Goat Milk Oligosaccharides Are Anti-Inflammatory in Rats with Hapten-Induced Colitis

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ABSTRACT Oligosaccharides are included among the anti-inflammatory components of milk because of their probiotic properties and their capacity to act as receptors of microorganisms. Here the intestinal anti-inflammatory effect of goat milk oligosaccharides (OS) was assessed in trinitrobenezesulfonic (T) acid-induced colitis in rats. Rats were randomly assigned to three different groups. Two groups (T and OS) of colitic rats and a control group (C) were studied. Group OS received 500 mg/kg-d of goat milk oligosaccharides orally, starting 2 d before the colitis induction until d 6, and groups T and C received the vehicle. When compared with the T group, the OS group showed decreased anoxia and body weight loss; reduced bowel wall thickening and longitudinal extent of necrotic lesions; downregulated colonic expression of interleukin 1β, inducible nitric oxide synthase, cyclooxygenase 2, and mucin 3; and increased trefoil factor 3. Thus, goat milk oligosaccharides have anti-inflammatory effects in rats with experimental colitis and may be useful in the management of inflammatory bowel disease. J. Nutr. 136: 672–676, 2006.

KEY WORDS: · colon · goat milk · inflammatory bowel disease · intestine · oligosaccharides

For over 30 years it has been accepted that breast-fed infants are more protected against infectious agents than formula-fed infants. Human milk oligosaccharides (OSs) are thought to be among the agents responsible for this effect because of their ability to stimulate the growth of bifidobacteria in the gastrointestinal tract while protecting against enteric pathogens (1–5). In fact, several clinical trials have demonstrated in the past few years that OSs can be considered prebiotics (i.e., food ingredients that have the potential to benefit the host by selectively stimulating the growth of desired host organisms in the gastrointestinal tract) (8–11). On the other hand, human milk OSs are structurally similar to those present in intestinal cell glycolipids and glycoproteins, and therefore they can act as receptors for microorganisms, constituting an additional defense mechanism (6,7,12).

Because of these properties, human milk OSs have also been included among the anti-inflammatory components of human milk and have been proposed to prevent or treat inflammatory diseases such as necrotizing enterocolitis or inflammatory bowel disease (IBD) (13,14).

IBD comprises two different but closely related conditions, ulcerative colitis and Crohn’s disease. The hallmark of IBD is chronic and relapsing inflammation of the intestine, but there are important differences from Crohn’s disease with regard to pathophysiology and treatment. Thus, ulcerative colitis affects the large intestine at the mucosal level, whereas Crohn’s disease is characterized by transmural inflammation and may involve any segment of the gastrointestinal tract, although the majority of cases show ileocolonic involvement. IBD is an important health problem because of its effect on the patient’s quality of life and because of its high prevalence, which has increased in the last few years (15,16). Despite intense investigative efforts, the cause of IBD is essentially unknown. However, it has become clear that the intestinal flora are determinant in the development of events that ultimately are involved in relapses (17). Thus, IBD probably represents an uncontrolled and exacerbated response to luminal antigens that are innocuous for the normal population.

Although IBD can often be successfully managed pharmacologically, the drugs used, such as corticoids, aminosalicylates, or azathioprine, have many serious adverse effects that limit their application. Hence, the search for new treatments with few adverse effects is much warranted (16). One such strategy involves the use of prebiotics and/or probiotics to modulate intestinal flora to promote the growth of host-friendly bacteria and inhibit the proliferation of potentially harmful microor-
gangnisms (18), although other mechanisms may be involved (19).

OSs are the third most abundant component of human milk after lactose and fat (6). Their concentration varies between 5 and 8 g/L (14). Human milk contains more OSs than ruminant milk, and these OSs are very heterogeneous; >100 different structures have been described. Recently, the concentration of OSs in human, bovine, caprine, and ovine milk was studied (14). Even though the concentration of goat milk OSs is low compared with human milk (0.25–0.30 g/L), it is higher than in milk of bovine and ovine origin (0.03–0.06 and 0.02–0.04 g/L, respectively).

That study also showed that the OS profile of goat milk is most similar to that of human milk. In fact, a larger amount and variety of acidic OS structures were identified in goat milk than in cow and sheep milk. (14). Therefore, goat milk OSs could be included in infant formulas to improve the nutrition of infants.

The aim of this study was to investigate the anti-inflammatory effect of goat milk OSs in a model of experimental colonic inflammation induced by the hapten, trimitrobenzenesulfonic acid (TNBS), in rats.

**MATERIALS AND METHODS**

Except where indicated, all reagents and primers were obtained from Sigma. Taq polymerase was purchased from Amersham Biosciences. Antibodies were purchased from Santa Cruz Biotechnology and Sigma.

