Bifidobacterium breve and *Streptococcus thermophilus* Secretion Products Enhance T Helper 1 Immune Response and Intestinal Barrier in Mice

SANDRINE MÉNARD,* DAVID LAHARIE,† CORINNE ASENSIO,† TERESITA VIDAL-MARTINEZ,* CÉLINE CANDALH,* ANNE RULLIER,‡ FRANK ZERBIB,† FRANCIS MÉGRAUD,† TAMARA MATYSIAK-BUDNIK,* AND MARTINE HEYMAN*,[1]

INSERM EMI 0212, Faculté Cochin-Necker, Paris, France; †Laboratoire de Bactériologie, Université Victor Segalen Bordeaux II, Bordeaux, France; and ‡Service d'Anatomo-Pathologie, Hopital Pellegrin, Bordeaux, France

Lactic acid bacteria or their secretion products can modulate immune responses differently in normal and inflammatory conditions. This comparative study analyzes the effect of oral administration of living lactic acid bacteria, or their conditioned media, on the epithelial and immune functions of colitis-prone C57BL/6 IL-10-deficient mice. Mice were untreated (control) or infected with *Helicobacter hepaticus* with or without oral treatment with living bacteria, *Bifidobacterium breve* C50 and *Streptococcus thermophilus* 065 (LB), or their culture-conditioned media (CM). Histology, cytokine mRNA, electrical resistance, and barrier capacity of colonic samples as well as cytokine secretion by mesenteric lymph node (MLN) cells were studied. *Helicobacter hepaticus* mice developed only mild colitis, which was not modified in LB or CM groups. In the CM (but not the LB) group, the colonic barrier was reinforced as compared to the other groups, as evidenced by decreased horseradish peroxidase (HRP) transcytosis and mannitol fluxes and increased electrical resistance. In MLN, the percentage of $CD4^+$ and $CD8^+$ T cells secreting IFNγ was significantly higher in CM (2.06% and 1.98%, respectively) mice than in *H. hepaticus* (1.1% and 0.47%, $P < 0.05$) or control mice. In addition, the nonspecific stimulation of IFNγ, TNFα, and IL-12 secretion by MLN cells was significantly higher in the CM group as compared to the other groups. In the absence of severe colitis, *Bifidobacterium breve* C50- and *Streptococcus thermophilus* 065-conditioned media can reinforce intestinal barrier capacity and stimulate Th1 immune response, highlighting the involvement of lactic acid bacteria–derived components in host defense. Exp Biol Med 230:749–756, 2005

Key words: probiotics; adjuvants; mucosal immunity; intestinal function

Lactic acid bacteria seem to have different effects on immune responses depending on whether they interact with their host in physiological or pathological conditions. Indeed, although they strengthen specific and innate immune responses in healthy individuals, they seem to down-regulate proinflammatory cytokine secretion in pathological conditions such as inflammatory bowel diseases. In physiological conditions, lactic acid bacteria stimulate the IgA secretory immune response to potentially noxious luminal pathogens, and some studies have shown that they could enhance systemic immune response in case of vaccination (1, 2).

In contrast with these immunostimulatory effects in physiological conditions, in inflammatory conditions, particularly in inflammatory bowel diseases in humans, an attenuation of proinflammatory cytokine secretion and tissue damage has been reported with a selected set of probiotic bacteria (3, 4), although no consensus has yet been reached regarding their efficacy (5). The most convincing effect was observed with the prevention of relapse in chronic pouchitis by oral VSL#3 treatment (6, 7). The beneficial effects of probiotics in inflammation could result from their combined capacity to improve intestinal barrier function and to limit inflammatory responses. Reinforcement of the intestinal barrier capacity has been shown both *in vitro*, using intestinal epithelial cell lines (8, 9), and *in vivo*, in animal models of gastroenteritis or colitis (10, 11).

The mechanisms of action of probiotics are not completely understood. In particular, it is not clear whether

S. Ménard was recipient of a fellowship from the "Ministère délégué à la Recherche." The work was partly supported by Blédina SA.

[1] To whom correspondence should be addressed at INSERM EMI 0212, Faculté Cochin Necker, 156 rue de Vaugirard, 75730 Paris, France. E-mail: heyman@necker.fr

Received June 8, 2005.
Accepted July 19, 2005.

1535-3702/05/23010-0749$15.00

their effects are related to the presence of the living bacteria or rather to the presence of some soluble bacterial factors.

IL-10–deficient mice are frequently used as a model of colitis (12) with chronic intestinal inflammation from an uncontrolled immune response induced by enteric antigens (13), which contributes to the impaired intestinal barrier function frequently observed in murine models of colitis (9) and in patients with Crohn's disease (14).

Our aim was to use this model to study the effect of two lactic bacteria, *Streptococcus thermophilus* 065 and *Bifidobacterium breve* C50, on inflammatory conditions often observed in IL-10–deficient mice and their capacity to modify the immune and intestinal barrier functions in these mice. Special emphasis was placed on the comparison between the effect of bacterial secretion products and that of living bacteria.

Materials and Methods

Preparation of Bacteria-Conditioned Media. The two lactic acid bacterial strains, *Bifidobacterium breve* C50 and *Streptococcus thermophilus* 065, provided by Bledina-SA (Steenvoorde, France), were cultured on brain-heart (BH) medium or lactose-enriched M20 medium, respectively, for 24 hrs in anaerobic conditions. Bacteria were pelleted, extensively washed, and placed for 48 hrs in a simplified medium consisting of RPMI containing 20% fetal calf serum (FCS) and 3% inulin (*B. breve*) or 3% D-glucose (*S. thermophilus*). Bacterial conditioned media (CM) corresponding to 5×10^5 and 8×10^5 CFU/ml of *S. thermophilus* and *B. breve*, respectively, were ultracentrifuged (100,000 *g*), filtrated on 0.22-μm membrane, and frozen (–80°C) until use.

