

Probiotic Bacteria Enhance Murine and Human Intestinal Epithelial Barrier Function

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Background & Aims: The probiotic compound, VSL#3, is efficacious as maintenance therapy in pouchitis and ulcerative colitis. The aim of this study was to determine the efficacy of VSL#3 as a primary therapy in the treatment of colitis in the interleukin (IL)-10 gene-deficient mouse. Mechanisms of action of VSL#3 were investigated in T₈₄ monolayers. **Methods:** IL-10 gene-deficient and control mice received 2.8×10^8 colony-forming units per day of VSL#3 for 4 weeks. Colons were removed and analyzed for cytokine production, epithelial barrier function, and inflammation. VSL#3 or conditioned media was applied directly to T₈₄ monolayers. **Results:** Treatment of IL-10 gene-deficient mice with VSL#3 resulted in normalization of colonic physiologic function and barrier integrity in conjunction with a reduction in mucosal secretion of tumor necrosis factor α and interferon γ and an improvement in histologic disease. In vitro studies showed that epithelial barrier function and resistance to *Salmonella* invasion could be enhanced by exposure to a proteinaceous soluble factor secreted by the bacteria found in the VSL#3 compound. **Conclusions:** Oral administration of VSL#3 was effective as primary therapy in IL-10 gene-deficient mice, and had a direct effect on epithelial barrier function.

The most widely accepted theory on the pathogenesis of Crohn's disease is that it occurs as a result of an aggressive immune response to the resident microflora of the gastrointestinal tract. A role for bacteria in the pathogenesis of Crohn's disease is supported by clinical findings whereby the disease improves when luminal bacterial concentrations are decreased by a variety of techniques, including antibiotics, bowel rest, decontamination, and lavage treatment.¹⁻³ Furthermore, evidence from several experimental models has shown that when animals are raised under sterile conditions, intestinal inflammation does not develop, again suggesting a role for luminal flora in the initiation and perpetuation of colitis.⁴⁻⁶

Probiotics have been defined as living organisms in food and dietary supplements which, upon ingestion, improve the health of the host beyond their inherent basic nutrition.⁷ Results from human clinical trials have confirmed therapeutic effects of selected strains of microbes in viral- and bacterial-induced intestinal infections and in antibiotic-induced diarrhea.⁸⁻¹¹ Recently, studies have shown that certain Lactobacilli strains appear to have protective immunomodulating properties and are able to induce a systemic Th2 response.¹²⁻¹⁴ In addition, *Lactobacilli* sp. have the ability to inhibit the adhesion of pathogenic bacteria to the intestinal wall¹⁵ and also to restore permeability defects induced by cow's milk in weanling rats.¹⁶ Taken together, these results suggest that probiotic bacteria may be effective in the treatment of Crohn's disease.

The probiotic compound, VSL#3 (VSL Pharmaceuticals, Gaithersburg, MD), contains 9×10^{10} colony-forming units (cfu)/g of viable, lyophilized bifidobacteria (*Bifidobacterium longum*, *B. infantis*, and *B. breve*), 8×10^{10} lactobacilli (*L. acidophilus*, *L. casei*, *L. delbrueckii* subsp. *L. bulgaricus*, and *L. plantarium*), and 20×10^{10} of *Streptococcus salivarius* subsp. *Thermophilus*. This probiotic combination has shown efficacy in the maintenance treatment of pouchitis,¹⁷ ulcerative colitis,¹⁸ and in preventing postoperative recurrence of Crohn's disease.¹⁹ The use of VSL#3 as a primary therapy for inflammatory bowel disease has not been investigated.

Interleukin (IL)-10 gene-deficient mice housed in conventional conditions develop a patchy, chronic colitis that is similar to human Crohn's disease.²⁰ We have previously shown that administration of *Lactobacillus* sp.

Abbreviations used in this paper: cfu, colony-forming unit; CM, conditioned media; ELISA, enzyme-linked immunosorbent assay; G, tissue ion conductance; IFN- γ , interferon γ ; IL, interleukin; Isc, short-circuit current; LPS, lipopolysaccharide; TNF- α , tumor necrosis factor α .

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0016-5085/01/\$35.00

doi:10.1053/gast.2001.27224

from birth prevents the development of spontaneous colitis in IL-10 gene-deficient mice²¹ and that antibiotic treatment was effective in treating established disease.²² However, the treatment of IL-10 gene-deficient mice with a single species of *Lactobacillus* after colitis had become established was not as effective.²¹ Thus, the aim of this study was to determine if a combination of probiotic bacteria as found in the VSL#3 compound was efficacious in the treatment of colitis in the IL-10 gene-deficient mouse, and also to examine the mechanism of the VSL#3 action on epithelial cell function in T₈₄ monolayers.

Methods

Animals

Homozygous IL-10 gene-deficient mice, generated on a 129 Sv/Ev background, and normal 129 Sv/Ev controls were housed behind a barrier under specific pathogen-free conditions. All provisions for the facility were sterilized using an autoclave. Nonautoclavable supplies were sprayed with disinfectant and introduced into the facility through a high-efficiency particulate air (HEPA)-filtered air-lock. The facility's air was HEPA-filtered. The mice were housed in micro isolator cages covered with tight-fitting lids that contained a spun-polyester fiber filter. The mice had ad libitum access to autoclaved (sterile) 9% fat rodent blocs and sterile filtered water. The facility's sanitation was verified by Health Sciences Lab Animal Services at the University of Alberta (Edmonton, Alberta, Canada). Sentinel Balb/c mice were housed in the animal room and examined for known bacterial, parasitic, and viral pathogens. Bacterial cultures, parasitologic examinations, serologic tracking profiles, and histologic stains, all negative for known murine viral and bacterial pathogens, indicated that the barrier was intact.

VSL#3 Therapy

VSL#3 therapy was initiated in IL-10 gene-deficient mice after inflammation had become established. Eight-week-old control and IL-10 gene-deficient mice received a daily oral gavage for 4 weeks of 2.8×10^8 cfu/mL VSL#3 dissolved in saline, and studied at 12 weeks of age. Colons were removed and examined for bacterial content, epithelial ion transport function, cytokine secretion, and histologic injury score. Control groups received saline gavage only.

Measurement of Colonic Bacteria

Colons were removed aseptically. For the purposes of measuring total bacterial content, a 2-cm segment of each region was removed, weighed, and homogenized in 5 mL of sterile phosphate-buffered saline (PBS). To measure for mucosal adherent bacteria, and any translocated bacteria, a 2-cm segment of intestine immediately adjacent to the segment described above was removed, vigorously washed once with 10 mL of sterile PBS, and then homogenized in 5 mL PBS. Serial

100-fold dilutions of both homogenates were plated on *Lactobacilli* sp. MRS agar for quantification of *Lactobacillus* and *Bifidobacterium* sp. (Difco, Detroit, MI). Bacterial counts are reported as log₁₀ colony-forming units per gram colonic tissue (cfu/g).

