Lactobacillus casei prevents the upregulation of ICAM-1 expression and leukocyte recruitment in experimental colitis

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Submitted 20 September 2005; accepted in final form 13 July 2006

Angulo, Sandra, Marta Llopis, María Antolín, Meritxell Gironella, Miquel Sans, Juan Ramon Malagelada, Josep Maria Piqué, Francisco Guarner, and Julián Panés. Lactobacillus casei prevents the upregulation of ICAM-1 expression and leukocyte recruitment in experimental colitis. Am J Physiol Gastrointest Liver Physiol 291: G1155–G1162, 2006. First published August 10, 2006; doi:10.1152/ajpgi.00446.2005.—Lactobacillus casei has been shown to attenuate the severity of experimental colitis. The objective of the present study was to determine whether the effects of L. casei on colitis are related to modulation of leukocyte recruitment into the inflamed intestine. Rats with a colonic segment excluded from fecal transit were surgically prepared. The segment was decontaminated with antibiotics and recolonized with normal flora isolated from the inflamed rat colon, associated or not to Enterobacteriaceae, and in some animals with intestinal microorganisms such as Enterococcus faecalis. L. casei demonstrated to be effective preventing the inhibition and invasion of adherent-invasive E. coli to human intestinal epithelial cells isolated from CD patients (16). A previous study has demonstrated that luminal bacteria may play an important role in inflammatory bowel diseases (13), in experimental and human inflammatory lesions (15, 30), in pouchitis (11), in inhibition of gastric colonization by Helicobacter pylori (4), and in lactose intolerance, food allergy, and constipation (19). Promising probiotic strains include the members of genera Lactobacillus, Bifidobacterium, and Enterococcus, some strains of Escherichia coli, and nonbacterial microorganisms such as Saccharomyces boulardii (14). Multiple mechanisms have been suggested to explain the protective effects of probiotics, including inhibition of bacterial growth, reduction of pathogen adherence, improvement of epithelial barrier integrity, and modulation of immune function (17).

The etiology of inflammatory bowel diseases (IBD) is unknown. Genetic factors as well as environmental triggers seem to play a substantial role in its pathogenesis. Among those environmental triggers, bacterial and viral organisms have received considerable attention (35). There is increasing evidence that luminal bacteria may play an important role in initiating and maintaining the inflammatory process in the colon (36). Models of spontaneous colitis in IL-10- or IL-2-deficient mice fail to develop inflammatory changes in the colon if the animals are raised in a germ-free environment but develop a spontaneous colitis when they are colonized with bacteria (37). Rats that are transgenic for the human HLA-B27 gene develop gastritis, colitis, and arthritis when raised in a specific pathogen-free environment, whereas in a sterile environment HLA-B27 transgenic rats remain free of colitis, gastritis, and arthritis (29). In human Crohn’s disease (CD), inflammatory lesions appear at sites with the highest concentration of luminal aerobic and anaerobic bacteria, such as the colon and the terminal ileum, and in these patients the concentration of Bifidobacteria has been shown to be reduced (10). Moreover, a reduction in intestinal bacterial load with broad-spectrum antibiotics has proved to be effective in intestinal inflammation in animal models of IBD and in patients with CD (5, 42). Overall, these studies suggest that an immune response against antigens of the normal resident flora is a key aspect of IBD pathophysiology (23).

Probiotics are defined as viable microorganisms that exhibit a beneficial effect on the health of the host by improving its intestinal microbial balance (17) or, more broadly, as “living micro-organisms, which upon ingestion in certain numbers, exert health effects beyond those inherent to basic nutrition” (14). Various studies have demonstrated the safety and efficacy of probiotic organisms in the treatment or prevention of certain infectious diarrheal diseases (13), in experimental and human IBD (15, 30), in pouchitis (11), in inhibition of gastric colonization by Helicobacter pylori (4), and in lactose intolerance, food allergy, and constipation (19). Promising probiotic strains include the members of genera Lactobacillus, Bifidobacterium, and Enterococcus, some strains of Escherichia coli, and nonbacterial microorganisms such as Saccharomyces boulardii (17). Multiple mechanisms have been suggested to explain the protective effects of probiotics, including inhibition of bacterial growth, reduction of pathogen adherence, improvement of epithelial barrier integrity, and modulation of immune function (34).