Experimental Protocol. Forty-eight female IL-10–deficient C57BL/6 mice (aged 5 weeks at the beginning of experimental protocol) were used. The animals were housed under specific pathogen-free conditions for 2 weeks before being transferred to a conventional litter and fed a standard nonsterile diet. Our aim was to analyze the effect of a 10-week oral treatment with a mixture of either the two living lactic acid bacteria (*B. breve* and *S. thermophilus*) or their corresponding culture-conditioned media on the appearance of colitis and changes in the intestinal barrier and immune response. Experiments were conducted in accordance with the ethical guidelines of the French Veterinary Department.

The control group comprised IL-10–deficient mice ($n = 12$) that, from the age of 6 weeks, received a daily placebo treatment consisting of 200 μl of phosphate-buffered saline (PBS) administered by oral gavage (using a stainless steel feeding tube) for 10 weeks. The *H. hepaticus* group comprised IL-10–deficient mice ($n = 12$) that were infected with *H. hepaticus* (strain CCUG 33637, ATCC 51448) by two oral gavages with 2×10^9 CFU in 500 μl at the age of 5 weeks. One week later, the treatment protocol commenced, during which the animals received a daily placebo consisting of 200 μl of PBS for 10 weeks. The CM group consisted of *H. hepaticus*–infected IL-10–deficient mice ($n = 12$) that were treated daily for 10 weeks by oral gavage with 200 μl of a mixture (1/1) of CM obtained from *B. breve* and *S. thermophilus*. These CM were prepared as already described (15). The LB group consisted of 12 *H. hepaticus*–infected IL-10–deficient mice that received 5×10^5 CFU of a mixture (1/1) of living bacteria (LB), *B. breve* and *S. thermophilus*, by oral gavage for 10 weeks. At the end of the treatment protocol, all of the mice (aged 16 weeks) were sacrificed to collect colon samples and intestinal lymph nodes. Distal colon fragments were fixed in 4% formaldehyde for histologic examination, and additional fragments were stored at –80°C before extraction of mRNAs.

H. hepaticus infection was confirmed in all infected mice by seminested polymerase chain reaction (PCR) in stool samples. After DNA extraction from the fecal samples using the QIAamp DNA stool minikit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions, a PCR was performed using primers F_2 16S and R_4 16S, detecting conserved bacterial 16S ribosomal DNA (16). These two oligonucleotides produced an amplified product of 1490 bp; 2.5 μl of the fecal DNA preparation was added to a 25 μl (final volume) reaction mixture containing ×1 reaction buffer (with 15 m*M* $MgCl_2$), 0.5 μ*M* each of the two primers, 200 μ*M* each deoxynucleotide, 0.1 μg/μl of bovine serum albumin, and 0.025 U/μl of *Taq* polymerase (Promega, Charbonnières les Bains, France). The following conditions were used for amplification: an initial denaturation at 94°C for 5 mins was followed by 40 cycles (denaturation at 94°C for 30 secs, annealing at 55°C for 30 secs, elongation at 72°C for 1 min 20 secs), and a final elongation at 72°C for 7 mins. After purification with a Sephacryl S-400 high-resolution (Amersham Biosciences, Saclay, France) column, a second set of primers was used for the amplification of a *Helicobacter* spp specific 16S ribosomal DNA fragment. These two oligonucleotides, 5′-GCTATGACGGGTATCC-3′ (C_{97}) and 5′-GATTTTACCCCTACACCA-3′ (C_{98}), produced an amplified product of 398 bp. The reaction mixture was the same as that used for the first amplification but without bovine serum albumin. After a denaturation at 94°C for 5 mins, 40 cycles (denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and elongation at 72°C for 30 secs) and a last elongation at 72°C for 7 mins were performed. PCR was still positive in all infected mice 4 weeks after infection.

Clinical and Histological Examination. *Weight Gain.* Mice were weighed twice a week, and their general behavior was recorded.

Histological Analysis. Tissues were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin. Sections were examined blind by a single pathologist (A.R.). Histological findings were reported using a microscopic score ranging from 0 to 4 as previously described (17). This score is based on the degree of epithelial layer erosion, goblet cell depletion, and inflammatory cell infiltrate; (0, normal; 1,

minimal evidence of inflammatory infiltrate; 2, significant evidence of inflammatory infiltrate, e.g., cryptitis, crypt abscesses; 3, significant evidence of inflammatory infiltrate with goblet cell depletion; 4, significant evidence of inflammatory infiltrate with erosion of the mucosa).

Preparation of Soluble H. hepaticus *and* E. coli *Soluble Extracts.* A soluble extract of *Escherichia coli* was prepared from cultures of *E. coli* (strain isolated from human microflora). The bacteria were harvested and washed extensively in PBS, then lysed by sonication at 4°C. Cell debris were removed by centrifugation at 8000 *g* for 30 mins; the supernatant was sterile filtered, and protein concentration was determined to be 1.2 mg/ml. Soluble *H. hepaticus* was prepared from cultures of *H. hepaticus* (strain CCUG 33637, ATCC 51448) according to the same protocol, leading to a final protein concentration of 1 mg/ml.