Epithelial Function

A separate group of mice (n = 10) were used for the study of epithelial transport function. Mice were killed by cervical dislocation, and a segment of colon was removed. The mucosa was mounted in Lucite chambers exposing mucosal and serosal surfaces to 10 mL of oxygenated Krebs buffer (in mmol/L: 115 NaCl, 8 KCl, 1.25 CaCl₂, 1.2 MgCl₂, 2 KH₂PO₄, 225 NaHCO₃; pH 7.35). The buffers were maintained at 37°C by a heated water jacket and circulated by CO₂/O₂. Fructose (10 mmol/L) was added to the serosal and mucosa sides. For measurement of basal mannitol fluxes, 1 mmol/L of mannitol with 10 μCi [³H] was added to the mucosal side. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current (Isc) with an automatic voltage clamp (DVC 1000 World Precision Instruments, New Haven, CT), except for 5–10 seconds every 5 minutes when PD was measured by removing the voltage clamp. Tissue ion conductance (G) was calculated from PD and Isc according to Ohm's Law.²³ PD is expressed as millivolts (mV), Isc as microamperes per square centimeter (μA/cm²), and G as millisiemens/cm². Baseline Isc and G were measured after a 20-minute equilibration period. Increases in Isc were induced by addition of the adenylate cyclase-activating agent, forskolin (10⁻⁵ mol/L), to the serosal surface. Epithelial responsiveness was defined as the maximal increase in Isc to occur within 5 minutes of exposure to the secretagogue.

Mucosal Cytokine Secretion

Colon organ cultures were prepared from control mice (n = 6), IL-10 gene-deficient mice (n = 6), and mice receiving VSL#3 therapy (n = 6). Colons were removed, flushed with cold PBS, and cut into 2-mm squares. Each square was washed and suspended in tissue culture wells (Falcon 3046; Becton Dickinson Labware, Lincoln Park, NJ) in RPMI-1640 supplemented with 10% fetal calf serum, 50 mmol/L 2-β-mercaptoethanol, penicillin (100 U/mL), streptomycin (100 U/mL), and the presence or absence of lipopolysaccharide (LPS). Cultures were incubated at 37°C in 5% CO₂ for 6 hours for measurement of tumor necrosis factor α (TNF-α) and 24 hours for measurement of interferon γ (IFN-γ). Supernatants were harvested and stored at -70°C for analysis of cytokine levels. TNF-α and IFN-γ levels in cell supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits (Medicorp, Montreal, Quebec).

Histologic Injury Grading

Mice were sacrificed using sodium pentobarbital (160 mg/kg) after 4 weeks of treatment (n = 8). Colons were

Table 1. Histologic Injury Scoring System

Group	Description	Score
Enterocytes		
Normal	Rare intraepithelial lymphocytes	0
Mild	Intraepithelial neutrophils	1
Moderate	Mucosal necrosis and/or luminal pus	2
Severe	Necrosis muscularis mucosa	3
Epithelial hyperplasia		
Normal		0
Mild		1
Moderate		2
Pseudopolyps		3
Lamina propria mononuclear infiltrate		
Normal	One small lymphoid aggregate	0
Slightly increased	More than one small aggregate	1
Markedly increased	Large aggregates and/or greatly increased single cells	2
Lamina propria neutrophil infiltrate		
Normal	No extravascular presence	0
Slightly increased	Single neutrophils	1
Markedly increased	Cell aggregates	2

NOTE. Minimal injury = 0; maximal injury = 10.
Data from Saverymuttu et al.²⁴

harvested and fixed in 10% phosphate-buffered formalin. These samples were paraffin-embedded in toto, sectioned at 4 μm , and stained with H&E for light microscopic examination. The slides were reviewed in a blinded fashion by 2 pathologists (J.D. and L.D.J.) and were assigned a histologic score for intestinal inflammation using a scheme adapted from Saverymuttu et al.²⁴ as detailed in Table 1. Histologic grades (ranging from 0 to 10) represent the numerical sum of 4 scoring criteria: mucosal ulceration, epithelial hyperplasia, lamina propria mononuclear infiltration, and lamina propria neutrophilic infiltration.

T₈₄ Cell Culture Studies

T₈₄ cells at passages 30–34 were grown as monolayers in a 1:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 medium supplemented with 15 mmol/L Na⁺-HEPES buffer, pH 7.5, 14 mmol/L NaHCO₃, and 5% newborn calf serum. For subculture, a cell suspension was obtained from confluent monolayers by exposing the monolayers to 0.25% trypsin and 0.9 mmol/L EDTA in Ca⁺ and Mg⁺ free PBS. Cells were seeded at a density of 1×10^6 cells/1.13 cm² polycarbonate tissue culture–treated filter and maintained at 37°C in a 5% CO₂ atmosphere. Cultures were refed daily with fresh media.

To qualitatively determine whether the T₈₄ cells had reached confluence, formed tight junctions, and established cell polarity, the electrical conductance and the spontaneous potential across the monolayer were determined using an

EVOM voltohmmeter and an STX-2 electrode set (World Precision Instruments, Sarasota, FL). To determine the effect of VSL#3 compound on epithelial function, monolayers were exposed to bacteria for varying lengths of time and then mounted in Ussing chambers for measurement of I_{sc}, PD, and mannitol permeability. The epithelial response to heat-killed VSL#3 (95°C for 30 minutes) was also determined. To determine if an active soluble compound was released into the media from bacteria, VSL#3 was incubated in cell culture media and the bacteria then removed by filtration. This conditioned media was placed on fresh T₈₄ monolayers for 6 hours. To determine if the active compound was heat labile, conditioned media was heated to 100°C for 15 minutes before being applied to T₈₄ monolayers, again for 6 hours. Serial 100-fold dilutions of conditioned media, and media containing heat-killed bacteria, were plated on brain-heart infusion media to ensure the absence of viable bacteria.

Other bacteria examined were *L. reuteri*, *E. coli* DH5 α (ATCC 43892), and *Streptococcus bovis* (ATCC 9809).

Proteinase K treatment. Conditioned media was prepared as above and divided into 2 equal aliquots. One aliquot was incubated with 45 μg of proteinase K for 30 minutes at 37°C and then with phenylmethylsulfonylchloride (final concentration = 1 mmol/L) to terminate further proteolysis. The other aliquot served as a sham control. Treated and nontreated conditioned media was placed onto T₈₄ monolayers for 6 hours. To control for effects of proteinase K and phenylmethylsulfonylchloride, cell culture media was treated in an identical fashion and then placed onto T₈₄ monolayers.

Cycloheximide studies. To determine if the monolayer response was dependent on protein synthesis, conditioned media was applied to monolayers in the presence and absence of cycloheximide (5 $\mu\text{g}/\text{mL}$).

Salmonella dublin invasion of T₈₄ monolayers. To determine if prior exposure of T₈₄ monolayers to VSL#3 provided any protection against subsequent invasive bacteria, T₈₄ monolayers were incubated with apical VSL#3 for 6 hours. Bacteria were then removed, monolayers rinsed twice with PBS, and *Salmonella dublin* applied in an invasion assay. Briefly, *S. dublin* strain Lane (ATCC #15480) was stored in 20% (wt/vol) skim milk at –70°C. Bacteria were inoculated at 0.18% (vol/vol) into 25 mL of Tryptone Soy Broth (Difco #0370-17-3) and grown statically overnight (18–20 hours) at 37°C before being used in T₈₄ invasion assays. For invasion assays, cultures of *S. dublin* were centrifuged at 2000g for 10 minutes, and then resuspended in PBS. Bacteria were diluted in serum-free cell culture media to 2×10^6 cfu/mL. This suspension (0.5 mL) was applied to the apical surface of a Transwell insert, and 1.75 mL of serum-free cell culture media was applied to the basolateral surface. T₈₄ cells were incubated at 37°C for 1 hour. The *Salmonella* suspension was then removed and bacterial numbers determined by plating onto Tryptone Soy Agar (Difco #0369-17) to establish the growth of the bacteria over the 1-hour incubation period and also to ensure the absence of any viable VSL#3. Extracellular *Salmo-*

nella were killed by washing the T₈₄ monolayers twice with serum-free culture media containing 50 µg/mL of the non-membrane permeant antibiotic gentamicin, followed by incubation for 2 hours at 37°C in the gentamicin media. Initial studies showed that this was sufficient time to kill all extracellular and adherent bacteria (data not shown). Cell culture media was then removed and cells washed twice with PBS. *Salmonella* invasion was determined by incubating the Transwell insert membranes for 5 minutes in sterile water containing 0.01% (wt/vol) Triton-X 100 so as to release intracellular bacteria. In preliminary experiments, this treatment was found to release all intracellular bacteria and to not affect bacterial viability (data not shown). The suspension was plated onto Tryptone Soy Agar plates and cfus determined after a 24-hour incubation. Invasion was expressed as a percentage of the number of *Salmonella* internalized by the monolayer to the total number of *Salmonella* exposed to the monolayer after 1-hour incubation. The ability of *E. coli* DH5α (ATCC 43892) to prevent subsequent *Salmonella* invasion was also studied.