In the present study, we used the probiotic bacteria Lactobacillus casei, which has been proven effective in improving murine chronic IBD and is associated with downregulation of proinflammatory cytokines such as IL-6 and IFN-γ in lamina propria mononuclear cells (22). Moreover, L. casei has been demonstrated to be effective preventing the inhibition and invasion of adherent-invasive E. coli to human intestinal epithelial cells isolated from CD patients (16). A previous study showed that colonization of an isolated colonic segment by L. casei attenuated the lesion induced by 2,4,6-trinitrobenzenesulfonic acid (TNBS) in Sprague-Dawley rats and significantly

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reduced bacterial translocation to the mesenteric lymph nodes, liver, and spleen (21). In the present study, we used L. casei before the induction of colitis by TNBS in rats with a colonic segment surgically excluded from fecal transit. The excluded colonic segment allowed radical elimination of commensal flora and recolonization with preselected bacterial species. Using this in vivo model, we characterized the effect of L. casei administration on the expression of endothelial cell adhesion molecules and leukocyte-endothelial interactions in the colonic microcirculation and evaluated their protective effect.

MATERIALS AND METHODS

Animals. Pathogen-free male Sprague-Dawley rats weighing 260–320 g and free of all pathogens listed in Federation of European Laboratory Animal Science Association Working Group on Health Monitoring of Rodent and Rabbit colonies guidelines were purchased from Charles River Laboratories (Lyon, France). Rats were maintained in a restricted-access room with a controlled temperature of 22°C, humidity of 50 ± 5%, and 12:12-h light-dark cycle and were housed in rack-mounted cages with a maximum of five rats before surgery and placed individually after surgery. Standard rodent chow pellets (Maintenance A-04, Panlab, Tarragona, Spain) and filtered tap water were provided ad libitum. Principles of laboratory animal care (National Institutes of Health, Pub. No. 86-23, Revised 1985) and the guidelines of procedures for animal experiments from the Generalitat de Catalunya were followed. All the experimental protocols with animals were approved by the Ethics Committee for animal experimentation of the University of Barcelona on July 16, 2002.

Surgical procedures and induction of colitis. Rats with a colonic segment excluded from fecal transit were randomly distributed into three groups: control, colitis, and colitis-L. casei (n = 5–7 animals per group and per experiment). An excluded colonic segment was surgically prepared as previously described (21). In ketamine-xylazine-anesthetized rats (90 mg/kg ketamine (Parke-Davis, Ann Arbor, MI) and 10 mg/kg xylazine (Bayer, Leverkusen, Germany)), the abdomen was opened through a midline laparotomy. The terminal ileum at the left side of the abdominal wall using 4-0 Vicryl suture (Ethicom, 2000 Nordested, FRG). The continuity of the gastrointestinal tract was achieved by an end-to-side ileocolonic anastomosis using 7-0 Vicryl (Dista, Alcobendas, Spain). Rats were then allowed to recover.

One day after surgery, rats were lightly anesthetized with ketamine-xylazine, and the lumen of the excluded segment was thoroughly flushed with a solution of broad-spectrum antibiotics (imipenem and vancomycin) and kept in the lumen for 3 h. Thereafter, the lumen was abundantly rinsed with 60 ml of sterile saline to eliminate the antibiotics. Then, 3 ml of a suspension of aerobic bacteria were inoculated into the segment lumen. The suspension was prepared as described below and included Enterobacter aerogenes, Klebsiella pneumoniae, and Streptococcus viridans for the colitis and control groups and the same strains plus L. casei strain DN-114 001 for the colitis-L. casei group. Colostomies were immediately closed by ligature after inoculation.

On day 3, colostomies were reopened and lumen mucin aggregates were removed by rinsing with 10 ml of Schaedler reduction medium (Bio Méries, Marcy l’Etoile, France) to avoid obstruction. Thereafter, 3 ml of anaerobic bacteria suspension were inoculated. The suspension was prepared as described below and included Bacteroides fragilis and Bacteroides disastonis for the colitis and control group and the same strains plus L. casei strain DN-114 001 for the colitis-L. casei group. Colostomies were immediately closed by ligature. To obtain a proper anaerobic viability and colonization, oxygen-intolerant anaerobes had to be inoculated following aerobic inoculation, thus allowing the latter to metabolize the oxygen present in the isolated intestinal loop. The resulting free-oxygen environment was then appropriated for the survival of anaerobic bacteria.

