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ORIGINAL ARTICLE

Intestinal anti-inflammatory activity of dietary fiber (*Plantago ovata* seeds) in HLA-B27 transgenic rats

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Abstract— Background & aims: Dietary fiber has been proven to be beneficial in maintaining remission in human ulcerative colitis, an effect related with an increased luminal production of short-chain fatty acids (SCFA). The aim of the present study was to further investigate the mechanisms involved in the intestinal anti-inflammatory effects of dietary fiber in an experimental model of rat colitis.

Methods: HLA-B27 transgenic rats (8–10 weeks old) were fed a fiber-supplemented diet (5% *Plantago ovata* seeds) for 13 weeks before evaluation of the colonic inflammatory status, both histologically and biochemically. The luminal colonic production of SCFA was quantified. In vitro studies were also performed to test the interaction between two SCFA (butyrate and propionate) as inhibitors of cytokine production in THP-1 cells.

Results: Dietary fiber supplementation ameliorated the development of colonic inflammation in transgenic rats as evidenced by an improvement of intestinal cytoarchitecture. This effect was associated with a decrease in some of the pro-inflammatory mediators involved in the inflammatory process: nitric oxide, leukotriene B_4, tumor necrosis factor α (TNFα). The intestinal contents from fiber-treated colitic rats showed a significant higher production of SCFA, butyrate and propionate, than non-treated colitic animals. In vitro studies revealed a synergistic inhibitory effect of butyrate and propionate on TNFα production.

Conclusions: Dietary fiber supplementation ameliorated colonic damage in HLA-B27 transgenic rats. This effects was associated with an increased production of SCFA, which can act synergistically in inhibiting the production of pro-inflammatory mediators.

Key words: inflammatory bowel disease; dietary fiber; HLA-B27 transgenic rat; short-chain fatty acids; TNFα

Introduction

The term inflammatory bowel disease (IBD) comprises two closely related pathologies, ulcerative colitis and Crohn's disease, which are characterized by chronic and spontaneously relapsing inflammation of the gut. Although much progress has been made in understanding the pathogenesis of human IBD, its precise etiology still remains unknown and involves a number of factors including genetic, environmental, microbial and immunological (1). Thus, an exacerbated inflammatory response of the intestine results from an inappropriate reaction towards a luminal agent, most probably driven by the intestinal microflora (2), which up-regulates the synthesis and release of different pro-inflammatory mediators, including reactive oxygen and nitrogen metabolites, eicosanoids, platelet activating factor and cytokines (3). Several experimental models in laboratory animals have been developed in order to reproduce this situation and have provided many important clues about the pathogenesis of IBD and the possible pharmacological strategies to be used in these intestinal conditions (2, 4). In this effect, it has been reported that transgenic rats expressing the HLA-B27 and the β_2-microglubulin genes develop spontaneous gastrointestinal inflammation that shares many of the clinical manifestations of the human disease, including colonic mucosal permeability changes and enhancement of nitric oxide (NO) metabolism that have been involved in the pathogenesis of this experimental model of colitis (5–8). However, the histological features of colitis in HLA-B27 rats more closely resemble ulcerative colitis than Crohn's disease (9). In addition, the colonic microflora has been described to play a key role in the development of the intestinal inflammatory status; thus these transgenic rats do not develop colitis under germ-free conditions, but if transferred into specific pathogen-free or conventional conditions they develop colitis (10).

[1] Both authors contributed equally to the supervision of this study.

