A). Effects were more pronounced, although not significantly different, in EcN-stimulated cultures. Therefore, no observation periods >8 h were included in this assay. In contrast to PM or EcN, infection with S. dublin (10⁴ CFU/ml) resulted in a significant reduction (30–50% dead cells/field; with 10⁴ CFU/ml E. coli or the PM. First, control experiments were performed to determine the number of washes required to ensure complete removal of E. coli or the PM from culture media before infection with S. dublin (10⁴ CFU/ml). Three serial washes with PBS (pH 7; 37°C) were found to effectively remove probiotics used for preincubation, with essentially no CFU remaining after this approach (data not shown). Furthermore, serial washes had no nonspecific effect on monolayer permeability or cytokine secretion (data not shown). On the basis of these results, three washes were used in subsequent experiments. Preincubation of IEC with E. coli had no effect on S. dublin-induced alterations in TER, whereas preincubation with the PM significantly diminished subsequent S. dublin-induced reduction in T84 monolayer TER (Fig. 5A), indicating
P < 0.01) in the number of viable cells (Fig. 6A). Coincubation of cells with an increasing concentration of PM (Fig. 6B) or E. coli (Fig. 6C) and constant amounts of S. dublin (10^4 CFU/ml) significantly increased cell survival.

Whereas preincubation with and subsequent washout of EcN had no effect on S. dublin-induced IEC damage, preincubation and subsequent wash off performed with the PM significantly diminished cell death induced by subsequent incubation with S. dublin. These effects were less pronounced than those observed after incubation with live organisms (Fig. 6D). These data indicate that dilution by nonpathogenic bacteria is an important mechanism in intestinal epithelial cell protection. In addition, a factor secreted by the PM protects epithelial cells in the absence of living organisms.

Effect of the PM and E. coli Nissle on stimulation with nonbacterial components. This protective effect is less effective when a nonbacterial cellular stressor is applied to the cell cultures. As shown in Fig. 7A, incubation of the cells with the nonbacterial oxidative cellular stressor monochloramine (NH_2Cl; 2 mmol) resulted in a significant decrease in TER. These effects were diminished, in part, by incubation with the PM but not with EcN (Fig. 7A).

Furthermore, the effect of stimulation of cultures with proinflammatory cytokines, such as TNF-α or IL-1β, was not altered by incubation with gram-positive or gram-negative organisms. As shown in Fig. 7B, cytokine-induced IL-8 secretion from IEC was unchanged in cultures infected with either.

Effect of E. coli and organisms of the PM on mucin gene expression. As assessed by real-time PCR, unstimulated HT-29 cells express MUC1, MUC2, MUC3, and MUC5AC. Whereas no significant changes were observed for MUC1, following stimulation with gram-positive organisms, MUC2, MUC3, and MUC5AC genes were significantly (P < 0.05) induced. Alterations in MUC2 and MUC5AC were more prominent than
those observed for MUC3. MUC2, MUC3, and MUC5AC were induced 3.5-, 3-, and 4.5-fold, respectively. Incubation with *E. coli* showed only some alteration in mucin expression without reaching the level of significance. The levels of GAPDH were not altered by bacterial stimulation (Fig. 8A).

Expression of mucin was also assessed using Western blot analysis. HT-29 cells express mucins 2, 3, and 5AC. Consistent with the mRNA data, a significant increase in mucin protein expression was detected when cells were incubated with gram-positive organisms. Protein levels were increased 2.2 (MUC2)-, 2 (MUC3)-, and 3.5-fold (MUC5AC). No significant alterations of mucin protein were seen in *E. coli*-infected cultures. The induction of mucin gene and antigen expression by organisms of the PM was also concentration dependent. At an optical density at 600 nm (OD600) of 0.3, induction of mucins was 58 (MUC2), 49 (MUC3), and 42% (MUC5AC) of the amount detected when performing the experiments at an OD600 of 1.