On day 6 after surgery, L. casei colonization in the isolated segment was controlled in all rats by microbiological culture, as described below. Colonization of the nonprobiotic inoculated flora was determined in randomly selected rats, since their ability to colonize the intestinal lumen has already been described in the same experimental model in a previous study (21). The luminal mucus was again removed by rinsing with up to 10 ml of Schaedler broth reduction medium (37°C). One-milliliter aliquots of the effluent were carefully collected in sterile plastic syringes, avoiding contact with air. They were immediately processed for microbiological culture to assess bacterial colonization. The microbiological washing outcomes were semiquantitative as the volume required for flushing removal of mucin aggregates fluctuated. This fluctuation depends on the size of the mucus, consistency, and adherence to the colonic mucosa. The removal of mucin aggregates is of vital importance because they may plug the colonic segment, impeding further liquid flow.

Thereafter, following effluent sampling, colitis was induced in rats from colitis and colitis-L. casei groups by an intraluminal instillation of 3 ml of TNBS solution (45 mg/ml in 10% ethanol) (TNBS from Sigma, St. Louis, MO) and ethanol (from Panreac, Barcelona, Spain). On day 7 after surgery, leukocyte-endothelial cell interactions were assessed in vivo in colonic venules by intravital microscopy or expression of endothelial ICAM-1 or VCAM-1 was measured by the dual-labeled antibody technique.

Bacterial suspensions. Bacteria used in the present study were kindly provided by the Digestive System Research Unit, University Hospital Vall d’Hebron.

Bacterial suspensions for inoculation were prepared from cultures of each individual strain. Nonprobiotic strains used in this protocol had been previously isolated by microbiological culture from colonic wall homogenates of rats subjected to acute TNBS colitis (8). These isolates include gram-positive and gram-negative aerotolerant and facultative aerobes and gram-negative obligate anaerobes that are commonly found among predominant species of the rat microflora: E. aerogenes, K. pneumoniae, S. viridans, B. fragilis, and B. disastonis (8). Aerotolerant bacteria were grown in liquid culture medium at 37°C under a controlled atmosphere (5% CO2): L. casei and S. viridans were grown in De Man Rogosa Sharp medium (MRS; Difco, Detroit, MI), and E. aerogenes and K. pneumoniae were grown in a 1% tryptone peptone medium (Difco), 0.5% NaCl, and 0.5% yeast extract (Difco). B. fragilis and B. disastonis were grown in Schaedler medium (Difco) for 48 h at 37°C in an anaerobic atmosphere (Anaerocult A Merck, Darmstadt, Germany). At the end of the phase of exponential growth, bacterial cultures were stopped. The final aerobic inoculum concentration for the three groups of rats was 6 × 106 colony-forming units (CFU)/ml. In the control and colitis groups, 1 ml of a 6 × 106 CFU/ml bacterial culture for each of the three bacteria (E. aerogenes, K. pneumoniae, and S. viridans) were mixed, with a final volume of 3 ml. In the colitis-L. casei group, 0.75 ml of a 6 × 106 CFU/ml bacterial culture for each of the four bacteria (E. aerogenes, K. pneumoniae, and S. viridans plus L. casei) were mixed, again with a final volume of 3 ml. Volumes of 3 ml per rat were instilled into the lumen of the excluded colonic segment on day 1.

The final anaerobic inoculum concentration for the three groups of rats was 5 × 107 CFU/ml. In the control and colitis groups, 1.5 ml of
a 5 × 10^6 CFU/ml bacterial culture for each of the two bacteria (\textit{B. fragilis} and \textit{B. disastoris}) were mixed, with a final volume of 3 ml. In the colitis-\textit{L. casei} group, 1 ml of a 5 × 10^5 CFU/ml bacterial culture for each of the three bacteria (\textit{B. fragilis} and \textit{B. disastoris}, with the \textit{L. casei} strain) were mixed, again with a final volume of 3 ml. Volumes of 3 ml per rat were instilled into the lumen of the excluded colonic segment on day 3. The concentration of anaerobic was slightly lower than aerobic bacteria because strict anaerobes such as \textit{B. fragilis} and \textit{B. disastoris} have a slower growth rate and the final bacterial concentration was also slightly lower in this case. In the present study, probiotic bacteria were not given in higher concentrations, because the aim was to determine whether \textit{L. casei} was able to compete with other enteric bacteria of the common flora at the same concentration. Llopis and colleagues (21) demonstrated that \textit{L. casei} is not only able to colonize competing with other common enteric bacteria but is also able to display its beneficial effect in in vivo studies.