The intestinal epithelium itself acts as a defence against invasion by luminal toxins and bacteria; however, this barrier is weakened or destroyed in IBD, leading to increased mucosal permeability and the subsequent translocation of endotoxins and antigens (11). SCFA, mainly acetate, propionate and butyrate, which are produced in the large bowel by the anaerobic bacterial fermentation of undigested dietary carbohydrates and fiber polysaccharides, have been proposed to actively contribute to the maintenance of colonic homeostasis, butyrate being considered as the major fuel source for the colonocyte (12). In fact, it has been suggested that a diminished β-oxidation of luminal butyrate to CO_2 and ketones, which results in energy deficiency within colonic epithelial cells, could contribute to the pathogenesis of ulcerative colitis (13). In addition, several authors have reported decreased fecal concentrations of SCFA in patients with ulcerative colitis and in the cotton-top tamarin model of idiopathic colitis (14, 15). All these facts suggest that restoration of luminal levels of butyrate may facilitate the recovery of the inflamed mucosa, as previously described (16, 17). An earlier study has reported the efficacy and safety of oral administration of a SCFA substrate (fermentable dietary fiber from *Plantago ovata* seeds) in patients with ulcerative colitis. This beneficial effect exerted by long-term *Plantago ovata* oral administration was associated with increased butyrate concentrations in the distal colon (18).

The aim of the present study was to further elucidate the probable mechanisms involved in the beneficial effects of *Plantago ovata* seeds in the treatment of IBD. We tested the effects of a dietary fiber-supplemented diet (5% *Plantago ovata* seeds) in HLA-B27 transgenic rats. Special attention was paid to its effects on the production of some of the mediators involved in the inflammatory response, such as tumor necrosis factor α (TNFα), leukotriene B_4 (LTB_4) and NO. In vitro experiments were also performed in order to gain knowledge about the mechanisms of action involved in the intestinal anti-inflammatory activity of dietary fiber, focusing on the effect of the SCFA, butyrate and propionate, on the production and release of TNFα in THP-1 cells.

Materials and methods

This study was carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals' as promulgated by the National Institute of Health.

Chemicals and isotopes

All chemicals, including lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were obtained from Sigma Chemical (Madrid, Spain), unless otherwise stated. L-[2,3,4,5-^{3}H]arginine monohydrochloride was purchased from Amersham Iberica (Barcelona, Spain). 2-Methylvaleric acid was obtained from Fluka (Madrid, Spain). *Plantago ovata* seeds were provided by Madaus S.A. (Barcelona, Spain). Kits for rat and human TNFα and leukotriene B_4 (LTB_4) assays were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Animals, diets and experimental design

Female HLA-B27 transgenic rats expressing human HLA-B27 and β2-microglobulin genes and Fisher 344 (F344) rats were purchased from Taconic Laboratory (Germantown, NY, USA) at 8–9 weeks of age. The rats were housed individually under specific pathogen-free conditions in makrolon cages, maintained in an air-conditioned atmosphere with a 12 h light–12 h dark cycle and provided with free access to rodent chow and tap water. After 1 week of acclimatization, the transgenic rats were randomly divided into two groups ($n=6$): the transgenic control and the fiber-treated groups; and the F344 rats ($n=10$) were assigned to the normal control group. The transgenic and the normal control groups received the standard diet and the other (treated group) was fed a fiber-supplemented diet for 13 weeks, when colonic inflammatory status was evaluated. The fiber-supplemented diet was prepared by adding 5 *g* of *Plantago ovata* seeds to 95 *g* of pulverised standard diet for rodents Panlab A04 (Panlab, Barcelona, Spain). The composition of the standard diet (g/100 *g* total dry weight) was: protein 19.5, fat 3.1, carbohydrates 67.8, fiber 4.4, minerals 5.0. The fiber in standard diet was mainly composed by cellulose.

Assessment of colonic damage

The body weight, food intake and stool consistency were recorded every 2 days. Stool consistency was scored according to the criteria described by Kerr et al. (8), which assigns a value of 1 for each of the following characteristics: pale color, visible mucus, softness, and fecal material adherent to the perianal area; a value of 2 for stool that does not have normal texture or normal shape; a value of 3 for liquid stool, and a value of 4 for the presence of visible blood; the total score was summed for each animal. On the last day, the rats were sacrificed with an overdose of halothane. The colon was removed and placed on an ice-cold plate, the luminal contents were collected for SCFA quantification (see below) and the colonic segment was cleaned of fat and mesentery, and blotted on filter paper. Each specimen was weighed and its length measured under a constant load (2 *g*). The colon was longitudinally opened and evaluated for macroscopically visible damage by two observers unaware of the treatment, according to the characteristics of colonic inflammation in HLA-B27 transgenic rats described by Aiko and Grisham (6), i.e. colonic hyperaemia, dilatation and bowel wall thickening, as well as lymph node swelling. The colon was