Determination of IL-8 Secretion

To assess epithelial response to *Salmonella* invasion and VSL#3 exposure, T₈₄ monolayers were exposed to bacteria, and IL-8 release into culture media was measured. IL-8 protein was measured with ELISA as follows: 96-well Maxisorp ELISA plates (Nunc, Rochester, NY) were coated with 4 µg/mL capture monoclonal anti-IL-8 antibody (R&D Systems, Minneapolis, MN) in PBS (pH 7.4) overnight. Plates were then blocked overnight (5% sucrose, 0.05% sodium azide, 1% bovine serum albumin in PBS, pH 7.4). Plates were washed 4 times between all steps with 0.05% Tween-20 PBS, pH 7.4. One hundred microliters of samples and standards (0–4000 pg/mL human recombinant IL-8; R&D Systems) were incubated in the plates overnight. Biotinylated polyclonal anti-IL-8 antibody (R&D Systems) was added (20 ng/mL in PBS, pH 7.4), and plates were incubated for 2 hours. One hundred microliters Streptavidin-HRP (Southern Biotechnology Associates, Birmingham, AL) was added for 1 hour followed by development with 100 µL TMBS (Calbiochem, San Diego, CA). Reaction was stopped with acid (0.5 mol/L H₂SO₄), and plates were read immediately at 450 nm using an ELISA plate reader (UV max; Molecular Devices, Sunnyvale, CA). All steps were performed at room temperature. ELISA was sensitive to <30 pg/mL.

Statistical Analysis

Data are expressed as means ± SEM. Data were tested for normality of distribution, and analyses were performed using the statistical software SigmaStat (Jandel Corporation, San Rafael, CA). Differences between means were evaluated using analysis of variance or paired *t* tests where appropriate. Specific differences were tested using the Student-Newman-Keuls test. Statistical evaluations of the significance of the differences in mean log₁₀ viable bacterial counts obtained from intestinal homogenates were performed using the Student *t* test.

Results

In Vivo Mouse Studies

Colonic bacteria. To confirm that the oral administration of the VSL#3 compound resulted in viable organisms reaching the colon, total and adherent levels of *Lactobacillus* spp. and *Bifidobacteria* spp. were measured in control and IL-10 gene-deficient mice after 4 weeks of treatment. There was no difference between controls and IL-10 gene-deficient mice in the total amount of *Lactobacillus* and *Bifidobacteria* (Figure 1A). However, IL-10 gene-deficient mice had significantly less amounts of adherent *Lactobacillus* and *Bifidobacteria* compared with controls (Figure 1B). Treatment of both control and IL-10 gene-deficient mice resulted in a significant in-

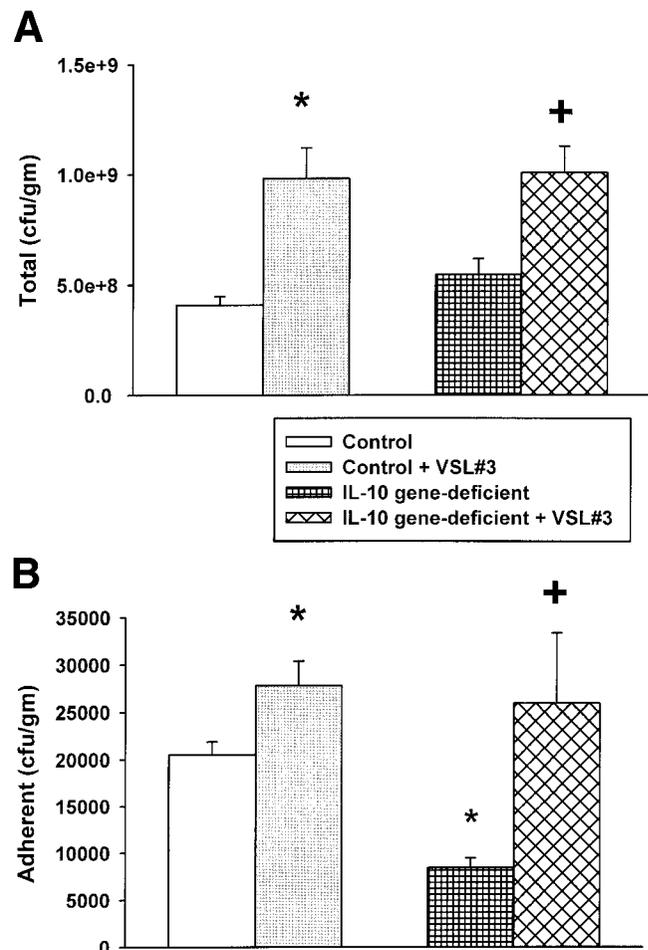


Figure 1. Colonic mucosal (A) total and (B) adherent levels of *Lactobacillus* spp. and *Bifidobacterium* spp. in IL-10 gene-deficient mice ($n = 6$) and age-matched controls ($n = 6$). IL-10 gene-deficient mice had significantly reduced levels of adherent *Lactobacillus* spp. and *Bifidobacterium* spp. compared with controls. Both control mice and IL-10 gene-deficient mice receiving VSL#3 therapy showed increased levels of (A) total and (B) adherent *Lactobacillus* spp. and *Bifidobacterium* spp. * $P < 0.05$ compared with age-matched control. + $P < 0.05$ compared with IL-10 gene-deficient mice.

crease in total and adherent *Lactobacillus* spp. and *Bifidobacteria* spp. bacteria (Figure 1). These data show that the oral administration of VSL#3 resulted in increased numbers of viable organisms in the colon in both control and IL-10 gene-deficient mice, confirming findings in human subjects consuming this product.^{17,18}

Intestinal cytokine secretion. *Mucosal TNF- α secretion.* Previous studies have suggested that the presence of certain strains of probiotic bacteria can alter tissue cytokine secretion from a proinflammatory to an anti-inflammatory profile.²⁵ Compared with controls, IL-10 gene-deficient mice spontaneously secreted higher amounts of TNF- α from both the ileum ($P \leq 0.05$; Figure 2A) and the colon ($P \leq 0.02$; Figure 2B). Colonic tissue from IL-10 gene-deficient mice responded to the presence of LPS with an enhanced release of TNF- α ($P \leq 0.01$), suggesting the presence of monocytes and active inflammation. In contrast, although ileal tissue from IL-10 gene-deficient mice showed a 2-fold increase in spontaneous basal TNF- α secretion compared with controls, this tissue did not respond to LPS with a significant increase in TNF- α secretion. There was also no evidence of histologic inflammation in this region. Control mice did not respond to the presence of LPS with an increase in TNF- α secretion in either the ileum or the colon.