Signaling mechanisms associated with alterations in TER and IL-8 secretion. To further characterize signaling pathways involved in probiotic-evoked effects, T84 and HT-29 cells were preincubated with inhibitors of the MAPK p38 or p42/44

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Fig. 5. Effect of pretreatment with live bacteria on T84 monolayer resistance and IL-8 secretion. A: T84 monolayers were infected with 10⁴ CFU/ml of *S. dublin* alone or preincubated with *E. coli* and organisms of the PM before infection with 10⁴ CFU/ml of *S. dublin*. Alterations in TER were measured at the indicated time points and calculated as %unstimulated controls. B: T84 and HT-29 cells were infected with 10⁴ CFU/ml of *S. dublin* alone or preincubated with 10⁴ CFU/ml *E. coli* or organisms of the PM and subsequently stimulated with *S. dublin* for 8 h. IL-8 secretion into cell supernatants was evaluated by ELISA. Values represent means ± SD of *n* = 5 independent assays.
for 90 min and subsequently incubated with $10^4$ CFU/ml of *E. coli* or *S. dublin* as indicated. Each inhibitor reduced IL-8 secretion induced by microorganisms. The inhibitory effects of SB-203580 were more pronounced than those of PD-98059. However, none of the inhibitors completely blocked the induced IL-8 secretion, suggesting the involvement of additional mechanisms of activation (Fig. 9A).

The increase in TER observed after incubation with the PM or media conditioned by the PM was significantly ($P < 0.01$) diminished in cultures that had been pretreated with either MAPK inhibitor (Fig. 9B). Consistent with these latter observations, as shown in Fig. 9C, media conditioned by the PM activated p42/44 MAPK as well as p38 in T84 cells, suggesting that the increase in TER observed after incubation with the conditioned media may be mediated by MAPK pathways. MAPK activation was comparable with the activation detected following incubation with living organisms of the PM. Increases in mucin expression were significantly diminished by
both the ERK1/2 inhibitor and, to a lesser extent, the p38 inhibitor, indicating that MAPK is also involved in mucin production induced by organisms of the PM in IEC (Fig. 9D).

Characterization of the soluble factor in PM-conditioned media. To further characterize the nature of the putative soluble factor released by the PM, conditioned media were treated with DNAse or protease, heat inactivated (Fig. 10A), filtered (Fig. 10B), or applied at different pH (Fig. 10C). After treatment with DNAse (200 U/ml), the protective effect of PM-conditioned media was diminished, although not significantly, indicating that bacterial DNA might have some protective or immunomodulatory effect but is not the major protective factor in PM-conditioned media. In contrast, treatment with protease (1% wt/wt) significantly diminished the protective effect of preconditioned media. In addition, the factor appeared to be heat sensitive, because no protective effect was observed when...
METHODS. Expression levels were calculated as mucin RNA/GAPDH expression and are depicted in %expression in unstimulated controls.

Mucin (MUC) expression was quantified as described in MATERIALS AND METHODS. HT-29 monolayer. A: HT-29 monolayers were infected for 6 h as indicated. Mucin (MUC) expression was quantified as described in MATERIALS AND METHODS. Expression levels were calculated as mucin RNA/GAPDH expression and are depicted in %expression in unstimulated controls. B: effect of the PM and EcN on mucin protein expression in HT-29 cells. Lysates from cultured cells treated with the microbes for 12 h were subjected to Western blot analysis (20 µg/lane). Arrows show the top of the separating gel; bars show size markers. Values represent means ± SD of n = 5 independent assays; *P < 0.05.

Fig. 8. Effect of 10⁵ CFU/ml of the PM or EcN on mucin expression in the HT-29 monolayer. A: HT-29 monolayers were infected for 6 h as indicated. Mucin (MUC) expression was quantified as described in MATERIALS AND METHODS. Expression levels were calculated as mucin RNA/GAPDH expression and are depicted in %expression in unstimulated controls. B: effect of the PM and EcN on mucin protein expression in HT-29 cells. Lysates from cultured cells treated with the microbes for 12 h were subjected to Western blot analysis (20 µg/lane). Arrows show the top of the separating gel; bars show size markers. Values represent means ± SD of n = 5 independent assays; *P < 0.05.

heat-inactivated medium (boiling for 15 min) was applied to cell cultures. With the use of filter devices specific for different nominal molecular weight limits (3, 10, 30, and 50 kDa), a significant decrease in the protective capacity was observed only when a membrane excluding molecules >50 kDa was used, suggesting that the putative factor is likely in excess of that size (Fig. 10B). Furthermore, alterations of the pH of PM-conditioned media, which averaged 5.5, also diminished the protective capacities of that medium (Fig. 10C). Of note and as expected, more extreme alterations of pH were cytotoxic (data not shown).