subsequently sectioned to be used for histology and biochemical determinations. One sample was immediately processed for the measurement of TNFα and LTB_4 levels, and the other two were frozen by immersion in liquid nitrogen and stored at −80°C until analysis for myeloperoxidase (MPO) and nitric oxide synthase (NOS) activities. All biochemical measurements were completed within 1 week from the time of sample collection and were performed in duplicate. For histological analysis, one fragment was embedded in paraffin and sections 5 μm thick were obtained and stained with hematoxylin and eosin. The histological damage was scored on a scale ranging from 0 to 4 according to the criteria reported by Rath et al. (19). Two pathologist observers (AC and MDL) unaware of the treatment conditions performed the evaluation and recorded all the parameters of histological damage.

MPO activity was measured according to the technique described by Krawisz et al. (20); the results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1 μmol hydrogen peroxide/min at 25°C. Colonic samples for TNFα and LTB_4 determinations were immediately weighed, minced on an ice-cold plate and suspended in a tube with 10 mmol/l sodium phosphate buffer (pH 7.4) (1/5 weight/vol). The tubes were placed in a shaking water bath (37°C) for 20 min and centrifuged at 9000 *g* for 30 s at 4°C; the supernatants were subsequently frozen at −80°C until assay. TNFα was quantified by enzyme-linked immunoabsorbent assay and the results were expressed as pg/g wet tissue. LTB_4 was determined by enzyme-immunoassay and the results expressed as ng/g wet tissue. Colonic NOS activity was determined by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline (21). For this purpose, the intestinal samples were homogenised (1/5 weight/vol) for 60 s in 10 mmol/l HEPES (pH 7.4) containing sucrose (0.32 mol/l), EDTA (100 μmol/l), leupeptin (22 μmol/l) and phenylmethhylsulphonyl fluoride (5.7 mmol/l); the resulting homogenate was centrifuged at 10,000 *g* for 10 min at 4°C, and the supernatants assayed for protein content according to the method proposed by Bradford (22). Samples (40 μg protein) were incubated at room temperature for 30 min in the presence of NADPH (1 mmol/l), calmodulin (0.3 μmol/l), tetrahydrobiopterin (10 μmol/l), $CaCl_2.2H_2O$ (2 mmol/l), L,-valine (10 mmol/l) (to inhibit non-specific arginase activity) and L-[^{3}H]arginine (100 μmol/1, 10 mCi/l). Incubations were terminated by the addition of 1 ml HEPES (20 mmol/l, pH 5.5) containing 1 mmol/l EGTA and 1 mmol/l EDTA. L-[^{3}H]citrulline was separated from arginine by adding 1.5 ml 1/1 suspension of Dowex (50 W) in water. Formation of citrulline in the presence of excess of L-NAME (10 mmol/l) was also determined in order to be able to subtract background citrulline formation. Radioactivity was measured in supernatants by liquid scintillation counting and the results were expressed as pmol L-citrulline/30 min per mg protein.