**Microbiological studies.** Samples of washings were collected under sterile conditions before the induction of colitis. Serial decimal dilution was also slightly lower in this case. In the present study, \textit{B. disastoris} (5% \textit{CO}_2) and selected for colony color and morphology. After incubation, the colonies were counted and further identified by biochemical methods (Gram’s staining, catalase, and oxidase) and API 50 CH gallery (Bio Mérieux, l’Etoile, France) for the identification of \textit{L. casei}.

**In vivo assessment of leukocyte-endothelial cell interactions in colonic venules.** Leukocyte-endothelial cell interactions in colonic submucosa and lamina propria venules were characterized by intravital microscopy in the excluded colonic segment. For intravital microscopy studies, rats were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) ip, and the left jugular vein and the right carotid artery were cannulated with polyethylene-10 tubing (Portex, Hyte, UK).

To measure VCAM-1 expression, 20 μg of 125I-5F10 were administered and 5 μg of 131I-UPC-10 were added to the mixture. For assessment of endothelial ICAM-1, a mixture of 5 μg of 125I-1A29 and 245 μg of unlabeled 1A29 was used, and 5 μg of 131I-P-23 were added to the mixture. The mixture of binding and nonbinding MAbs was administered through the jugular vein catheter. The injected activity in each experiment was calculated by counting a 5-μl sample of the mixture containing the radiolabeled MAbs. After injection, the MAbs were allowed to circulate for 5 min, and the animals were exsanguinated. The accumulated activity of each MAb in an organ was expressed as nanograms of binding antibody per gram of tissue. The formula used to calculate VCAM-1 and ICAM-1 expression was as follows: endothelial expression = [(cpm 125I organ g^{-1} cpm 125I injected^{-1}) – (cpm 131I organ g^{-1} cpm 131I Injected^{-1})] × (cpm 251I in plasma)/(cpm 131I in plasma) mg injected MAb, where cpm is counts per minute. This formula was modified from the original method (26) to correct the tissue accumulation of nonbinding MAb for the relative plasma levels of binding and nonbinding MAbs (18).

**Pathology studies.** After rat euthanasia, the isolated segment was extracted and opened longitudinally. A picture was taken of each segment, and a numerical code was assigned. The extent of tissue damage was evaluated by using the image management software Leica IM 50-Measurement (Leica Imaging Systems, Cambridge, UK).

The extent of the damaged area was expressed as the percentage of total mucosal surface in the excluded colonic segment. The degree of inflammation of the colon was graded semiquantitatively from 0 to 11, taking into account (1) loss of mucosal architecture (score 0–3); 2) crypt abscess formation (score 0–3); 3) muscle thickening (score 0–3); 4) statistical significance was set at \( P < 0.05 \).

**RESULTS**

A microbiological control of probiotic and nonprobiotic inoculated bacteria was performed in rats before the induction

\[ \text{VENULAR BLOOD FLOW (VBF)} \]

was estimated from the mean of the velocity of three FFV using the empirical relationship of VBF = FFV/1.6 (6). Venular wall shear rate (\( \gamma \)) was calculated, assuming cylindrical geometry, using the Newtonian definition of \( \gamma = 8 \) (VBF/\( D \)) (20).