DISCUSSION

In the present study, we have shown that in contrast to a pathogenic strain (S. dublin) and a gram-negative commensal (E. coli Nissle 1917), a complex mixture of probiotic organisms did not induce IL-8 secretion from intestinal epithelial cells. These results are consistent with previous studies showing the absence of IL-8 gene induction or secretion of that cytokine by the PM (25, 32). Coculture of IEC with gram-positive organisms of the PM and E. coli or S. dublin significantly reduced the induction of IL-8 secretion by the gram-negative bacteria compared with cultures infected with E. coli or S. dublin alone. This effect may have in vivo relevance in which a number of microbes compete for access to intestinal epithelial cells. Interestingly, cells were also protected, although to a lesser extent, when organisms of the PM were washed off before the application of S. dublin. In addition, a less pronounced but comparable effect was observed when IEC infected with S. dublin were cultured in medium conditioned by the PM.

These results might reflect competition for IEC cell surface receptors by products of the probiotics, membrane-stabilizing effects, or interference with proinflammatory intracellular pathways. However, as indicated by the data obtained with the cytokines IL-1β and TNF-α, proinflammatory pathways of the IEC are not globally muted. Shedding of epithelial receptors by microorganisms might be one of the major mechanisms responsible for the observed effects of PM. Candidate receptors include members of the TLR family, which have been shown to mediate effects induced by microbial compounds (36). Signaling through these receptors in IEC involves MAPK including p42/44 or p38 (5), and specific inhibitors of these kinases in the present study reduced E. coli- and S. dublin-induced IL-8 secretion. Of note, cell wall components from gram-positive organisms, which are present in the PM, have been identified as ligands for TLR2 (50).

Restoration or stabilization of the physical barrier function is an important goal in the treatment of intestinal inflammatory disorders. Incubation of IEC with S. dublin resulted in a rapid, significant decrease in TER with some decrease also observed following incubation with E. coli. In contrast, the PM increased TER when added alone and stabilized TER when cocultured with S. dublin. Similar effects of the PM on TER in IEC monolayers have also been described by Madsen (32), showing an increase in TER following a 6-h incubation period.

PM was found to significantly diminish Salmonella-induced alterations in the cellular cytoskeleton (22, 23), including the distribution of the tight-junction protein ZO-1. Although the mechanisms by which Salmonella induces dysfunction of tight junctions have not been defined in detail, it appears that the gross disruption of epithelia associated with shedding of enterocytes and loss of the luminal barrier involves a number of effects on the cytoskeleton (7, 23). Stabilization of the cytoskeleton as observed in this study might be important in the preservation of the epithelial architecture and, thereby, in the conservation of the intestinal barrier. Of note, effects on alterations in TER induced by the nonbacterial stressor monochloramine were less pronounced. Monochloramine is an oxidant that is known to increase epithelial permeability, i.e., by disruption of the cytoskeleton (1, 46).
Results from this study and previous work (32) indicate that this membrane-stabilizing effect is mediated, at least in part, by a soluble, heat labile substance that is pH dependant. Further characterization of that substance identified it to be protease sensitive, with an estimated size in excess of 50 kDa. The factor is therefore most likely of protein nature. However, DNase treatment of conditioned media had also potentially reduced the protective capacity of conditioned media. Immunmodulatory actions of bacterial DNA motifs have been recently described, and the application of these motifs in inflammatory conditions seems to be beneficial. Therefore, the effects of the PM-conditioned media may be due, in part, to bacterial DNA.

The MAPK p42/44 and p38 appear to be involved in membrane stabilization induced by the PM. These intracellular signaling intermediates participate also in growth factor-induced increase in TER (2, 27). PM-conditioned media was also found to activate the MAPK p42/44 and p38, indicating that the effect of the soluble factor is mediated, at least in part, by the MAPK pathway. Further characterization of this factor and other factors released from probiotic organisms is needed to exploit their beneficial effects. In an earlier report (49), factors...
recovered from lactobacillus-conditioned cell culture media had been shown to reduce cytokine-induced apoptosis and alter the activation of Akt and p38 MAPK.