After 4 weeks of VSL#3 treatment, basal ($P \leq 0.05$) and LPS-stimulated ($P \leq 0.01$) TNF- α secretion was significantly reduced in the ileum and colon of IL-10 gene-deficient mice as compared with untreated IL-10 mice. Interestingly, colons from control mice also showed a reduction in basal and LPS-stimulated TNF- α secretion ($P \leq 0.05$) after treatment with VSL#3. There was no effect of VSL#3 on cytokine secretion from the ileum of control mice. Thus, although the bacterial load of the colon was significantly increased in control and IL-10 gene-deficient mice, both groups responded to the increased total load with a decrease in TNF- α secretion.

Mucosal IFN- γ secretion. IL-10 gene-deficient mice showed a similar increase in spontaneous secretion of IFN- γ compared with controls in both ileum (Figure 3A) and colon (Figure 3B). In contrast to the results seen with TNF- α , IFN- γ secretion was stimulated by LPS in both the ileum and colon of IL-10 gene-deficient mice. Control mice did not respond to LPS stimulation.

VSL#3 treatment normalized basal IFN- γ secretion in the ileum (Figure 3A), but not in the colon (Figure 3B) in IL-10 gene-deficient mice. However, LPS-stimulated IFN- γ secretion was reduced in both ileum and colon by VSL#3 treatment. Control mice receiving VSL#3 also showed a reduction in IFN- γ secretion in the colon.

These data would suggest that the microflora present

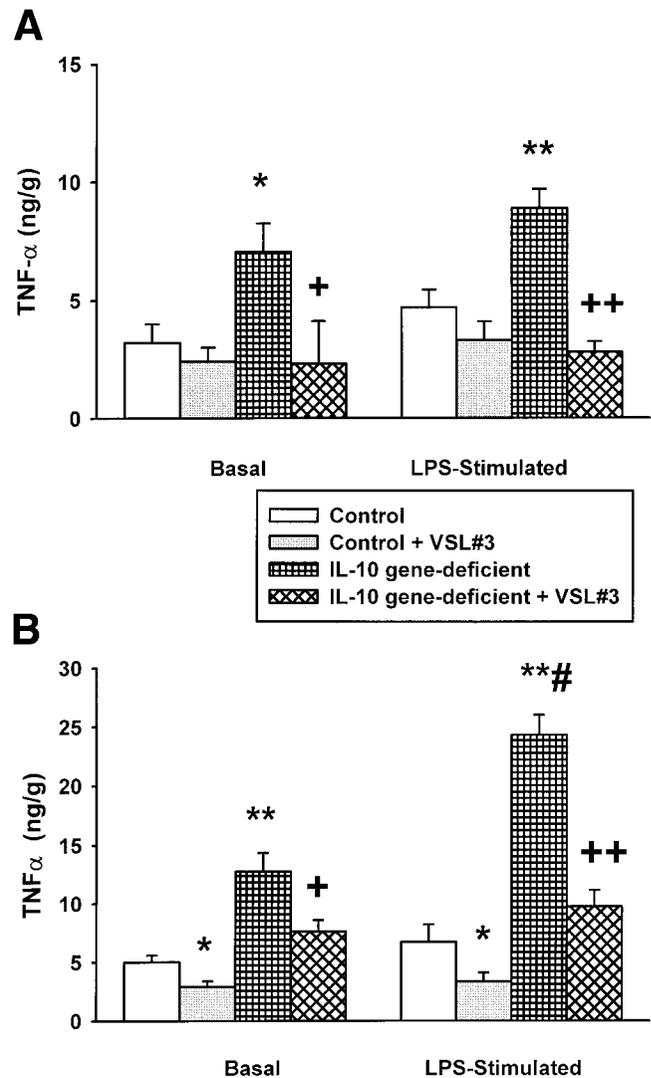


Figure 2. Effect of VSL#3 on TNF- α secretion from (A) ileum and (B) colon. Under basal conditions, IL-10 gene-deficient mice ($n = 8$) secreted significantly higher amounts of TNF- α compared with controls ($n = 8$) in both the ileum and colon. Colons from IL-10 gene-deficient mice responded to LPS with a significant increase in TNF- α secretion. After 4 weeks of VSL#3 treatment, basal and LPS-stimulated secretion of TNF- α was reduced in the ileum and colon of IL-10 gene-deficient mice. Control mice treated with VSL#3 ($n = 8$) showed a reduction of basal and LPS-stimulated TNF- α secretion in the colon. * $P < 0.05$ compared with age-matched control. ** $P < 0.02$ compared with age-matched control. + $P < 0.05$ compared with IL-10 gene-deficient mice. ++ $P < 0.01$ compared with IL-10 gene-deficient mice. # $P < 0.01$ compared with basal secretion.

in the lumen can modulate immune cytokine responses, both under normal and inflammatory conditions. Furthermore, even in the absence of the regulatory cytokine, IL-10, the intestinal mucosa in IL-10 gene-deficient mice was able to respond to the presence of the *Lactobacillus* and *Bifidobacteria* strains found in the VSL#3 mixture with a down-regulation of a proinflammatory immune response.

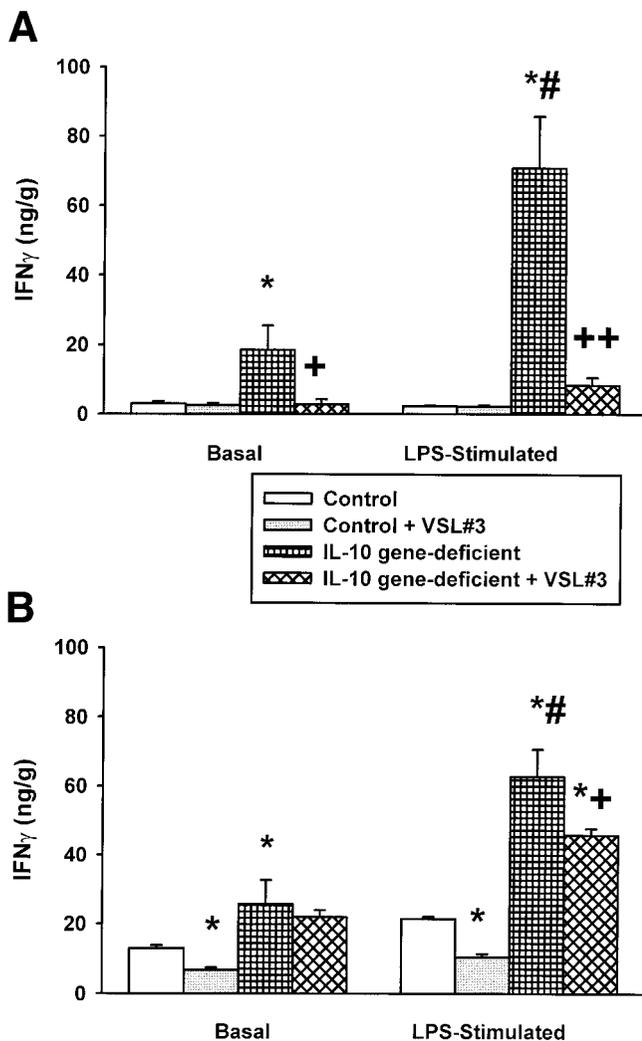


Figure 3. Effect of VSL#3 on IFN- γ secretion from (A) ileum and (B) colon. Under basal conditions, IL-10 gene-deficient mice ($n = 8$) secreted significantly higher amounts of IFN- γ compared with controls ($n = 8$) in both the ileum and colon. The ileum and colon from IL-10 gene-deficient mice responded to LPS with a significant increase in IFN- γ secretion. After 4 weeks of VSL#3 treatment, secretion of basal and LPS-stimulated IFN- γ was reduced in ileum of IL-10 gene-deficient mice. Basal secretion of IFN- γ in the colon was not altered by VSL#3 treatment, but LPS-stimulated secretion was significantly reduced. Control mice treated with VSL#3 ($n = 8$) showed a reduction of IFN- γ secretion in the colon. * $P < 0.05$ compared with age-matched control. + $P < 0.05$ compared with IL-10 gene-deficient mice. ++ $P < 0.01$ compared with IL-10 gene-deficient mice. # $P < 0.02$ compared with basal secretion.