The LPS content of the bacterial supernatants after sonication, as measured using the Limulus amebocyte lysate determination (Charles River, Orléans, France) was 8.6 μg/ml for *E. coli* and <1 ng/ml for *H. hepaticus.*

Activation Status of Mesenteric Lymph Node Cells. Mesenteric lymph nodes (MLNs) were collected at sacrifice, and single-cell suspensions were prepared in Cerottini culture medium (DMEM glutamax supplemented with 8% heat-inactivated fetal calf serum; asparagine, 36 mg/liter; arginine, 116 mg/liter; folic acid, 10 mg/liter; HEPES, 1 g/liter; β-mercaptoethanol, 0.05 m*M*; penicillin, 100 U/ml; streptomycin, 100 μg/ml; fungizone, 1 μg/ml). Generally, the total cell count was approximately 20×10^6 cells/mouse, which was then adjusted to 2×10^6 cells/ml in culture medium.

Cytokine Secretion: Enzyme-Linked Immunosorbent Assay (ELISA) and Flow Cytometry. *ELISA.* MLN cells were seeded on 24-well culture plates in duplicate at 2×10^6 cells/well in 1 ml of Cerottini culture medium with or without stimulation by soluble *H. hepaticus* (1 μg/ml) or soluble *E. coli* (1 μg/ml) in duplicate. After 72 hrs, supernatants were collected and frozen at −20°C until assayed. Three proinflammatory cytokines, IFNγ, TNFα, and IL-12, were tested using duoset ELISA kits (R&D, Abingdon, UK).

Flow Cytometry. IFNγ secretion by MLN cells in basal conditions without stimulation was assessed by flow cytometry using the mouse IFNγ secretion assay (Miltenyi Biotec, Paris, France). MLN cells were seeded for 16 hrs on 24-well culture plates at 10^6 cells/well in 1 ml RPMI containing 5% mouse serum. The cells were then washed in cold buffer, and capture antibody against IFNγ/CD45 was added for 5 mins on ice, after which the samples were incubated for 45 mins at 37°C under slow continuous rotation to allow cytokine secretion. Samples were washed and treated with anti-IFNγ APC-conjugated detection antibody for 10 mins on ice. The cells were then counterstained with monoclonal antibody against CD3-PE, CD4-FITC or CD3-PE, CD8-FITC. A total of ~100,000 mesenteric lymph node cells/mouse were passed in the flow cytometer (FACS Calibur and CELLQuest software, Becton-Dickinson, Pont de Claix, France) to count the IFNγ-secreting cells in the selected $CD3^+CD4^+$ and $CD3^+CD8^+$ cell populations. Gated cells comprised about 30,000 cells (CD4 or CD8), and the absolute number of IFNγ-secreting cells varied from 20 (control mice) to 1400 (CM mice).

Activation Markers on T Cells. CD44 and CD62L markers were used to measure T-cell activation. MLN cells were stained with anti-CD3-PE and anti-CD44-FITC antibodies (Becton Dickinson, Pont de Claix, France). CD62L staining was achieved using a biotinylated anti-CD62L antibody, which was detected by a PE-cyanin7-streptavidin conjugate (Becton Dickinson).

Activation Status in Colonic Mucosa: Quantitative PCR Analysis. Distal colonic fragments (3 to 5 mm) were placed in RNA later (Ambion, Austin, TX) and stored at −80°C until use. The fragments were placed in RLT buffer (Qiagen) and homogenized using a Fast Prep apparatus. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Three micrograms of total RNA were reverse-transcribed for 75 mins at 37°C in a reaction mixture containing 50 μg/ml of random hexamers (Promega), 10 IU/μl of Moloney murine leukemia virus reverse transcriptase (M-MLV RT), ×1 RT buffer (250 m*M* Tris-HCl, pH 8.3; 375 m*M* KCl, 15 m*M* $MgCl_2$), 0.01 *M* dithiothreitol, 125 μ*M* of each dNTP, and 2 IU/μl of RNaseOUT (Invitrogen, Cergy Pontoise, France). After denaturation of the reverse transcriptase, cDNAs were stored at −20°C.

IL-12 mRNA was quantified by real-time RT-PCR using the corresponding primers and taqman probes provided by Applied Biosystems (Assay-on-demand) and the TaqMan Universal PCR Master Mix (Applied Biosystems, Courtaboeuf, France). IFNγ expression was quantified using SYBR green PCR Master Mix (Applied Biosystems) and 900 n*M* of the forward and reverse primers (18). The PCR reaction was carried out using an ABI PRISM 7700 sequence detection system. After an initial step at 95°C for 10 mins, 40 cycles were performed as follows: denaturation at 95°C for 15 secs and annealing and extension at 60°C for 1 min. Data were referred to the expression of TATA box binding protein by calculating $2^{-\Delta CT}$, with ΔCT the difference in threshold cycles of target and housekeeping genes. All results are expressed as arbitrary units (AU).

Epithelial Barrier Function of the Colonic Mucosa. Distal colon fragments from all mice were opened along the mesenteric border, layered on a Millipore filter (8-μm pores), and mounted as flat sheets in Ussing chambers with an exposed surface area of 0.2 cm^2. They were bathed on both sides with 1.5 ml of Ringer solution containing 5 m*M* mannitol, which was continuously thermostated, circulated, oxygenated, and maintained at pH 7.4 with 5% CO_2/95% O_2. The mucosal and serosal bathing solutions were connected via agar bridges to calomel electrodes for measurement of the transepithelial

potential difference (PD) and to Ag-AgCl electrodes for current (ΔI) application. The tissue was kept under open-circuit conditions, and electrical measurements were performed using a DVC 1000 voltage/current clamp (World Precision Instruments, Aston, UK). The tissue was regularly clamped at 1 mV to measure ΔI and to calculate the electrical resistance (R) according to Ohm's law ($\Delta PD = R \times \Delta I$).