**Goat milk OS isolation, quantification, and characterization.** Isolation, quantification, and characterization of goat milk OSs was carried out as described elsewhere (14). Briefly, milk used in this project was pasteurized skimmed goat milk supplied by Puleva Biotech S.A..

To isolate the OS fraction, a two-stage tangential ultrafiltration-nanofiltration process was selected (14). Multilayer ceramic Ceram Inside membranes (TAMI Industries) made of ZrO2–TiO2, with three channels (25 cm long) of 3.6-mm hydraulic diameter, membrane area of 0.019 m², and molecular weight cutoffs of 50 and 1 kDa, respectively, were used. There were two separate, consecutive and continuous, dialysis steps. The cumulated permeate from the first stage was collected and used as initial retenate in the second step. The retenate from the second step, containing the OS fraction, was lyophilized. Quantification of OSs was performed by high-pH anion-exchange chromatography with pulsed amperometric detection with use of a Carbo Pac PA-1 column (250 × 4.6 mm i.d.) connected to a Dionex system equipped with a pulsed amperometric detector and a Foxys Jr. fraction collector (Isco Inc.). Solutions of OS standards (Sigma) were used to identify and quantify OS peaks obtained in the chromatograms.

A product containing >80% of the original OS content, only 5% (wt/wt) of lactose and virtually salt free, was obtained and used to carry out all the experiments. Major OS structures found in the product were the acidic OS 6-sialyl-lactose, 3-sialyl-lactose, disialyl-lactose, and N-glycolylneuraminic-lactose, and the neutral OS 3-galactosyl-lactose, lacto-N-hexose, and N-acetylgalactosaminyl-lactose.

**Animals.** Female Wistar rats (175–225 g) obtained from the Laboratory Animal Service of the University of Granada were housed in Makrolon cages (Bayer) and maintained in our laboratory in air-conditioned animal quarters with a 12-h light-dark cycle. The rats had free access to tap water and were fed a commercial diet of Panlab AO4 (Panlab). Diet composition (wt/wt) was 15.4% protein, 2.9% fat, 60% carbohydrates, 3.9% fiber, 3.3% mineral, and 12% moisture. The study was carried out in accordance with the Directive for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes of the European Union (86/609/EEC).

**Induction of colitis.** Colitis was induced by the method of Morris et al. (20) with minor modifications. Briefly, rats were deprived of food overnight and anesthetized with halothane. Under these conditions, the rats were given 10 mL of TNBS dissolved in 25 mL of 50% ethanol (vol/vol) by means of a Teton cannula inserted 8 cm through the anus. The rats were kept in a head-down position for an additional 30 s and returned to their cages.

**Experimental design.** The rats were randomly assigned to three different groups (n = 6). Two groups (T and OS) received the TNBS challenge to induce colitis as described above, whereas a control group (C) was given 0.25 mL of PBS (vehicle). Group OS received 500 mg/kg of the OSs in 1% methylcellulose, and T group was given PBS in an aqueous solution, 20°C, Sigma orally, starting 2 d before the TNBS challenge, whereas groups T and C received the vehicle. The rats were treated for 6 d after the TNBS challenge. An esophageal catheter was used to deliver all treatments. Food intake, water intake, and body weight were determined every day.

**Assessment of colonic damage.** The rats were killed by cervical dislocation, and the entire colon was removed and placed on an ice-cold plate, cleaned of fat and connective tissue, and fixed on filter paper. Each specimen was weighed and its length measured under a constant load (2 g). The large intestine was longitudinally opened and scored for visible damage on a scale of 0–25 according to the following criteria: adhesions (0–3), obstruction (0–2), thickening (0–2), hyperemia (0–3), fibrosis (0–3), necrosis (0–5), and scarring and deformation (0–7) (21). The colon was subsequently divided longitudinally into several pieces for biochemical determinations. The fragments were immediately frozen in liquid nitrogen. All the samples were kept at −80°C until used.

**Western blot.** The colon levels of cyclooxygenase 2 (COX2) and inducible oxide nitric synthase (iNOS) were determined by immunoblotting. Colonic samples were homogenized in lysis buffer (0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100 in PBS) with protease inhibitors (1,10-phenanthroline, phenylmethylsulfonylfluoride, and aprotinin). The supernatants obtained after centrifugation (7000 × g; 10 min at 4°C) were boiled for 4 min in Laemmli buffer, separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with the corresponding antibodies. The bands were detected by enhanced chemiluminescence (PerkinElmer) and quantified with NIH software (Scion Image). After transferring the samples to nitrocellulose membranes, equal loading was checked routinely by Coomassie blue staining.