Epithelial ionic function. In vitro and in vivo studies have shown that proinflammatory cytokines can alter epithelial transport and barrier functions.^{26–29} Thus, we examined colonic epithelium from IL-10 gene-deficient mice in Ussing chambers to determine if these mice exhibited physiologic alterations in colonic ionic function. As seen in Figure 4, colons from IL-10 gene-deficient mice showed significant reductions in basal Isc (Figure 4A) and PD (Figure 4B) compared with age-

matched controls. In addition, colonic tissues showed a 90% reduction in Isc response to forskolin, suggesting a severe impairment in adenosine 3',5'-cyclic monophosphate-dependent active chloride secretion (Figure 4C). After 4 weeks of VSL#3 treatment, baseline Isc, PD, and Δ Isc response to forskolin were all normalized. Physiologic transport function of colonic tissue from control mice was not affected by VSL#3 treatment.

Epithelial Barrier Function

We have previously shown that colons from IL-10 gene-deficient mice show increased permeability as measured by in vivo perfusion.³⁰ Measurements made in colonic tissues from IL-10 gene-deficient mice mounted in Ussing chambers confirmed this in vivo data, showing a significant increase in unidirectional mannitol fluxes as compared with control mice (Figure 4D). After 4 weeks of VSL#3 therapy, mannitol fluxes were completely normalized in the IL-10 gene-deficient mice. Interestingly, although physiologic ionic function was not affected in control mice by VSL#3 treatment, mannitol fluxes were reduced, suggesting that the species of microflora present in the colon can directly alter colonic permeability.

Histologic and Morphologic Analysis

In our animal care facility, all IL-10 gene-deficient mice develop colonic inflammation by 8 weeks of age.²¹ Thus, this study examined mice at 8 weeks of age to ascertain the effectiveness of the VSL#3 compound in attenuating established colitis. As seen in Table 2, by 12 weeks of age, IL-10 gene-deficient mice demonstrated a decrease in body weight coupled with an increased wet weight of the colon, indicative of chronic inflammation that can be characterized by increased muscularis mucosa and edema.³¹ Histologic analysis of the colons showed that 100% of the IL-10 gene-deficient mice showed histologic inflammation. Generally, the colonic epithelium was disrupted in a patchy fashion by either regions of erosion and tissue necrosis, or deeper ulcerations beyond the level of the muscularis propria. Patchy transmural acute and chronic inflammation was evident and intraepithelial neutrophils and lymphocytes were present throughout in a patchy distribution. Collecting of neutrophils and lymphocytes were present in the lamina propria and muscularis mucosa outside the realm of the erosions and ulcerations as well. Moderate to marked epithelial hyperplasia was present. IL-10 gene-deficient mice receiving VSL#3 therapy showed a reduction in mucosal ulceration, epithelial hyperplasia, and mononuclear and neutrophilic infiltrate into the lamina propria. Coupled with this improvement in all histologic parameters, those mice receiving VSL#3 therapy also showed a

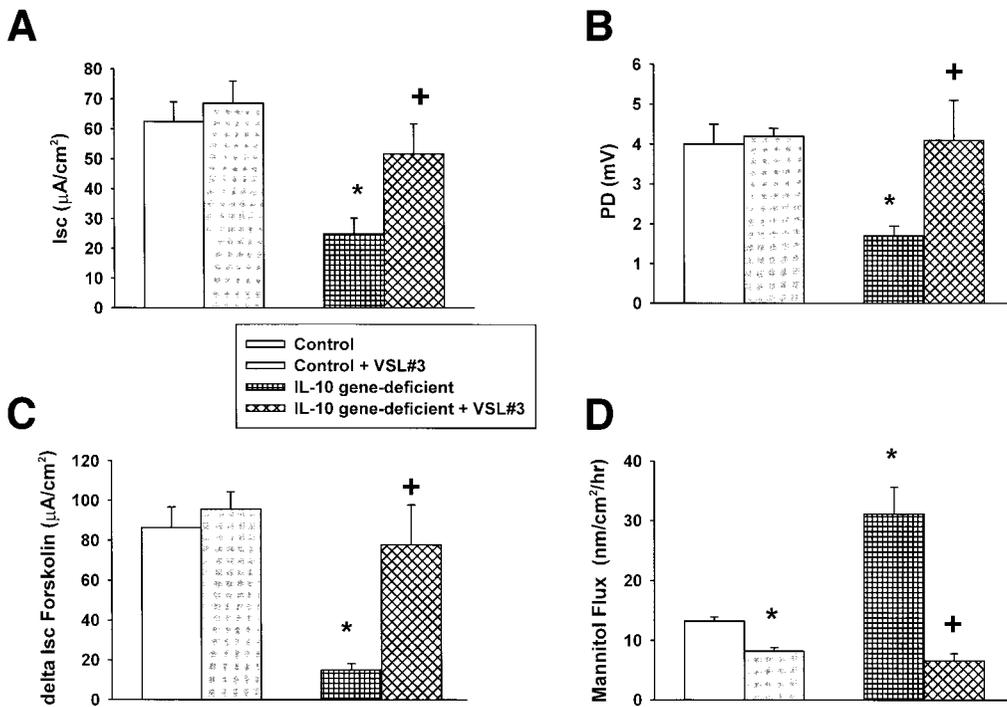


Figure 4. Effects of VSL#3 treatment on colonic electrical parameters and barrier function in control (n = 10) and IL-10-deficient mice (n = 14). IL-10-deficient mice had significantly reduced (A) I_{sc}, (B) PD, and (C) Δ I_{sc} response to forskolin, coupled with an increase in unidirectional (D) mannitol fluxes. Four weeks of VSL#3 therapy normalized all parameters in IL-10 gene-deficient mice. Control mice treated with VSL#3 (n = 6) showed a decrease in mannitol fluxes (D). *P < 0.05 compared with age-matched control. +P < 0.01 compared with IL-10 gene-deficient mice.

decrease in the wet weight of the colon and a resultant decrease in the ratio of weight/weight to length (Table 2).

T₈₄ Monolayer Experiments

Having observed that VSL#3 treatment of both control mice and IL-10 gene-deficient mice resulted in an enhancement of epithelial barrier function, we next sought to determine if the VSL#3 compound was having a direct effect on epithelial function, or, conversely, was the beneficial effect of VSL#3 caused by a down-regulation of proinflammatory cytokine secretion from immune

cells. To answer this question, we applied VSL#3 directly to the apical surface of monolayers and monitored monolayer resistance. As seen in Figure 5, 6 hours after monolayer exposure to VSL#3, resistance had increased. By 8 hours, the increased resistance plateaued. pH of the media began to decrease after 8 hours of incubation (data not shown). Thus, to avoid the complications of pH effects, further studies were limited to a maximum of 6 hours of incubation.