SCFA quantification in colonic contents

Colonic contents were obtained and homogenized with $NaHCO_3$ (150 mmol/l, pH 7.8) (1/5 weight/vol) in a nitrogen atmosphere. Aliquots of 10 ml from each fecal sample were then incubated in duplicate for 24 h at 37°C either in the presence of *Plantago ovata* seeds (at a final concentration of 10 *g*/l) or in the absence of substrate (control incubations). Incubation was terminated by freezing and specimens were stored at −80°C until gas–liquid chromatographic analysis of the SCFA. Briefly, sulphuric acid was added to fecal homogenates and they were centrifuged at 10,000*g* for 5 min at 4°C and SCFA from supernatants were extracted in ethylether. After this, 2 μl of the sample was injected splitless into a CarloErba 8060 gas chromatograph equipped with a 250 μm (internal diameter), 30 m INNOWAX capillary column with a film thickness of 0.25 μm, associated with a Mass Detector Platform II (Micromass, UK). Carrier and make up gas were both helium, each with a flow rate of 1 ml/min. Injection temperature was 200°C. The initial oven temperature was 80°C; this was maintained for 2 min before the temperature was raised 5°C/min to 200°C. Acetate, propionate and butyrate concentrations were calculated from areas of peaks (internal standard was 2-methylvaleric acid) automatically calculated using Masslynx program version 2.1 from Fisons connected on-line to the mass detector.

Inhibition of cytokine production in cell cultures

The human monocytic cell line THP-1 was obtained from the Cell Culture Unit of the University of Granada (Granada, Spain) (ECACC reference number: 88081201) and used to assay the effects of the different SCFA on TNFα production. These cells were cultured in RPMI 1640 (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Life Technologies), 2 mmol/l L-glutamine and 0.05 mmol/l mercaptoethanol in a humidified 5% CO_2 atmosphere at 37°C. Cells were seeded onto 24-well plates at a density of 2×10^6 cells/ml and pre-incubated for 60 min with different concentrations of butyrate (0.1–8 mmol/l) or propionate (0.1–8 mmol/l) or mixtures of butyrate/propionate. Afterwards, cells were stimulated with LPS (1 μg/ml) and PMA (1 μmol/l). After 20 h, plates were centrifuged at 1000 *g* for 10 min and supernatants were collected and stored at −80°C for the determination of TNFα levels by ELISA. The percentage inhibition of cytokine production was calculated for every concentration of SCFA, both separately or in mixture of different proportions. At least four different experiments were performed in these assays. No cytotoxicity was detected in either of the SCFA studied or their mixtures, at any concentration assayed, as evidenced by the colorimetric MTT (tetrazolium) assay (23), which revealed a viability greater than 95% in all cases.

Statistical analysis

All results are expressed as the mean ± SEM. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and post hoc least significance tests. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, Maryland), with statistical significance set at $P<0.05$. In the in vitro studies, the interactions between butyrate and propionate on TNFα cell production were evaluated by an isobolographic analysis using mixtures of both SCFA (24). Statistically significant deviations from the line of additivity in the isobologram were analyzed as described by Tallarida et al. (25).

Results

Effect of fiber supplementation on colonic inflammation

Dietary fiber supplementation ameliorated the development of spontaneous intestinal inflammation in HLA-B27 transgenic rats, which was evident in the course of the experiment since treated rats showed a delay in the onset of diarrhoeic faeces. All rats were sacrificed 13 weeks after the beginning of the treatment (23 weeks old), when all transgenic rats from non-treated group presented completed unformed stool or diarrhoea. According to the criteria described by Kerr et al. (8), these rats showed a mean stool score of 3.3 ± 0.2. Thus, their stools were consistently pale, contained mucus, and were typically softer than those of F-344 rats, resulting in some of the rats in the presence of fecal material adherent to the perianal area. The corresponding stool score for transgenic fiber-treated rats was 1.8 ± 0.2 ($P<0.01$), as the stools were typically pale and contained mucus, but with a similar consistency to that of F-344 rats. The inflammatory status in HLA-B27 control rats was also observed macroscopically by colonic hyperaemia, dilatation and bowel wall thickening, as well as lymph node swelling, particularly in the ileocecal region. The colonic specimens from fiber-fed transgenic rats displayed similar characteristics, except one of the rats that showed no hyperaemia and another one without lymph node swelling.