Horseradish peroxidase (HRP; mol wt, 40 kDa) was used as a soluble protein marker of the transcellular transport pathway (transcytosis). Mannitol was used as a small molecular tracer (mol wt, 182 daltons) of the paracellular pathway. HRP (0.4 mg/ml), [^{3}H]HRP (37 kBq/ml) and [^{14}C]mannitol (12.2 kBq/ml) were simultaneously added to the mucosal compartment bathing intestinal fragments. Samples (800 μl) were taken from the serosal compartment at 10, 30, 50, 70, 90, and 110 mins and replaced by fresh Ringer solution. Intact HRP transport from the mucosal to the serosal compartment was determined by enzymatic assay (19) on 200 μl of serosal samples. Total HRP (intact + degraded = ^{3}H-equivalent HRP) and [^{14}C]mannitol fluxes were assessed on 500-μl serosal samples by counting the ^{3}H and ^{14}C radioactivity using liquid scintillation photometry (Kontron, Betamatic) after double-labeling correction, and are expressed as ng/hr·cm^2 and nmol/hr·cm^2, respectively. Degraded-HRP fluxes were calculated as total minus intact HRP fluxes. Mean steady-state fluxes obtained from 50 to 110 mins are presented.

Statistical Analysis. Statistical analysis was performed using the SAS package. The results are expressed as mean ± SE, and the comparison of different parameters among the groups was performed by using analysis of variance and nonparametric tests (Wilcoxon and Kruskal-Wallis). The general linear model procedure was used for multiple group-to-group comparisons. The differences were considered significant for $P < 0.05$.

Results

Weight Gain. There were slight differences in weight gain expressed as a percentage of initial weight among the different groups of mice during the experimental protocol. Figure 1A indicates that LB mice had the lowest growth rate. The treatment of *H. hepaticus*–infected mice with bacteria-conditioned medium seemed to improve the growth rate as compared to untreated infected mice.

Colonic Microscopic Score in Mice. Figure 1B shows the histological scores obtained in all of the mice. As compared with control mice (score 0.82 ± 0.18), *H. hepaticus*–infected mice had a small but significant increase in the inflammatory infiltrate (score 2.10 ± 0.40, $P < 0.002$), corresponding to mild colitis. This score was not modified in CM- or LB-treated mice (score 2.16 ± 0.29 and 2.28 ± 0.42, respectively).

Function of Mesenteric Lymph Node Cells.

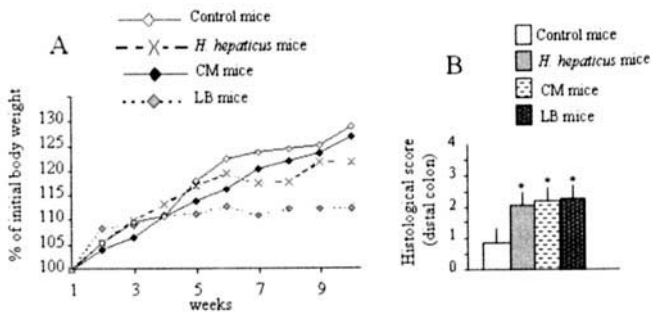

Figure 1. (A) Body weight curves of control IL-10–deficient mice, those infected with *H. hepaticus* alone (*H. hepaticus* mice), or those infected with *H. hepaticus* followed by treatment with either 5×10^5 CFU/day of living *B. breve* and *S. thermophilus* (LB mice) or their culture-conditioned media (CM mice). (B) Histological examination of mucosal lesions (grades 0 to 4) observed in distal colon. *H. hepaticus*–infected mice presented a significantly higher score than control mice. Treatment with CM or LB had no additional effect on histological score. *$P < 0.002$ as compared with control mice.

Spontaneous and Stimulated Cytokine Secretion as Measured by ELISA. The TNFα, IL-12, or IFNγ secretion after stimulation with soluble *H. hepaticus* extract did not differ from the basal secretion of these cytokines (data not shown). Figure 2 illustrates the secretion profiles of the three proinflammatory cytokines by MLN cells in basal or soluble *E. coli*–stimulated conditions. The basal secretion levels of IFNγ, TNFα, and IL-12 were low (< 70 pg/ml) and similar in all groups of mice. After stimulation with soluble *E. coli* extracts, these secretion levels were not significantly modified in control mice. However, in *H. hepaticus*–infected mice, *E. coli*–stimulated secretion of TNFα was higher (685 ± 209 pg/ml) than in control mice (184 ± 45 pg/ml, $P < 0.02$), and *E. coli*–stimulated secretion of IL-12 tended to be higher (197 ± 39 pg/ml) than in control mice (104 ± 25 pg/ml, $P = 0.08$), suggesting a higher susceptibility of macrophages/dendritic cells to nonspecific LPS activation in these mice. The most striking effect was an eightfold increase in *E. coli*–induced IFNγ secretion in the CM group (1594 ± 774 pg/ml) as compared with the control (88 ± 50 pg/ml), *H. hepaticus* (202 ± 107 pg/ml), and LB (0.6 ± 0.6 pg/ml) groups ($P < 0.005$), indicating a Th1 skewing of the immune response. Significantly higher TNFα (×1.6) and IL-12 (×1.5) secretion levels in response to the *E. coli* extract were also observed in CM mice (1090 ± 333 and 299 ± 74 pg/ml, respectively) as compared to *H. hepaticus* mice (685 ± 209 and 197 ± 39 pg/ml, $P < 0.05$), although to a much lesser extent than for IFNγ.