To further investigate the effects of VSL#3 on epithelial resistance, T₈₄ monolayers were studied in Ussing chambers after 2, 4, and 6 hours of exposure. As seen in

Table 2. Morphologic and Histologic Characteristics of 12-Week-Old Control Mice, IL-10 Gene-Deficient Mice, and IL-10 Gene-Deficient Mice Receiving VSL#3 for 4 Weeks

Group	Colonic physical parameters				Histologic score				
	Body wt (g)	Wt/weight (g)	Length (cm)	Wt/length (%)	Enterocytes (0–3 units)	Epithelial hyperplasia (0–3 units)	Lamina propria mononuclear infiltrate (0–2 units)	Lamina propria neutrophilic infiltrate (0–2 units)	Total (0–10)
Control (n = 8)	26.6 ± 0.6	0.16 ± 0.01	8.3 ± 0.2	1.9 ± 0.01	0.2 ± 0.1	0.2 ± 0.2	0.4 ± 0.2	0.6 ± 0.2	1.4 ± 0.6
IL-10 gene-deficient (n = 8)	20.5 ± 0.5 ^a	0.32 ± 0.06 ^a	8.1 ± 0.3	3.7 ± 0.5 ^a	2.0 ± 0.2 ^a	2.1 ± 0.2 ^a	1.7 ± 0.2 ^a	1.8 ± 0.1 ^a	7.6 ± 0.5 ^a
IL-10 gene-deficient + VSL#3 (n = 10)	23.7 ± 0.7 ^{a,b}	0.14 ± 0.01 ^b	7.6 ± 0.2	1.8 ± 0.03 ^b	0.7 ± 0.2 ^b	0.4 ± 0.2 ^b	0.8 ± 0.2 ^b	0.8 ± 0.2 ^b	2.7 ± 0.4 ^b

NOTE. Values are means ± SE.

^aP < 0.05 compared with control.

^bP < 0.05 compared with IL-10 gene-deficient mice.

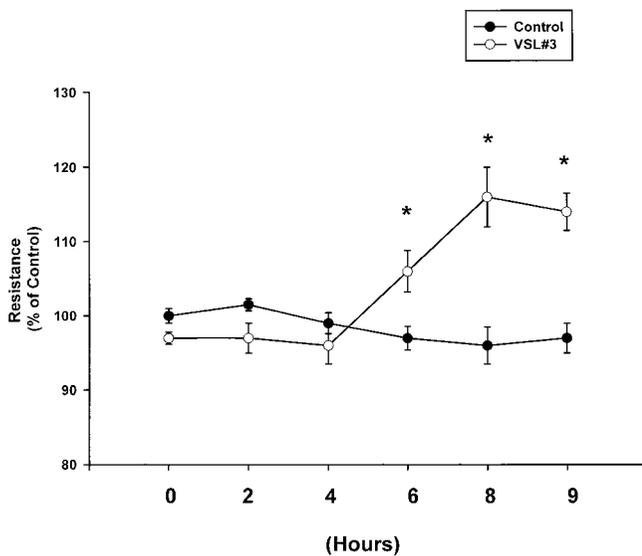


Figure 5. Effect of VSL#3 on T_{84} monolayer resistance. After 6 hours of exposure, resistance began to rise in monolayers. This increase plateaued by 8 hours ($n = 6$). * $P < 0.05$ compared with control monolayer.

Table 3, after 2 hours of exposure, there was no difference in conductance or mannitol permeability, although short circuit current was enhanced. After 4 hours, monolayers still did not show any change in monolayer conductance, but mannitol permeability and short circuit current were both decreasing. By 6 hours, both mannitol permeability and monolayer conductance were significantly decreased, and short circuit current had returned to normal values. To determine if live bacteria were required for the enhancement of barrier function, an experiment using heat-killed bacteria was performed. As can be seen in Table 3, heat-killed bacteria had no effect on either monolayer conductance, I_{sc} , or mannitol permeability.

To examine whether the response of the T_{84} monolayer was specific to the bacteria found in the VSL#3 com-

pound, or, conversely, occurred as a response to a certain concentration of bacteria, we exposed monolayers to similar amounts of the nonpathogenic gm-positive bacterial strains, *L. reuteri* (9.3×10^7 cfu), *S. bovis* (8×10^8 cfu), or a gm-negative nonpathogenic *E. coli* (DH5 α ; 4.7×10^7 cfu). *L. reuteri* and *E. coli* both increased monolayer permeability and conductance, whereas *S. bovis* had no effect on either parameter (Table 3). Thus, it would seem that the increase in resistance observed as a response to VSL#3 is specific to either one or more, or the combination, of the bacteria that comprise the VSL#3 compound. In addition, these data would also suggest that the monolayer response is not related to nonspecific cellular wall components associated with either gm-negative or gm-positive bacteria, as the 2 gm-positive compounds, *L. reuteri* and *S. bovis*, elicited different responses from the monolayer.

To determine if the effect of the VSL#3 compound on monolayer resistance was a result of a secretion of a soluble factor, we examined the response of monolayers to conditioned media. VSL#3 was incubated in cell culture media after which the bacteria were removed by filtration. This conditioned media was then placed on fresh T_{84} monolayers for 6 hours. As seen in Table 3, monolayers responded to conditioned media with a similar decrease in conductance and mannitol permeability, suggesting that a soluble factor was responsible for the enhancement in barrier integrity.

To further investigate the nature of this soluble factor, we treated the conditioned media for 15 minutes at 100°C to determine if the factor is heat labile. As seen in Figure 6, conditioned media (CM) reduced monolayer conductance and mannitol flux to approximately 54% of control values. Heat-inactivated CM had no effect on either monolayer conductance (Figure 6A) or mannitol

Table 3. Electrical and Permeability Parameters of T_{84} Monolayers Exposed to Bacteria

Group	Time of exposure (h)	Bacteria/well	G (mS/cm^2)	I_{sc} ($\mu A/cm^2$)	Mannitol ($nmol/h/cm^2$)
Control ($n = 8$)	0	0	1.5 ± 0.1	1.9 ± 0.1	6.8 ± 0.6
VSL#3 ($n = 9$)	2	7.4×10^7	1.1 ± 0.1	3.0 ± 0.2^a	5.8 ± 1.0
	4	2.5×10^7	1.2 ± 0.1	2.6 ± 0.1^a	3.8 ± 0.4
	6	6.8×10^7	0.6 ± 0.1^a	2.4 ± 0.1	2.4 ± 0.2^a
VSL#3 conditioned media ^b ($n = 3$)	6	0	0.8 ± 0.1^a	1.8 ± 0.2	3.2 ± 0.4^a
VSL#3 heat killed ($n = 3$)	6	8.9×10^7	1.8 ± 0.3	1.9 ± 0.3	5.9 ± 0.9
Gm-positive bacteria					
<i>Lactobacillus</i> ($n = 6$)	6	9.3×10^7	3.3 ± 0.4^a	2.1 ± 0.3	29.7 ± 8.2^a
<i>Streptococcus bovis</i> ($n = 6$)	6	1.3×10^8	1.0 ± 0.4	1.3 ± 0.4	7.9 ± 2.4
Gm-negative bacteria					
<i>E. coli</i> (DH5 α) (nonpathogenic; $n = 6$)	6	4.7×10^7	2.8 ± 0.3^a	1.3 ± 0.2	21.1 ± 6.8^a

^a $P < 0.05$ compared with controls.