The microscopic studies evidenced the intestinal anti-inflammatory effect exerted by dietary fiber (Fig. 1). Histological assessment of colonic samples from the transgenic control group revealed an increase in the bowel thickness with an extensive inflammatory cell infiltration, composed by neutrophils and mononuclear cells, and hiperplasia of crypt epithelial cells, with a score value of 3.4 ± 0.4. In addition, the goblet cells had emptied their mucin content in all specimens and the apical epithelial surface was necrosed in four of the six rats, caused by the inflammatory injury derived from the neutrophilic infiltration. However, histological analysis of the colonic specimens from transgenic rats treated with fiber revealed a preservation in the colonic cytoarchitecture when compared with controls, showing a score value of 1.8 ± 0.3 ($P<0.01$ vs HLA-B27 control group). Thus, fiber treatment attenuated the mucosal thickness and regenerated crypts, diminished oedema and the leukocyte infiltrate, which in turn was predominantly mononuclear and was restricted to the mucosa. The mucin content of the goblet cells was preserved in fiber-fed colitic rats and the mitotic activity was confined to the lower half of epithelium elongation in comparison with non-treated colitic rats.

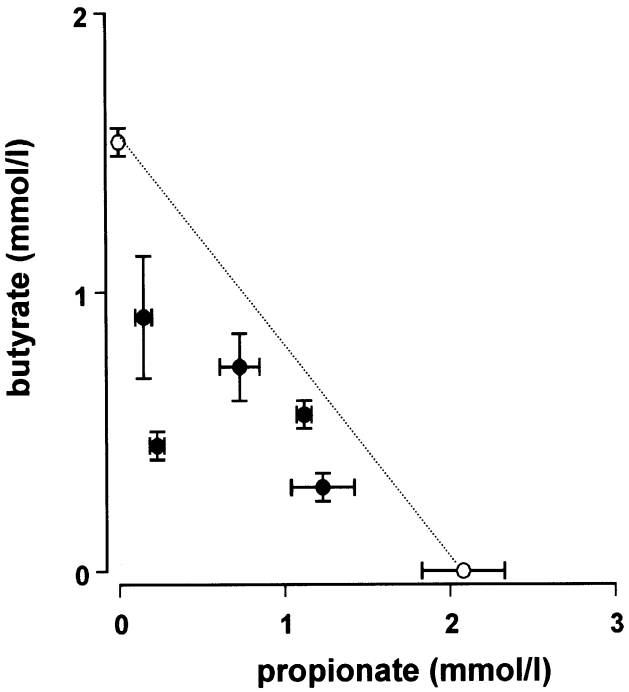

Fig. 1 Isobologram for the interaction between propionate and butyrate in inhibiting TNFα production in THP-1 cells when stimulated with LPS/PMA. Each solid circle represents the estimated concentration of SCFA present in the mixture when a 25% inhibition is achieved. The concentrations of pure propionate and pure butyrate inducing 25% inhibition are shown in open circles. The dotted line represents the line of additivity, and deviations below the line mean positive interactions. Results are expressed as means ± SEM. All points are significantly different ($P<0.05$) from the line of additivity (not shown).

The inflammatory status was also assessed biochemically by an increase in colonic MPO activity in comparison with F-344 rats (179 ± 19 vs 56 ± 10 U/g, $P<0.01$). MPO is a marker of neutrophil infiltration that has been reported to be enhanced in several experimental models of colitis, including spontaneous intestinal inflammation in HLA-B27 transgenic rats (6, 20). Colonic MPO activity in fiber-treated transgenic rats was similar to that obtained in the transgenic control group (176 ± 13 U/g, $P>0.1$). The colonic inflammation in HLA-B27 rats was also characterized by increased levels of colonic TNFα (Table 1), and LTB_4 (Table 1) as well as by a greater colonic NOS activity

Table 1 Nitric oxide synthase (NOS) activity, TNFα and LTB_4 levels in colon specimens from non-colitic F344 rats, HLA-B27 control colitic rats fed-control diet and HLA-B27 fiber colitic rats fed a fiber-supplemented diet (5% *Plantago ovata* seeds)

Group	NOS activity[1]	LTB_4(ng/g)	TNFα (pg/g)
F344 ($n=10$)	108.3±19.6	4.28±0.59	155.3±37.0
HLA-B27 control ($n=6$)	179.2±18.6**	11.41±1.78**	544.2±68.8**
HLA-B27 fiber ($n=6$)	110.9±23.9*	6.53±0.83*	298.3±22.1*

[1]NOS activity is expressed a pmol L-citrulline/mg protein-30 min. Data are expressed as mean±SEM.
*$P<0.05$ vs HLA-B27 control.
**$P<0.05$ vs F344.