**Endothelial VCAM-1 and ICAM-1 expression.** The monoclonal antibodies (MAb) used to measure VCAM-1 expression were 5F10, a murine IgG2a against rat VCAM-1 (33), and UPC-10, a nonbinding, murine IgG2a. The MAbs used to measure ICAM-1 expression were 1A29, a mouse IgG1 against rat ICAM-1 (41), and P-23, a murine IgG1 directed against human (but not rat) P-selectin. 5F10 was obtained from Biogen (Cambridge, MA) and UPC-10 was from Sigma Quimica. 1A29 and P-23 were scaled up and purified by protein A or G chromatography at Pharmacia Upjohn Laboratories (Kalamazzo, MI). The selective MAbs directed against VCAM-1 and 1A29 directed against ICAM-1 were labeled with 125I, whereas the nonbinding MAbs were labeled with 131I (Amersham Ibérica, Madrid, Spain). Radioiodination of the MAbs was performed by using the iodogen method as described previously (7, 27).

Animals were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) ip, and the left jugular vein and the right carotid artery were cannulated with polyethylene-10 tubing (Portex, Hyte, UK). To measure VCAM-1 expression, 20 μg of 125I-5F10 were administered and 5 μg of 131I-UPC-10 were added to the mixture. For assessment of endothelial ICAM-1, a mixture of 5 μg of 125I-1A29 and 245 μg of unlabeled 1A29 was used, and 5 μg of 131I-P-23 were added to the mixture. The mixture of binding and nonbinding MAbs was administered through the jugular vein catheter. The injected activity in each experiment was calculated by counting a 5-μl sample of the mixture containing the radiolabeled MAbs. After injection, the MAbs were allowed to circulate for 5 min, and the animals were exsanguinated. The accumulated activity of each MAb in an organ was expressed as nanograms of binding antibody per gram of tissue. The formula used to calculate VCAM-1 and ICAM-1 expression was as follows: endothelial expression = [(cpm 125I \text{organ g}^{-1} cpm 125I injected^{-1}) – (cpm 131I \text{organ g}^{-1} cpm 131I Injected^{-1})] × (cpm 251I in plasma)/(cpm 131I in plasma) mg injected MAb, where cpm is counts per minute. This formula was modified from the original method (26) to correct the tissue accumulation of nonbinding MAb for the relative plasma levels of binding and nonbinding MAbs (18).

**Pathology studies.** After rat euthanasia, the isolated segment was extracted and opened longitudinally. A picture was taken of each segment, and a numerical code was assigned. The extent of tissue damage was evaluated by using the image management software Leica IM 50-Measurement (Leica Imaging Systems, Cambridge, UK). The extent of the damaged area was expressed as the percentage of total mucosal surface in the excluded colonic segment. The degree of inflammation of the colon was graded semiquantitatively from 0 to 11, according to previously defined criteria of Appleyard and Wallace (1) and taking into account (1) loss of mucosal architecture (score 0–3); 2) cellular infiltration (score 0–3); 3) muscle thickening (score 0–3); 4) statistical significance was set at \( P < 0.05 \).

**RESULTS**

A microbiological control of probiotic and nonprobiotic inoculated bacteria was performed in rats before the induction
of colitis. *L. casei* was recovered in 100% of the rats in which the bacteria was inoculated (colitis-*L. casei* group), and, as expected, the probiotic was not recovered from any animal in the control and colitis groups.

Nonprobiotic bacteria were also recovered in 100% of the randomly selected rats analyzed, thus indicating that the strategy used for intestinal colonization was highly effective. Pooled bacterial recovery results from the three groups were as follows: *Enterobacteriaceae* (*K. pneumoniae* and *E. aerogenes*) were recovered from $2 \times 10^2$ to $2 \times 10^7$ CFU/ml of washing efflux. *Streptococcus* were recovered from $2 \times 10^2$ to $4 \times 10^7$ CFU/ml; and *Bacteroides* (*B. disastonis* and *B. fragilis*) were recovered from $1 \times 10^1$ to $4 \times 10^7$ CFU/ml. Presence of *L. casei* in the colitis-*L. casei* group ranged from $4 \times 10^1$ to $1.3 \times 10^5$ CFU/ml. The wide ranges of these values are most probably related to fluctuations in the flushing volumes used to remove mucin aggregates before sampling. As a consequence, microbiological washing outcomes were semiquantitative.