^bVSL#3 bacteria were grown in cell culture media for 6 hours and removed by filtration. The media was placed on new T_{84} monolayers for 6 hours.

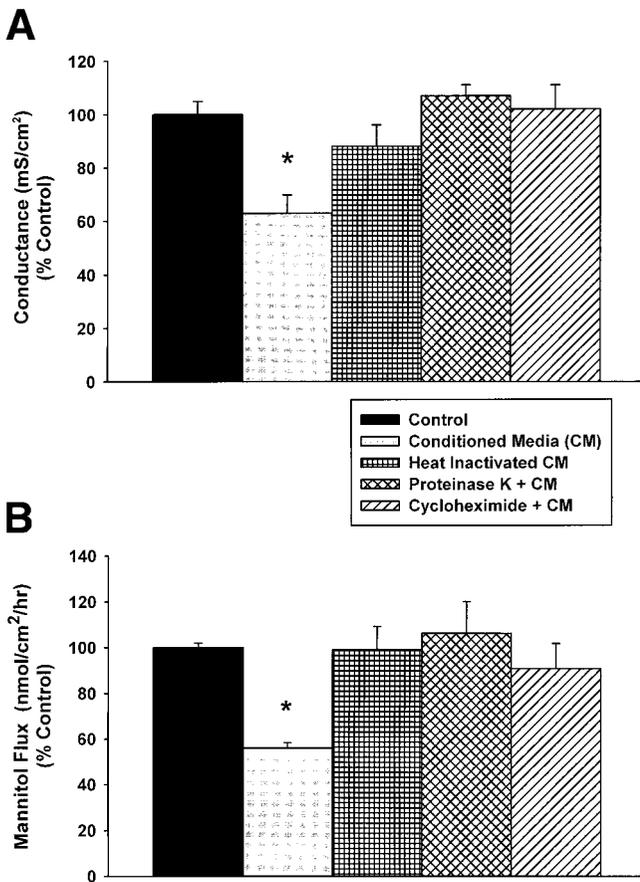


Figure 6. Conditioned media from VSL#3 compound reduced (A) conductance and (B) mannitol flux in T₈₄ monolayers. Heat inactivation (n = 6) and proteinase K treatment (n = 6) of the conditioned media blocked the effects on barrier function. Treating the monolayer with cycloheximide (n = 6) also blocked effects of the conditioned media. *P ≤ 0.05 compared with control monolayer.

flux (Figure 6B), suggesting that the active factor is heat labile.

We next sought to determine if the active factor was proteinaceous in nature. Figure 6 shows the effect of proteinase K treatment of conditioned media. Proteinase K treatment prevented the drop in conductance and mannitol flux, suggesting that the active factor is a protein.

Finally, to determine if protein synthesis was required for the epithelial response, we treated monolayers with cycloheximide (5 μg/mL) concurrently with the application of CM. Cycloheximide blocked the effect of CM on monolayer conductance and mannitol flux, suggesting that a soluble protein factor may initiate one or more intracellular signaling pathways within the epithelium that requires protein synthesis.

Effects of VSL#3 on *Salmonella dublin* Invasion

If bacteria in the VSL#3 compound were releasing a soluble factor that was responsible for enhancing epi-

thelial resistance, it would be expected that exposure to the VSL#3 compound may protect the epithelium against pathogenic bacterial invasion, even if the VSL#3 was removed. Indeed, recent studies have shown that some *Lactobacillus* strains secrete surface-active components that bind to various surfaces and act to inhibit adhesion of pathogenic bacteria such as *Enterococcus faecalis*.³² To examine whether the bacteria in the VSL#3 compound release similar factors, we exposed T₈₄ monolayers to increasing concentrations of VSL#3. The media containing the bacteria was removed, and the monolayers washed to ensure removal of all bacteria. *Salmonella dublin* was then applied to the monolayers and allowed to invade for 1 hour. As seen in Figure 7, pre-exposure of T₈₄ monolayers to VSL#3 resulted in a dose-dependent reduction of subsequent invasion by *S. dublin*. *S. dublin* viability was not affected by the epithelium pre-exposure to VSL#3, suggesting that the soluble factor was not acting as an antimicrobial compound (data not shown). In contrast, pre-exposure of the monolayers to *E. coli* (DH5α) had no effect on subsequent *S. dublin* invasion. These data support the concept that a soluble factor is secreted from the VSL#3 bacteria that may either enter the cell to signal an enhancement of barrier integrity, or conversely, bind to apical surface receptors to block *S. dublin* invasion.

Effects of VSL#3 on IL-8 Secretion

Infection of T₈₄ monolayers with invasive strains of bacteria such as *S. dublin* results in the coordinated

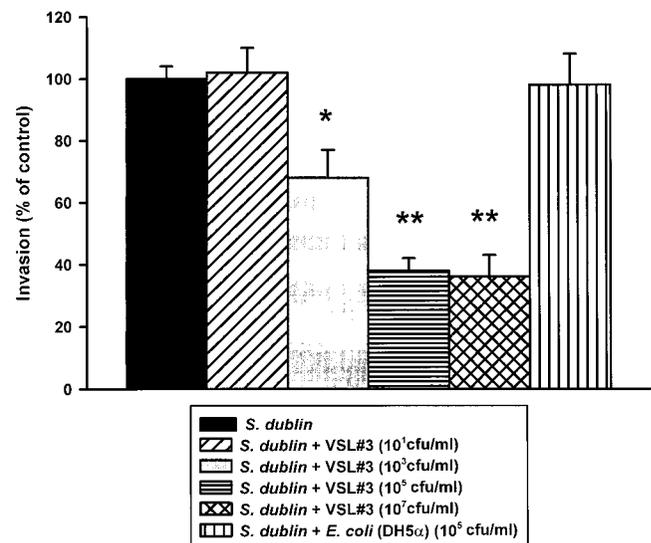


Figure 7. Pre-exposure of T₈₄ monolayers to VSL#3 resulted in a dose-dependent reduction in subsequent invasion of monolayers by *S. dublin*. In contrast, prior exposure of monolayers to *E. coli* (DH5α) had no effect on *S. dublin* invasion (n = 6). *P < 0.05 compared with control monolayer. **P < 0.02 compared with control monolayer.

expression and up-regulation of messenger RNA and secretion of IL-8.³³ In that the presence of VSL#3 resulted in a reduced ability of *S. dublin* to invade T₈₄ monolayers, we assessed IL-8 secretion in the presence and absence of increasing doses of *S. dublin* to determine if VSL#3 also down-regulated the ability of epithelial cells to signal the immune system. VSL#3 alone did not induce IL-8 secretion (data not shown). *S. dublin* increased IL-8 secretion in a dose-dependent manner (Table 4). Pre-exposure of monolayers to VSL#3 (10^5 cfu/mL) resulted in a modest reduction ($P \leq 0.05$) of IL-8 secretion at 10^5 and 10^6 cfu/mL *S. dublin*. At the highest dose of *S. dublin* examined (10^7 cfu/mL), VSL#3 had no effect on IL-8 secretion. This would suggest that although the probiotic bacteria found in VSL#3 are able to attenuate bacterial invasion, these bacteria do not alter the ability of the epithelial cell to activate a mucosal inflammatory reaction in response to a pathogenic bacterial invasion.