(Table 1) in comparison with non-colitic animals. Treatment of colitic rats with fiber resulted in a significant reduction of colonic TNFα and LTB_4 levels and in lower colonic NOS activity compared to non-treated transgenic rats, resulting in a normalization when compared with non-colitic F-344 rats ($P>0.1$).

Effect of fiber supplementation on SCFA production in HLA-B27 transgenic rats

When the colonic contents from transgenic control rats were incubated for 24 h in the absence or in the presence of fiber, a significant reduction in the levels of butyrate was observed compared to non-colitic F-344 rats ($P<0.05$, Table 2), whereas propionate levels were not significantly modified as a consequence of the inflammatory process. However, the intestinal contents obtained from the transgenic treated rats showed greater butyrate production, after incubation with or without fiber for 24 h, and higher levels of propionate only when incubated in the presence of fiber for 24 h, than those from transgenic control rats ($P<0.05$, Table 2). No significant modification in acetate levels was observed in any of the groups studied (Table 2).

In vitro effects of propionate and butyrate on TNFα production

THP-1 cells, when incubated in the presence of LPS plus PMA resulted in a significant increased TNFα production (2.88±0.58 μg/l; $P<0.01$) over baseline (0.23±0.05 μg/l). Although propionate and butyrate, at any of the concentrations assayed, did not affect the basal production of TNFα by these cells (not shown), both SCFA showed a concentration-dependent inhibitory effect on TNFα production after stimulation of the cells with LPS and PMA. The maximum inhibitory effects of propionate and butyrate were 35±3% and 55±2%, respectively. The IC_{50} value of butyrate was 4.14±0.41 mmol/l, whereas the corresponding value for propionate was higher than 8 mmol/l (not determined).

When different propionate/butyrate mixtures (4:1, 2:1 1:1, 1:2 and 1:4) were incubated in the culture media, synergetic inhibitory effects on TNFα production were observed. To further analyze the interactions between both SCFA, isobolographic analysis were carried out from the curves obtained with the different concentration mixtures. The level of inhibition considered was 25% because propionate alone failed to inhibit more than 35% on the production of this cytokine at the higher concentration assayed (Fig. 2). The dotted line in Figure 2 represents the lines of additivity, so that deviations below this line mean positive interaction. It can be observed that all the points of the propionate/butyrate mixtures were significantly different from the line of additivity indicating that there were synergistic effects of both SCFA in inhibiting the production and/or release of TNFα in THP-1 cells. Of note, all the combinations assayed showed a significant increase in the maximum inhibitory effect on TNFα production in THP-1 cells, when compared with those obtained when both SCFA were assayed separately (Table 3).

Discussion

The present study supports previous studies that describe the beneficial effects of dietary fiber in intestinal inflammation, both in humans (18, 26, 27) and in experimental models of colitis (28–30). Thus, dietary supplementation of *Plantago ovata* seeds (5%) for 13 weeks to HLA-B27 transgenic rats resulted in a partial prevention of the development of the colonic inflammation that occurs in these rats (5). This anti-inflammatory effect was already evidenced in the course of the experiment since, after periodic evaluation of the stool

Table 2 Short-chain fatty acid (SCFA) production (μmol/g faeces) in the colonic contents obtained from non-colitic F344 rats, HLA-B27 control colitic rats fed-control diet and HLA-B27 fiber colitic rats fed a fiber-supplemented diet (5% *Plantago ovata* seeds) after 24 h incubation with or without fiber (10 g/l)