IFNγ Secretion by MLN Cells as Measured by Flow Cytometry. Both CD4$^+$ and CD8$^+$ T cells were studied. There was an increase in the number of CD4$^+$ T cells secreting IFNγ in the CM mice (2.06 ± 0.37%) as compared with the control (0.67 ± 0.19%, $P < 0.005$) or *H. hepaticus*–infected mice (1.1 ± 0.32%, $P < 0.05$) (Fig. 3A). The same tendency was observed for CD8$^+$ T cells secreting IFNγ (1.98 ± 0.52% in CM mice vs. 0.76 ±

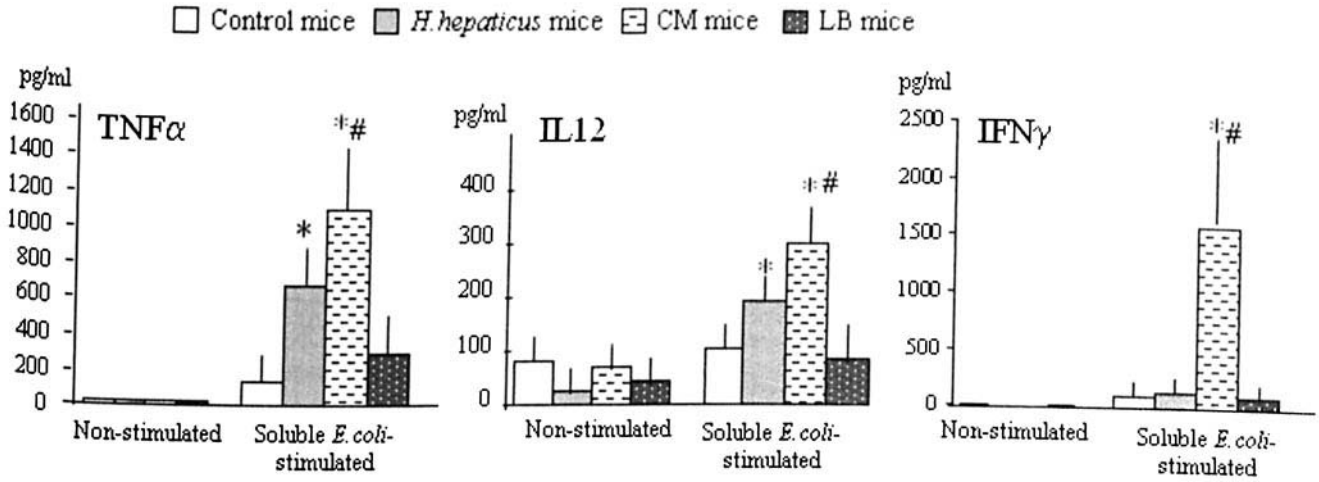

Figure 2. Cytokine secretion by mesenteric lymph node cells under basal conditions or after 72-hr stimulation with soluble *E. coli*. #Significantly different from *E. coli*–stimulated control and *H. hepaticus* mice ($P < 0.05$). *Significantly different from matched unstimulated cells ($P < 0.0001$).

0.26%, $P < 0.02$; and 0.47 ± 0.09%, $P < 0.01$; in control and *H. hepaticus* mice, respectively). In LB mice, the percentage of $CD4^+$ T cells secreting IFNγ (1.95 ± 0.67%) was higher than that observed in control mice (0.67 ± 0.19%, $P < 0.03$), but not in *H. hepaticus* mice (1.1 ± 0.32%). This difference was not observed for $CD8^+$ T cells (0.57 ± 0.16%, 0.76 ± 0.26%, and 0.46 ± 0.09% in LB, control, and *H. hepaticus* mice, respectively).

It is apparent that, although IFNγ secretion was significantly higher in $CD4^+$ T cells from both CM and LB mice than in *H. hepaticus* mice ($P < 0.03$), in $CD8^+$ T cells, such a secretion was significantly increased in CM mice only ($P < 0.05$) but not in LB mice.

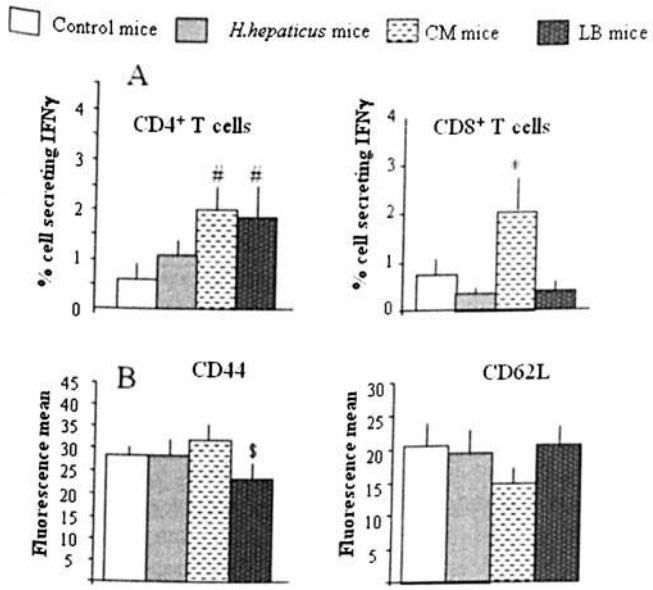

Figure 3. (A) Spontaneous IFNγ secretion by MLN cells measured by flow cytometry. Results are presented as the percentage of cells secreting IFNγ among the population of $CD4^+$ or $CD8^+$ T cells. (B) $CD4^+$ or $CD8^+$ T cell activation status measured by flow cytometry using the expression of CD44 and CD62L taken as activation markers. *Significantly different from control, *H. hepaticus*, and LB mice ($P < 0.05$). #Significantly different from control mice ($P < 0.03$).