Discussion

In the present study, we have shown that treatment of IL-10 gene-deficient mice with the probiotic compound, VSL#3, results in a normalization of colonic physiologic function and barrier integrity in conjunction with a reduction in mucosal levels of proinflammatory cytokines and a significant improvement in histologic disease. Furthermore, *in vitro* studies show that epithelial barrier function can be modulated by exposure to proteinaceous soluble factor(s) secreted by the probiotic bacteria found in the VSL#3 compound.

The luminal bacterial flora appears to play a major role in the initiation and perpetuation of chronic inflammatory bowel diseases in humans and in animal models.³⁴ Colitis in the IL-10 gene-deficient mouse is associated with altered colonic microflora colonization, and can be modulated by either antibiotic treatment or *Lactobacillus*

spp. treatment.^{21,22,35} However, while treatment of IL-10 gene-deficient mice with *Lactobacillus* spp. from birth attenuates disease occurrence, treatment of mice with established inflammation with *Lactobacillus* spp. alone is much less effective.²¹ The use of the VSL#3 compound in this study was much more effective than the use of a single *Lactobacillus* in improving colitis and normalizing epithelial function. The rationale behind the use of a probiotic compound containing high levels of a mixture of different bacterial species was to attempt to influence the microbial composition of the colon through a synergistic action of the different strains in the mixture. Indeed, one of the bacteria in this mixture, *L. delbruekii* subsp. *Bulgaricus* has been shown to enhance the mucus binding of *Bifidobacterium lactis* *in vitro*, suggesting that combinations of adherent probiotic strains can influence other strains' adhesion and activity in the human intestinal tract.³⁶ In addition, all strains used in the VSL#3 compound are highly resistant to bile and acid, do not degrade mucin *in vitro*, and survive passage into the colons in humans.¹⁸ In our study, mice receiving the VSL#3 compound showed a significant increase in levels of total and adherent *Lactobacillus* spp. and *Bifidobacteria* spp., confirming the passage of viable bacteria into the colon. Although there have been no human trials examining the ability of VSL#3 to induce remission in patients with inflammatory bowel disease, this compound has shown efficacy in the maintenance of remission in pouchitis,¹⁷ ulcerative colitis,¹⁸ and Crohn's disease.¹⁹ Our study is the first to show that using VSL#3 alone is effective in reducing inflammation and restoring epithelial function in a model of chronic colitis. Indeed, the altered colonic physiologic function as evidenced by reductions in Isc and a diminished adenosine 3',5'-cyclic monophosphate-dependent chloride secretion was normalized after VSL#3 therapy.

The mechanisms by which probiotics exert their effects *in vivo* have not been clearly defined. Numerous studies have characterized the ability of various strains of probiotics to alter the activity and cytokine expression of gut-associated lymphoid tissue and epithelial cells.³⁷⁻⁴⁰ In the IL-10 gene-deficient mouse, colonic inflammation is associated with high levels of mucosal IFN- γ and TNF- α production.^{30,41} When these mice are raised under germ-free conditions, mucosal production of IFN- γ and TNF- α are both normalized,³⁰ suggesting that colitis in the IL-10 gene-deficient mouse occurs as a consequence of a Th1-driven response to normal mucosal microflora. Treatment of both control and IL-10 gene-deficient mice with the VSL#3 compound decreased IFN- γ and TNF- α secretion. In that the treatment with

Table 4. Effect of Pretreatment of T₈₄ Monolayers With VSL#3 (10^5 cfu/mL) on *S. dublin*-Induced IL-8 Secretion

<i>S. dublin</i> (cfu/mL)	IL-8 (pg/mL)	
	<i>S. dublin</i>	<i>S. dublin</i> + VSL#3 ^a
10^4	0	0
10^5	15.4 \pm 3.2	0 ^b
10^6	632.3 \pm 6.2	560.1 \pm 8.1 ^b
10^7	775.2 \pm 9.7	795.6 \pm 10.4

NOTE. Values are means \pm SEM of quadruplicate samples determined by ELISA. Experiments were performed twice with similar results.

^aVSL#3 was constant at 10^5 cfu/mL.

^b $P < 0.05$ compared with *S. dublin* alone.

VSL#3 resulted in a substantial increase in the total load of bacteria in the colon in both groups, the observed reduction in proinflammatory cytokine secretion indicates that the intestine is able to discriminate and define selective responses to different nonpathogenic strains of bacteria. Our findings are similar to those of Haller et al.,³⁸ who showed that intestinal epithelial cells are able to differentiate between commensal nonpathogenic bacteria and deliver distinct cytokine responses to underlying immune cells in vitro.

Another mechanism by which probiotic bacteria may exert protection is through an enhancement of intestinal barrier function.¹⁶ The lumen of the intestine contains bacteria, bacterial products, and dietary antigens capable of initiating and sustaining inflammation. The normal intestinal epithelium provides a barrier relatively impermeable to these luminal constituents. The IL-10 gene-deficient mouse shows an increase in colonic permeability that is absent in mice raised under germ-free conditions.³⁰ VSL#3 treatment reduced colonic permeability in both IL-10 gene-deficient mice and control mice, suggesting that the type and quantity of bacterial species present in the colon modulate intestinal permeability. Although this reduction in intestinal permeability may have occurred partially as a result of a probiotic bacterial-induced reduction in proinflammatory cytokine release, the results from the T₈₄ monolayer experiments indicate that epithelial cells may also respond directly to either certain probiotic bacteria or to a secreted bacterial factor. Although the identity of this secreted factor is unknown, studies by Heinemann et al.³² have demonstrated that certain strains of *Lactobacillus* release surface-active components, which can inhibit adhesion of pathogenic bacteria. Whether these surface-active components can also act as signals to epithelial cells to reduce paracellular permeability is not known and is currently under investigation. However, our data showing that *S. dublin* invasion is reduced in T₈₄ monolayers after monolayer exposure to the VSL#3 compound supports the concept of a surface-acting proteinaceous component being released by a bacteria found in the VSL#3 mixture.

Another mechanism by which probiotic bacteria could protect epithelium is by receptor competition, whereby probiotics compete with microbial pathogens for a limited number of receptors present on the surface epithelium. Although this most certainly contributes to the beneficial effects of the VSL#3 probiotic compound in vivo, because our data showed a substantial increase in levels of adherent bacteria, the findings with the T₈₄ cells showed first, that conditioned media has the same effect as does live bacteria on monolayer permeability, and

second, that removal of VSL#3 bacteria before the invasion by *S. dublin* still resulted in protection from bacterial invasion, and certainly indicates that some secreted factor is responsible for additional direct effects on epithelium.

In conclusion, the VSL#3 mixture is highly efficacious in the treatment of colitis in the IL-10 gene-deficient mouse model of colitis. Furthermore, this is the first study to show that *Lactobacillus* spp. and/or *Bifidobacterium* spp. produce a proteinaceous factor(s) that directly alters epithelial permeability and protects against pathogenic bacterial invasion. The beneficial in vivo effects of this probiotic compound are most likely a result of a combination of: (1) the ability of certain *Lactobacilli* spp. to adhere to mucosal surfaces and inhibit the attachment of other pathogenic bacteria; (2) a secretion of a factor(s) that enhances barrier integrity; and (3) immunomodulatory effects on cells of the immune system.

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Received February 1, 2001. Accepted May 16, 2001.

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Supported by grants from Alberta Heritage Foundation for Medical Research, Crohn's and Colitis Foundation of Canada, and Gastrointestinal Liver Disease Research Group.