	Acetate		Propionate		Butyrate		Total SCFA	
	Without fiber	With fiber	Without fiber	With fiber	Without fiber	With fiber	Without fiber	With fiber
F344	1112±73	1562±196	195.8±11.1	253.2±37.8	98.5±4.5	168.3±19.6	1406±80	1983±151
HLA-B27 control	1364±337	991.2±36.1	260.0±17.4	178.7±10.1	47.5±6.6**	54.7±9.0**	1672±144	1295±193
HLA-B27 fiber	1162±89	872.2±49.8	297.5±59.5	298.2±37.2*	84.7±11*	109.5±13.7*	1545±99	1280±125

Data are expressed as mean±SEM. The production of each SCFA by colonic contents incubated with fiber was calculated as follows: total production of SCFA in the presence of fiber-production of SCFA in the absence of fiber.
*$P<0.05$ vs HLA-B27 control.
**$P<0.05$ vs Fischer 344.

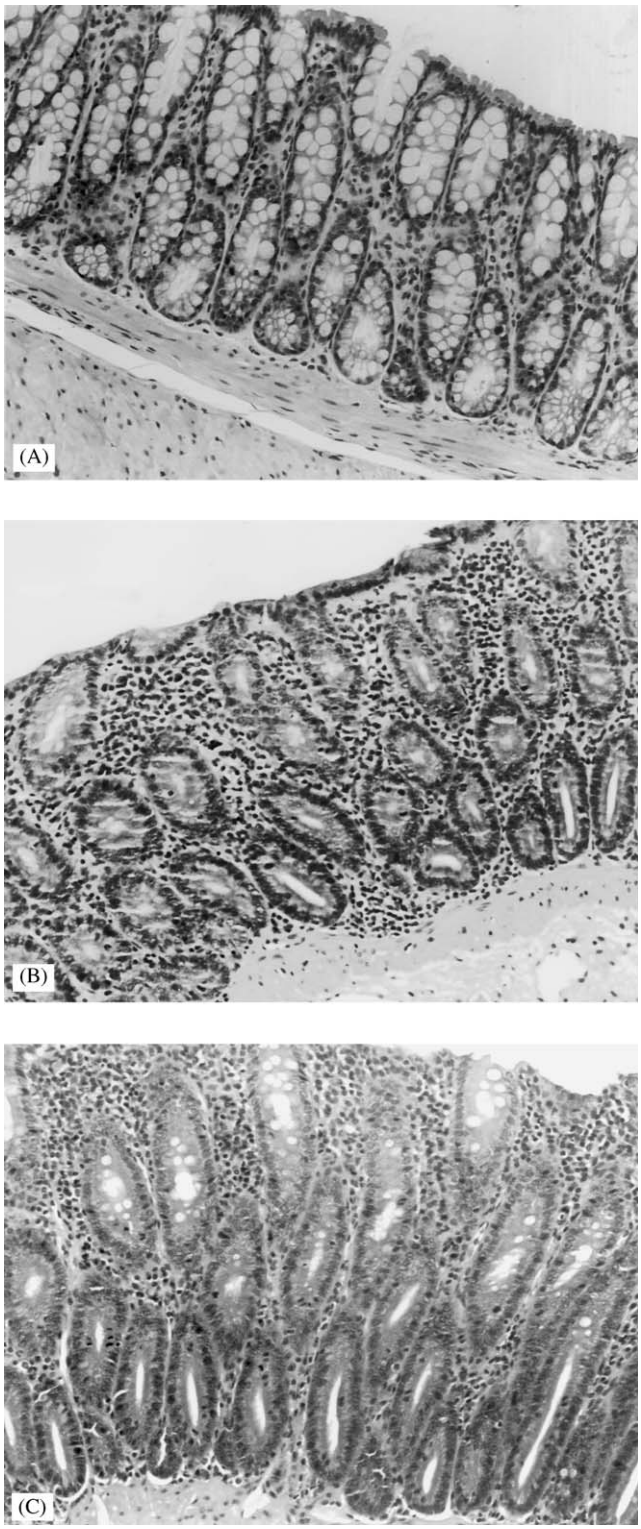

Fig. 2 Histological evidence of the anti-inflammatory activity of dietary fiber in HLA-B27 colitic rats (original magnification 200 ×). (A) Non-colitic Fischer 344 group showing the normal histology of the rat colon. (B) HLA-B27 control group showing dense inflammatory infiltrate of leukocytes in the lamina propria, with epithelial damage, necrosis of the surface cells, loss of mucin secretion and evident mitotic activity in the tubular glands. Erythrocyte extravasation and oedema is also observed. (C) HLA-B27 fiber-treated group showing amelioration in the inflammatory process with lesser infiltration of leukocytes, mainly mononuclear cells, reduction of oedema, preservation of mucin secretion and presence of goblet cells in the superficial portion of the glands and proliferative activity restricted to the bottom of the crypts.

Table 3 Parameters (IC_{25} and maximum percentage of inhibitory effect) of the inhibitory effects of propionate:butyrate mixtures on TNFα production in THP-1 cells stimulated with LPS plus PMA

Mixture	*n*	IC_{25} (propionate) (mmol/l)	IC_{25} (butyrate) (mmol/l)	E_{max} (inhibition)
4:1	5	1.23±0.19	0.30±0.05	59.9±1.5
2:1	5	1.12±0.06	0.56±0.03	71.1±1.2
1:1	6	0.73±0.12	0.73±0.12	71.7±0.9
1:2	6	0.23±0.02	0.45±0.02	73.7±0.6
1:4	5	0.15±0.01	0.91±0.22	75.2±1.3

IC_{25} (propionate) and IC_{25} (butyrate) reflect the estimated amount of propionate and butyrate, respectively, present in the mixture when a 25% inhibition in the TNFα production is achieved.