Expression of T Cell Activation Markers CD44 and CD62L by Flow Cytometry. T cell activation is associated with high expression of CD44 and low expression of CD62L. Figure 3B indicates that the expression of CD44 tended to be higher in CM mice, but the difference was statistically significant only for LB mice (31.5 ± 1.9% vs. 23.2 ± 2.4%, $P < 0.02$). There was a slight decrease in CD62L expression in CM mice as compared with the other groups, but the difference was not statistically significant ($P = 0.1$).

Expression of Cytokines by Quantitative PCR on Distal Colonic Mucosa. As shown in Figure 4, a significant increase in IFNγ mRNA expression was observed in distal colon of CM mice (2.4 ± 0.6 AU, $P < 0.001$) and LB mice (1.5 ± 0.8 AU, $P < 0.05$) as compared with *H. hepaticus* (0.5 ± 0.2 AU) and control mice (0.05 ± 0.02 AU). No significant variation in IL-12 expression was found.

Epithelial Barrier Function in Distal Colonic Mucosa. The parameters reflecting paracellular permeability (mannitol fluxes and electrical resistance) and transcellular macromolecular transport (HRP fluxes) in distal colon are reported in Figure 5. As compared with control mice, CM mice presented lower HRP transport as attested by the significant decrease in degraded-HRP fluxes ($P < 0.02$) and, to a lesser extent, intact-HRP fluxes ($P = 0.09$). In contrast, LB mice presented similar HRP fluxes as control or *H. hepaticus* mice. Intestinal permeability along the paracellular pathway was also significantly decreased in the CM group as compared with the control and *H. hepaticus* groups, as attested by a significant decrease in mannitol fluxes (74 ± 11 vs. 116 ± 10 and 109 ± 14 nmol/hr·cm^2, $P < 0.05$) correlating with a significant rise in electrical resistance R (53 ± 5 vs. 41 ± 4 and 40 ± 3 Ω·cm^2, $P < 0.05$) in this group of mice. Thus, at the colonic level, both transcellular permeability to macromolecules and paracellular diffusion of small molecules were decreased by treatment with CM (but not with LB), indicating that

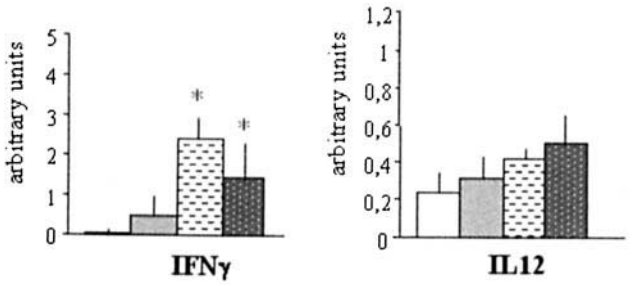

Figure 4. Quantification of cytokine mRNA in the distal colon as measured by real-time PCR and reported as arbitrary units (AU). IFNγ mRNA expression was significantly increased in CM mice ($P < 0.001$) and LB mice ($P < 0.05$) as compared with control and *H. hepaticus* mice. There were no statistically significant differences in IL-12 mRNA expression among the different groups.

bacterial secretion products are probably the active components involved in the improvement of intestinal barrier integrity.

Discussion

The present study shows that, in the absence of important inflammation, lactic acid bacteria–conditioned medium stimulates Th1 immune response and strengthens the colonic barrier, two effects likely to be beneficial in the maintenance of intestinal homeostasis and in immune defense. This study also indicates that both effects are stronger with the bacterial conditioned media than with the living bacteria.

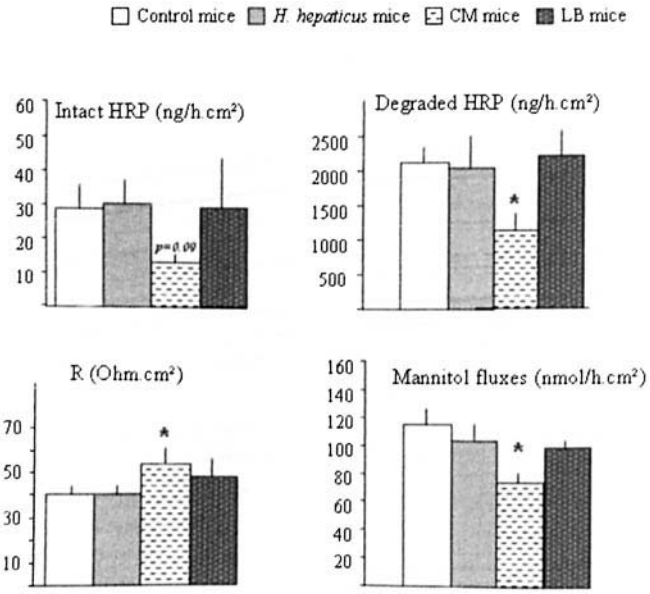

Figure 5. Measurement of colonic permeability in IL-10–deficient mice that either did or did not orally receive bacteria-conditioned media or living bacteria. Markers of transcellular (degraded-HRP fluxes) and paracellular (mannitol fluxes) permeability were significantly decreased in CM mice as compared with control and *H. hepaticus* mice. In parallel, a significant increase of electrical resistance ($P < 0.03$) and a tendency ($P = 0.09$) toward a decrease of intact-HRP fluxes in CM mice, as compared with the other groups, confirmed the reinforcement of the colonic barrier by bacteria-conditioned medium. No significant differences were observed in LB mice. *Significantly different from control and *H. hepaticus* groups ($P < 0.05$).