**Leukocyte-endothelial cell interactions in colonic venules.** Induction of colitis by administration of TNBS-ethanol in the excluded colonic segment colonized by normal rat colonic flora induced a pronounced increase in leukocyte-endothelial cell interactions (Figs. 1 and 2). There was a onefold increase in the flux of rolling leukocytes (rolling cells per minute) and in the number of rolling leukocytes (numbers of cells rolling in 100-μm length of venule). Few adherent leukocytes were present in venules of noninflamed colonic segments, and an eightfold increase in leukocyte adhesion was observed after administration of TNBS-ethanol in the excluded colonic segment. No differences in leukocyte rolling velocity, venular blood flow, or venular wall shear rate were observed between control and colitis group rats (data not shown).

Colonization of the excluded segment with *L. casei* and normal flora before the induction of colitis did not reduce the flux or number of rolling leukocytes below the level observed in the control group but markedly reduced (60%) the number of firmly adherent leukocytes to colonic venules (Fig. 2).

**Endothelial VCAM-1 and ICAM-1 expression.** Endothelial ICAM-1 and VCAM-1 expression significantly increased in the colonic endothelium in association with the induction of colitis. As shown in Fig. 3, under baseline conditions, VCAM-1 expression was low and markedly increased in the inflamed colon (3-fold). Expression of ICAM-1 was significantly higher than that of VCAM-1 under baseline conditions.

Fig. 1. Photographs of intravital microscopy showing leukocyte-endothelial cell interactions in a noncolitic rat (A–C) and a colitic rat (D–F). Photograph sequences have been taken at 0.3-s intervals; direction of flow is rightward. In the noncolitic rat, a single rolling leukocyte is seen, whereas in the venule of the colitic rat numerous rolling and 2 adherent (fixed) leukocytes are observed.
as estimated by the amount of antibody bound per gram of tissue. The magnitude of ICAM-1 upregulation in colitic animals was weaker than that of VCAM-1. Colonization of the colonic segment before the induction of colitis with the probiotic *L. casei* significantly attenuated the upregulation of ICAM-1, bringing those values to the levels observed in control noncolitic animals (Fig. 3B), but did not decrease expression of VCAM-1 (Fig. 3A).

To determine whether expression of ICAM-1 and VCAM-1 in the isolated colonic segment might be influenced in part by surgical manipulation, expression of these adhesion molecules was also measured in the proximal ileum and distal end colon. As shown in Table 1, in noncolitic control group animals, no significant differences were observed in the level of expression of ICAM-1 or VCAM-1 between the isolated colonic segment and adjacent segments. Furthermore, induction of colitis by TNBS induced an upregulation of these adhesion molecules

![Fig. 2. Leukocyte-endothelial cell interactions in the colon of 3 groups of rats: control, colitis, and colitis-*L. casei*. A: number of adherent leukocytes (cells/100 μm venule). B: flux of rolling leukocytes (cells/min). C: number of rolling leukocytes (cells/100 μm). *P < 0.05 vs. control; #P < 0.05 vs. colitis.](http://ajpgi.physiology.org/)

![Fig. 3. Expression of VCAM-1 (A) and ICAM-1 (B) in control, colitis, and colitis-*L. casei* rats. Results are expressed as ng MAb/g tissue. #P < 0.05 vs. control; *P < 0.05 vs. colitis (ICAM-1); P < 0.05 vs. control (VCAM-1).](http://ajpgi.physiology.org/)

**Table 1. ICAM-1 and VCAM-1 expression in the colitic isolated segment, noninflamed terminal colon, and ileum**

<table>
<thead>
<tr>
<th></th>
<th>Colitis Segment (TNBS)</th>
<th>Terminal Colon</th>
<th>Ileum</th>
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</thead>
<tbody>
<tr>
<td><strong>ICAM-1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>117.7±11.8</td>
<td>124.9±14.8</td>
<td>228.1±28.4</td>
</tr>
<tr>
<td>Colitis</td>
<td>180.8±16.1†</td>
<td>173.5±31.9</td>
<td>265.8±11.1</td>
</tr>
<tr>
<td>Colitis-<em>L. casei</em></td>
<td>117.2±17.8*</td>
<td>177.2±26.7</td>
<td>213.5±34.5</td>
</tr>
<tr>
<td><strong>VCAM-1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.0±0.8</td>
<td>3.9±0.5</td>
<td>5.2±0.4</td>
</tr>
<tr>
<td>Colitis</td>
<td>10.4±1.0*</td>
<td>3.7±0.4</td>
<td>6.5±0.7</td>
</tr>
<tr>
<td>Colitis-<em>L. casei</em></td>
<td>9.5±0.9*</td>
<td>4.6±0.6</td>
<td>7.0±0.8</td>
</tr>
</tbody>
</table>

Values are means ± SE in ng MAb/g tissue. TNBS, 2,4,6-trinitrobenzenesulfonic acid. *P < 0.05 vs. colitis, †P < 0.05 vs. control (ICAM-1); *P < 0.05 vs. control (VCAM-1)
only in the isolated loop, indicating that this process does not have a systemic component.