**RT-PCR.** RT-PCR was used to examine the expression of IL-1β, transforming growth factor β (TGF-β), and mucins (MUCs) 2, 3, and 4 as well as trefoil factor 3 (TFF3) in rat tissues. Total RNA was extracted with Triozol (Invitrogen). A total of 5 μg of RNA per sample was subjected to reverse transcription by use of the First-strand cDNA synthesis kit (Amersham Biosciences). PCR amplification was performed with 2 μL of cDNA for a final PCR volume of 25 μL. Rat primers were as follows: MUC2 (sense 5'-GCT CAA TTC CAG AAG GCC ACA G A-3'; antisense 5'-CCA GAT AAC AAT GAT GGC AGA GC-3'); MUC3 (sense 5'-CAC AAA GGC AAG AGT CCA CTG TCT AAG-3'; antisense 5'-GCT GAT GCA TTT GCC AGA G-3'); MUC4 (sense 5'-GCT AGA AGA AGA TGC TTA TTC-3'; antisense 5'-CCA GCC CAT GCC AGA CCT TC-3'); TGF8 (sense 5'-ATG GAC ACC AGA GCC AGC TTC TG-3'; antisense 5'-ACG GCC CCT TTC CCA AAT CTG-3'); TGF9 (sense 5'-GCT AAT GGT GCA CAC CCA C-3'); TGF10 (sense 5'-GCT AGA AGA TGC TTA TTC-3'; antisense 5'-CCA GCC CAT GCC AGA CCT TCC-3'). The cycle numbers and hybridization temperatures for each PCR were as follows: 23 cycles and 56°C (for MUC2, MUC3, and TFF3); 27 cycles and 57°C (for MUC4, TGF8, and IL-1β); and 17 cycles and 60°C (for the ribosomal 18S unit). To set up the PCR conditions, different amounts of colonic RNA from a pool of samples were amplified by use of different numbers of cycles (data not shown).

After PCR amplification, 5 μL of each reaction was resolved in 2.5% agarose gels. Bands were quantitated with NIH software (Scion Image).

**Northern blot analysis.** Equal amounts (30 μg) of total RNA were resolved by use of 1% agarose/formaldehyde gels, transferred to a nylon membrane (Hybond-XL, Amersham Pharmacia Biotech) and cross-linked by use of UV-Stratalinker (Stratagene). Prehybridization and hybridization with 32P-labeled probes were carried out in ULTRAhyb solution (Ambion) at 42°C. The intestinal IL-10 probe was obtained from a Bioimage fragment. The nucleotide sequence of human IL-10 has 84% homology with that of the rat IL-10. The construct was cut with SfiI and purified by electroleoration. A β-actin

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RESULTS

As expected, colitic rats (group T) experienced severe anorexia (7.4 ± 1.6 vs. 13.5 ± 0.6 g/(rat-d); P < 0.05) and loss of body weight (−3.3 ± 2.4% vs. +5.7 ± 1.1% after 6 d, P < 0.05) in comparison with control rats (group C). The colon was thickened and shortened, with areas of mucosal erosion and necrosis and submucosal fibrosis (Table 1). TNBS colitis was also characterized biochemically by an increase in colonic IL-1β (Fig. 1), iNOS, and COX2 (Fig. 2) and a decrease in MUC2 and MUC3 (Fig. 1), whereas IL-10 (Fig. 3), TFF3, and MUC4 (Fig. 1) were unchanged.

Pretreatment of rats with OSs prevented body weight loss because these rats had gained 4.0 ± 1.8% of their initial body weight on d 6 of colitis (P < 0.05 vs. group T). Rats pretreated with OS tended to consume slightly more food than those in group T (9.0 ± 1.1 vs. 7.4 ± 1.6 g/(rat-d)). Rats treated with OS also had significantly lower colonic weight, weight-to-length ratio, and extension of necrotic lesions than group T rats (Table 1). At a biochemical level, this was associated with lower levels of colon iNOS, COX2, and IL-1β in OS than in rats (Figs. 1 and 2). OS treatment augmented the expression of TFF3 (Figs. 1 and 2), while TGF-β, IL-10, MUC2, and MUC3 did not differ between the OS and T groups (Figs. 1 and 3).

DISCUSSION

The absence of curative drug treatments for IBD is probably related to the fact that the exact cause of this condition has not been identified. However, the colonic flora clearly play a role in the pathophysiology of IBD (17). Thus, the severity of experimental colitis is dramatically reduced when animals are reared in a germ-free environment, but not a specific pathogen-free environment (22). Furthermore, antibiotics such as metronidazole display anti-inflammatory effects in animal models of colitis (23). Apart from the use of antibiotics, the colonic microflora can be manipulated with the use of probiotics and prebiotics (i.e., health-promoting bacteria or products that favor the balance toward "anti-inflammatory" microflora). Although much is known about the pathogenic effects of many microorganisms on the intestine, the role of host-bacteria interactions in health and in IBD is comparatively obscure (17). However, it appears that prebiotics and probiotics may be beneficial in IBD, although controlled studies are needed in humans (18). Possible mechanisms include increased production of butyrate, reduction of the number of proinflammatory bacterial species, and stimulation of Toll-like receptors by bacterial CpG DNA.