Although IL-10–deficient mice have been widely used as a model of IBD (13), this model is highly conditioned by environmental and genetic factors, and spontaneous colitis is often mild and delayed. Successful induction of colitis in IL-10–deficient mice has been reported (10), but other studies have indicated that these mice do not develop severe colitis even after colonization with *H. hepaticus* (20). In our experimental setting, C57BL/6 IL-10–deficient mice infected by *H. hepaticus* developed only mild colitis, with limited histological damage.

As already mentioned, a clear dichotomy is observed between the anti-inflammatory effect of probiotics in inflammatory context and their immune stimulatory effects under physiological conditions. Indeed, lactic acid bacteria have the capacity to stimulate the immune response under basal conditions, both *in vitro* (15, 21) and *in vivo*, during vaccination in animals (1) or in humans (2). Lactic acid bacteria also stimulate secretory immune response to luminal pathogens. In addition, various species of Lactobacilli or Bifidobacteria have been shown to enhance the secretion of specific IgA, especially in case of rotavirus infection in children (22, 23). Nonspecific, anti-infectious mechanisms of defense also seem to be enhanced by ingestion of selected strains of lactic acid bacteria. In adults, the consumption of milk fermented with *B. bifidum* or *L. acidophilus* LA1 induced phagocytosis of *E. coli*, as assessed *in vitro* (24). The activation of the monocytes/macrophages (25) by ingestion of lactic acid bacteria may be responsible for the enhancement of innate immunity, an effect explained by the fact that cell walls or DNA of gram-positive bacteria are potent activators of toll-like receptors.

In contrast, lactic acid bacteria tend to moderate exacerbated cytokine secretion and to improve inflammatory conditions in humans (7) and in experimental models of colitis (10).

The experimental conditions achieved in the present study correspond to a subnormal rather than a pathological state because histological damage is moderate, and spontaneous proinflammatory cytokine secretion in intestinal mucosa and MLN cells is low. In these conditions, *B. breve* and *S. thermophilus* stimulated local Th1 immune response and reinforced the colonic epithelial barrier. Surprisingly, bacteria-conditioned media were more efficient in producing these effects than the living bacteria. Although viability of bacteria is considered a major aspect of probiotic activity, especially in lactose intolerance, there is emerging evidence that bacterial components or secretion products are involved in the effects on the immune system (15, 26). The mechanisms by which lactic acid bacteria or their conditioned media could act locally on intestinal and immune function were analyzed. First, the reactivity of intestinal immune cells in MLNs was tested under basal conditions and in response to specific (soluble *H. hepaticus*

extract) and nonspecific (soluble *E. coli* extract containing LPS) stimuli. In our experimental setting, the basal secretion of cytokines by MLN cells was negligible, confirming the low score of colitis. However, in mice receiving bacteria-conditioned medium, cells did not respond to specific antigens (*H. hepaticus)* but gained the capacity to respond to a nonspecific stimulus (*E. coli*), a phenomenon not observed in control or *H. hepaticus* mice. This was attested to by an eightfold increase in IFNγ secretion and to a lesser extent in TNFα and IL-12 secretion, by MLN cells of CM mice. A possible interpretation could be that CM mice received bacterial DNA present in bacteria-conditioned medium, a component shown to be immunostimulant and to act as adjuvant in healthy individuals (1, 27). The absence of effect of living bacteria could be because bacterial DNA in this case is not exposed in the intestinal lumen but hidden within the bacterial cells. Although bacterial DNA was shown to reduce inflammation in experimental colitis (26), under our subnormal conditions, this effect could be mainly immunostimulant.

In addition to polarizing T cells toward Th1 response, treatment with bacteria-conditioned medium had a positive effect on epithelial barrier, as evidenced by a reinforcement of the distal colonic barrier, both at the transcellular and paracellular levels. A decrease in HRP fluxes indicated lower transcytosis whereas decreased mannitol fluxes and increased electrical resistance reflected decreased paracellular diffusion and tight junctional complex reinforcement. These results are in agreement with those obtained in guinea pigs fed a dehydrated fermented (*B. breve* and *S. thermophilus)* milk (28) or in IL-10–deficient mice treated with VSL#3-conditioned medium (9).

In conclusion, in this context of mild inflammation, the administration of *B. breve*– and *S. thermophilus*–conditioned medium, in addition to reinforcing intestinal barrier function, strongly stimulated the Th1 immune system, a feature that may highlight the interest of lactic acid bacteria in immune defense and vaccination.