**Effect of L. casei pretreatment on severity of pathological changes.** The induction of colitis by administration of TNBS-ethanol-induced severe colonic damage, with gross inflammatory changes in 94.6% of the mucosal surface of the excluded colonic segment. Pretreatment with L. casei resulted in a significant reduction of the grossly damaged area (control group 0 ± 0%; colitis group 94.6 ± 3.7%, P < 0.05 vs. control group; colitis-L. casei group 75.6 ± 11.7%, P < 0.05 vs. control and colitis group). Evaluation of the histological severity of the colitis according to the Appleyard-Wallace score showed a marked increase in histological lesions associated with the development of colitis (control 0.25 ± 0.14 and colitis 5.60 ± 0.59, P < 0.01). Addition of L. casei to the bacterial inocula did not significantly decrease the overall histological severity score (4.92 ± 0.76) or any of the subscores.

**DISCUSSION**

In this study, we investigated whether the probiotic L. casei alters one of the key components of an inflammatory response, namely, the recruitment of immune cells into the inflamed organ, in an experimental model of transmural inflammation induced by TNBS. Previous experiments demonstrated that instillation of TNBS into a segment of bowel without luminal bacteria resulted in mild mucosal injury, whereas rats with a bowel segment colonized with aerobic and anaerobic bacteria showed a strong inflammatory reaction in response to the TNBS challenge (8). An interesting aspect also shown in the previous study is that bacteria exert their pathogenetic role within the first 24 h after administration of TNBS, that is, in the early stages of epithelial damage and inflammation (8).

Furthermore, a recent study using the same experimental model and bacterial strains employed in the present study documented a significant reduction in the colonic inflammatory damage induced by TNBS when the excluded segment was inoculated with L. casei and normal flora. Moreover, the addition of the probiotic significantly reduced the translocation of bacteria to the mesenteric lymph nodes, liver, and spleen (21).

In keeping with these observations, we also documented a significant reduction in the severity of gross colonic damage, and this reduction was of similar magnitude to that observed in the previous study. However, colonization by L. casei did not result in significant attenuation of the microscopic lesion. This discrepancy has also been noted in previous studies. For example, Pavan and colleagues (28) found that pretreatment with a L. plantarum or L. lactis strain had no effect on histological scores in mice with TNBS-induced colitis, whereas translocation of bacteria was significantly reduced. This may be related to the fact that histology score is a reflection of the most severe area of damage, and, in our experiments, areas of severe damage were still present in animals administered L. casei, but the area of ulceration was significantly reduced.

The present study was particularly focused on elucidating whether the modulation of the process of leukocyte recruitment was part of the effects of the probiotic treatment. Both experimental (31) and clinical studies (9) have demonstrated that selectively reducing the recruitment of leukocytes into the inflamed intestine affords a significant therapeutic benefit in IBD, and this effect may also be a contributor to the beneficial actions of some drugs in colitis such as cyclosporin A (40).

Recruitment of leukocytes from the vascular compartment to the extravascular space is a critical component of inflammation. Leukocyte trafficking through the vascular wall results from a complex series of interactions between leukocytes and endothelium, which include the following steps: rolling, activation, firm adhesion, and emigration (25).

An enhanced leukocyte recruitment in intestinal venules with significant increases in the numbers of leukocytes with rolling and adhesion interactions has been a constant finding in all experimental models of colitis (12, 31, 39).

These interactions are precisely regulated by several cell adhesion molecules expressed on the surface of endothelial cells and leukocytes (31). Our results show that leukocyte-endothelial cell interactions were attenuated in response to treatment with the probiotic L. casei, with a significant reduction of the number of firmly adherent leukocytes.