Goat milk OSs are good candidates to serve as effective prebiotic anti-inflammatory agents in IBD. They have been shown to (1) inhibit the adhesion of bacteria to the epithelial membrane, 2) reduce bacterial translocation in cell models, and 3) promote the selective growth of lactobacillus and bifidobacteria (1,2,6). Goat milk was initially selected as a source because of its relatively high concentration (as compared with cow milk) and the presence of branched OSs (14). Because of its presence in milk, these compounds are generally considered nontoxic.

TNBS-induced colitis showed the expected (24) characteristics of anorexia with loss of body weight, epithelial necrosis, bowel wall thickening, and colon shortening. Pretreatment of rats with 500 mg/(kg-d) of goat milk OSs resulted in protection from the colonic inflammation induced by the hapten TNBS. This beneficial effect was seen in higher body weight gain and lower anorexia and ameliorated macroscopic appearance (colonic weight-to-length ratio, necrotic extension). In addition, treatment was associated with a significantly lower expression of iNOS, COX2, and IL-1β. IL-1β is one of the predominant cytokines in rat TNBS colitis, and it is expressed at higher levels than TNF or interferon-γ, whereas iNOS is the main source of NO in intestinal inflammation. Both are thought to exert a proinflammatory role in TNBS colitis (25). COX2, the inducible form of cyclooxygenase, is also upregulated in IBD (26,27), but it is thought to play an anti-inflammatory role (28) because COX2 selective inhibition (i.e., without affecting COX1) does not affect or may even be detrimental to the patient (29,30). Thus, the downregulation of COX2 expression is a marker rather than a mechanism of anti-inflammatory effects of OS. We also examined the expression of the TGF-β/IL-10 axis but found no effect of OSs at this level. This is interesting because it reveals that the inhibitory effect of OS treatment on cytokine production was specific.

Because prebiotics have been claimed to modulate mucin expression in the intestine (31,32), we assessed the mucin expression profile by RT-PCR. Colonic inflammation was

### Table 1

<table>
<thead>
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<th>C (%)</th>
<th>T (%)</th>
<th>OS (%)</th>
</tr>
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<tbody>
<tr>
<td>Colon weight, g</td>
<td>1.0 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon length, cm</td>
<td>17.1 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.1 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.5 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon weight/length ratio, mg/cm</td>
<td>61.1 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>145.6 ± 14.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.6 ± 11.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Extent of necrosis, cm</td>
<td>—</td>
<td>2.5 (1.0-4.9)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 (0.5-2.0)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Damage score</td>
<td>—</td>
<td>9.0 (6.3-11.8)</td>
<td>5.8 (3.0-8.0)</td>
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<sup>1</sup> Values are mean ± SEM or medians (25%-75% quartiles), n = 6. Values in a row with superscripts without a common letter differ, P < 0.05.

<sup>2</sup> The group OS received goat milk oligosaccharides 500 mg/(kg-d) starting 2 d before the induction of colitis until d 6.
characterized by a significant decrease in MUC2 and MUC3 mRNA, whereas MUC4 was unchanged. These results are similar to those of previous reports (24), although it should be noted that changes in mucin expression may not be uniform in experimental colitis (21,33–36). Mucins are generally considered to serve a protective function in the intestine, and, in particular, MUC2 has been shown to be involved in protection against colonic inflammation (37). OS treatment significantly reduced the MUC3 transcript levels and did not significantly alter the expression of either MUC2 or MUC4. These results indicate that the mechanism of action of the OSs was unrelated to mucin expression levels. On the other hand, OS treatment was associated with marked upregulation of TFF3 expression, which is also goblet cell specific. TFF3 is a bioactive peptide involved in the maintenance of gastrointestinal tissue as well as in tissue repair, particularly at the epithelial level (38). Intracellular but not intraperitoneal administration of TFF3 is beneficial in experimental colitis induced by dextran sulfate sodium (39), and strains of Lactococcus lactis engineered to produce TFF3 in vivo also have therapeutic value in different models of colitis in mice (40). Thus, the fact that mRNA levels of TFF3, which is transcriptionally regulated (41), were increased by OS treatment indicates a possible mechanism of action.

In conclusion, goat milk OSs are anti-inflammatory when administered as a pretreatment in the TNBS model of rat colitis, a widely used preclinical model of IBD. Therefore, these compounds may be a valuable alternative to current therapies to treat IBD. Further studies are warranted to validate this approach.

**LITERATURE CITED**


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