1. Benyacoub J, Czarnecki-Maulden GL, Cavadini C, Sauthier T, Anderson RE, Schiffrin EJ, von derWeid T. Supplementation of food with *Enterococcus faecium* (SF68) stimulates immune functions in young dogs. J Nutr 133:1158–1162, 2003.
2. Isolauri E, Joensuu J, Suomalainen H, Luomala M, Vesikari T. Improved immunogenicity of oral D × RRV reassortant rotavirus vaccine by *Lactobacillus casei* GG. Vaccine 13:310–312, 1995.
3. Borruel N, Carol M, Casellas F, Antolin M, de Lara F, Espin E, Naval J, Guarner F, Malagelada JR. Increased mucosal tumour necrosis factor alpha production in Crohn's disease can be downregulated ex vivo by probiotic bacteria. Gut 51:659–664, 2002.
4. Gupta P, Andrew H, Kirschner BS, Guandalini S. Is Lactobacillus GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. J Pediatr Gastroenterol Nutr 31:453–457, 2000.
5. Prantera C, Scribano ML, Falasco G, Andreoli A, Luzi C. Ineffectiveness of probiotics in preventing recurrence after curative resection for Crohn's disease: a randomised controlled trial with Lactobacillus GG. Gut 51:405–409, 2002.
6. Gionchetti P, Rizzello F, Helwig U, Venturi A, Lammers KM, Brigidi P, Vitali B, Poggioli G, Miglioli M, Campieri M. Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. Gastroenterology 124:1202–1209, 2003.
7. Gionchetti P, Rizzello F, Venturi A, Brigidi P, Matteuzzi D, Bazzocchi G, Poggioli G, Miglioli M, Campieri M. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. Gastroenterology 119:305–309, 2000.
8. Resta-Lenert S, Barrett KE. Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive *Escherichia coli* (EIEC). Gut 52:988–997, 2003.
9. Madsen K, Cornish A, Soper P, McKaigney C, Jijon H, Yachimec C, Doyle J, Jewell L, De Simone C. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology 121:580–591, 2001.
10. Madsen KL, Doyle JS, Jewell LD, Tavernini MM, Fedorak RN. *Lactobacillus* species prevents colitis in interleukin 10 gene-deficient mice. Gastroenterology 116:1107–1114, 1999.
11. Isolauri E, Kaila M, Arvola T, Majamaa H, Rantala I, Virtanen E, Arvilommi H. Diet during rotavirus enteritis affects jejunal permeability to macromolecules in suckling rats. Pediatr Res 33:548–553, 1993.
12. Blumberg RS, Saubermann LJ, Strober W. Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. Curr Opin Immunol 11:648–656, 1999.
13. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10–deficient mice develop chronic enterocolitis. Cell 75:263–274, 1993.
14. Zareie M, Singh PK, Irvine EJ, Sherman PM, McKay DM, Perdue MH. Monocyte/macrophage activation by normal bacteria and bacterial products: implications for altered epithelial function in Crohn's disease. Am J Pathol 158:1101–1109, 2001.
15. Menard S, Candalh C, Bambou JC, Terpend K, Cerf-Bensussan N, Heyman M. Lactic acid bacteria secrete metabolites retaining anti-inflammatory properties after intestinal transport. Gut 53:821–828, 2004.
16. Fox JG, Dewhirst FE, Shen Z, Feng Y, Taylor NS, Paster BJ, Ericson RL, Lau CN, Correa P, Araya JC, Roa I. Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. Gastroenterology 114:755–763, 1998.
17. McCarthy J, O'Mahony L, O'Callaghan L, Sheil B, Vaughan EE, Fitzsimons N, Fitzgibbon J, O'Sullivan GC, Kiely B, Collins JK, Shanahan F. Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. Gut 52:975–980, 2003.
18. Xia D, Sanders A, Shah M, Bickerstaff A, Orosz C. Real-time polymerase chain reaction analysis reveals an evolution of cytokine mRNA production in allograft acceptor mice. Transplantation 72:907–914, 2001.
19. Maehly AC, Chance B. The assay of catalases and peroxidases. In: Glick D, Ed. Methods of Biochemical Analysis (Vol. 1). New York: Wiley Interscience, pp357–398, 1954.
20. Dieleman LA, Arends A, Tonkonogy SL, Goerres MS, Craft DW, Grenther W, Sellon RK, Balish E, Sartor RB. *Helicobacter hepaticus* does not induce or potentiate colitis in interleukin-10–deficient mice. Infect Immun 68:5107–5113, 2000.
21. Pelto L, Salminen SJ, Isolauri E. Lactobacillus GG modulates milk-induced immune inflammatory response in milk hypersensitive adults. Nutr Today 31(Suppl):45S–46S, 1998.
22. Kaila M, Isolauri E, Soppi E, Virtanen E, Laine S, Arvilommi H. Enhancement of the circulating antibody secreting cell response in human diarrhea by a human Lactobacillus strain. Pediatr Res 32:141–144, 1992.
23. Kaila M, Isolauri E, Saxelin M, Arvilommi H, Vesikari T. Viable versus inactivated Lactobacillus strain GG in acute rotavirus diarrhoea. Arch Dis Child 72:51–53, 1995.

24. Schiffrin EJ, Rochat F, Link-Amster H, Aeschlimann JM, Donnet-Hughes A. Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. J Dairy Sci 78:491–497, 1995.
25. Miettinen M, Vuopio Varkila J, Varkila K. Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. Infect Immun 64:5403–5405, 1996.
26. Rachmilewitz D, Katakura K, Karmeli F, Hayashi T, Reinus C, Rudensky B, Akira S, Takeda K, Lee J, Takabayashi K, Raz E. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. Gastroenterology 126:520–528, 2004.
27. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. Proc Natl Acad Sci U S A 93:2879–2883, 1996.
28. Terpend K, Blaton MA, Candalh C, Wal JM, Pochart P, Heyman M. Intestinal barrier function and cow's milk sensitization in guinea-pigs fed milk or fermented milk. J Pediatr Gastroenterol Nutr 28:191–198, 1998.