The present results also suggest that apart from a direct effect, protective effects of the PM might accrue by competition for limiting access of the pathogens to the epithelial surface. Such a defensive layer might consist of the organisms themselves or induction of mucins. In our study, incubation of epithelial cells with PM induced the expression of several mucins. Expression of mucins, as detected in the mRNA and protein level, has been shown to be paralleled by mucin secretion. Mucin expression in epithelial cells has been suggested to play a role in modulating the adhesion of live organisms to the epithelial surface as well as bacterial components such as LPS (11, 26, 51). Adherence of microbes such as some lactobacilli with subsequent mucin secretion has also been reported by others (28) during the preparation of this manuscript. Although data presented herein suggest that this effect is mediated, at least in part, by MAPK, specific receptors and pathways need to be defined.

These mechanisms might facilitate the reestablishment of intestinal homeostasis and the preservation of epithelial barrier function, representing key elements in a therapeutic approach to intestinal disorders including IBD. Although most studies on the molecular basis of bacterial-epithelial cross-talk have been performed with enteric pathogens, promising results in the use

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**Fig. 10.** Characterization of the soluble factor released by the PM. **A:** T84 monolayers were incubated in PM-conditioned medium (1) or conditioned medium that has been DNase (2) or protease (3) treated or was heat inactivated (4) and were coinfected with 10⁴ CFU/ml of S. dublin. TER was measured at the time points indicated. Values are given as %unstimulated controls and represent means ± SD of n = 3 independent experiments. **B:** T84 cells were incubated in medium conditioned by the PM for 12 h with subsequent application to size-specific filter devices. Cells were then infected with 10⁴ CFU/ml of S. dublin. TER was measured at the time points indicated. Values are given as %unstimulated controls and represent means ± SD of n = 3. **C:** T84 cells were incubated in medium conditioned by the PM for 12 h with subsequent adjustment of the pH as indicated. Cells were then infected with 10⁴ CFU/ml of S. dublin. TER was measured at the time points indicated. Values are given as %unstimulated controls and represent means ± SD of n = 3.
of probiotics as a preventative approach to maintain the balance of the intestinal microflora have been reported (for a review, see Refs. 32 and 44). Early studies used _E. coli_ Nissle, whereas recent reports (24, 33, 41) have also described the beneficial effects of _Lactobacilli, Bifidobacteria_, and _Streptococci_. In animal studies, inflammatory changes associated with methotrexate-induced colitis in rats were prevented by the administration of lactobacillus strains _Reuteri_ and _Plantarum_ (35). Moreover, _Lactobacilli_ decreased the intestinal myeloperoxidase concentration, a marker of inflammation. In addition, the translocation of luminal microorganisms to extra-intestinal sites was significantly reduced, and a significant decrease in plasma endotoxin levels was observed. Administration of _Lactobacillus_ strains reduced intestinal inflammation in IL-10-deficient mice, which spontaneously develop enterocolitis (30, 31, 42). The PM has been reported to be more effective than a single _Lactobacillus_ in normalizing epithelial function and improving colitis (52). This has been attributed to the synergistic action of the individual strains in the mixture. In theory, adherent strains of that mixture might improve the mucus binding, adhesion, and activity of others (40). In humans, the PM has shown promising results when administered in trials assessing their effects in the maintenance of remission of pouchitis (17), UC (47), and in the prevention of recurrence of Crohn’s disease (4).

Results from this study provide insight into the mechanisms that may contribute to these clinical effects. As indicated, commensal organisms affect the intestinal epithelium in a direct and indirect manner. Prevention of pathogen access to the IEC by some organisms may complement mechanisms that strengthen or reestablish the epithelial barrier function. Functional capacities might be explained by species-specific mechanisms of action. Further understanding of the molecular foundation of the relationship between microbes and the host should clarify the contribution of these microorganisms to host physiology and allow enhanced application of these approaches as therapy.

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