Treatment with L. casei did not impact the first phase of leukocyte-endothelial cell interactions, namely leukocyte rolling. Lack of effect of L. casei treatment on rolling interactions probably does not limit the anti-inflammatory action of this treatment, because interfering with this initial cellular interaction in the setting of TNBS-induced colitis does not affect subsequent steps of leukocyte recruitment, such as firm adhesion to the venular endothelium and emigration (32).

Firm adhesion is the crucial determinant of the infiltration of an organ by inflammatory cells. In fact, it has been shown that 80% of cells that adhere for a period of 30 s or longer finally emigrate into the interstitium of an organ (24), and this was the time period used in the present study to categorize a leukocyte as firmly adherent.

The different steps of leukocyte recruitment are governed by adhesion molecules belonging to different families. Thus the initial rolling interactions are mediated by selectins and their ligands, whereas firm adhesion is mediated by integrins on the surface of leukocytes and immunoglobulin superfamily molecules on the endothelium (25). Because intravital microscopy studies revealed that the probiotic L. casei significantly reduces leukocyte adhesion, without having an impact on rolling interactions, to further explore the mechanistic basis for the effects of the probiotic strain on intestinal inflammation, we concentrated our efforts on endothelial cell adhesion molecules involved in firm adhesion.

In that regard, previous studies using the TNBS model of colitis showed that immunoneutralization of ICAM-1 attenuates, but does not completely abrogate, leukocyte adhesion, whereas VCAM-1 blockade decreases leukocyte adhesion in colitic rats to levels similar to those of noncolitic animals (31). Therefore, we studied the effects of L. casei on expression of these two adhesion molecules in colonic inflammation. Interestingly, expression of ICAM-1 was downregulated by treatment with the probiotic, although VCAM-1 expression was unaffected. Therefore, it is conceivable that the reduction of leukocyte adhesion in response to treatment with L. casei is related to the downregulation of ICAM-1. Although this may appear to contradict our previous observations, suggesting a crucial role for VCAM-1 in the pathogenesis of colitis induced by TNBS, it can be explained by an important difference between our present and previous studies in the time point chosen. In the present study we assessed parameters of muco-
PROBIOTICS AFFECT INFLAMMATORY RESPONSE IN COLITIS

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sal inflammation 1 day after TNBS administration. This may represent a more acute phase of the colonic inflammatory reaction, and ICAM-1 is known to play a determining role in acute inflammatory conditions. However, VCAM-1 is more relevant as a mediator of chronic inflammatory diseases in which recruitment of lymphocytes, as opposed to neutrophils, is the main cellular event.

Our study has several limitations. First, in this model, we assessed inflammatory changes within the first 24 h after TNBS administration and, therefore, may not accurately represent the chronic colitis of human IBD. We did not extend this study for a longer period of time because we observed an increment of bacterial contamination together with a reduction in the recovery of the probiotic bacteria. Second, probiotics have been shown to be able to affect various components of the inflammatory cascade: coculture with L. casei strain significantly reduces TNF-α release in normal and inflamed mucosa from patients with CD (3) and also reduces IL-8 release via TNF-α independent pathways (2). Therefore, we cannot establish a cause-effect relationship between the downregulation of ICAM-1 and reduction of leukocyte adhesion, because other components of the inflammatory response such as a decrease in leukocyte activating or chemotactic signals may also contribute to reduce leukocyte recruitment in our model.

The use of probiotics in the clinical setting remains controversial, and their mechanism of action has not been fully clarified. We believe our study sheds a beam of light on the mechanistic basis for testing probiotics in a clinical setting. Issues to be resolved include the preparation to use, mode of delivery, and interactions between probiotic bacteria and other traditional medications. Further trials are needed to establish the precise role of probiotic bacteria in the treatment of IBD.

ACKNOWLEDGMENTS

The authors thank Montserrat Casellas, Milagros Gallart, Carmen Alastrue, and Fidelma Greaves for technical assistance in the analytical procedures.

GRANTS

This work was supported by Ministerio de Ciencia y Tecnología Grant SAF2002-02211 and Instituto de Salud Carlos III Grant C03/02. S. Angulo is a recipient of a grant from the Ministerio de Ciencia y Tecnología.

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