consistency, fiber-fed transgenic animals were assigned a lower stool score than non-treated transgenic rats during the 13 weeks that the experiment lasted. A previous study demonstrated a direct correlation between loose stools/diarrhoea and histopathological and biochemical evidence of active colitis in this model of spontaneously developing IBD (6). In fact, diarrhoea is a prominent symptom of IBD, which has been correlated with impaired barrier function that probably results in altered intestinal permeability (31, 32). This alteration in mucosal permeability probably facilitates the introduction of antigenic or infectious agents through the intestinal mucosa leading to an exacerbated immune response (2).

In the present study, dietary fiber from *Plantago ovata* seeds was incorporated to the standard diet, which contained cellulose in its composition as the main source of fiber. Previous studies have shown that cellulose, when incorporated in the rat diet, does not alter the normal evolution of the spontaneous inflammation developed in HLA-B27 transgenic rats. In fact, this type of fiber it is used as a negative control to compare the efficacy of different dietary strategies in intestinal inflammation (28). In consequence, although an interaction between both types of fiber cannot be ruled out, the contribution of cellulose to the beneficial effect exerted by the Plantago ovata supplemented diet may be of limited importance.

The intestinal anti-inflammatory effect exerted by dietary fiber was confirmed histologically after microscopic evaluation of colonic specimens once the rats were sacrificed. Thus, an amelioration of the histological lesions that characterize this experimental model of rat colitis was observed. Most of the samples from colitic-treated rats (5 of 6) showed a lower impact of the inflammatory process in comparison with non-treated transgenic rats. In this effect, dietary fiber treatment resulted in a preservation of the intestinal epithelium, as evidenced by the presence of mature goblet cells with increased mucin content and a lower oedema and granulocyte infiltration in comparison with non-treated colitic animals. Surprisingly, the intestinal anti-inflammatory effect was not associated with a decrease in MPO activity, a marker of neutrophil infiltration that

has been described to be upregulated in several experimental models of intestinal inflammation (6, 20). The lack of an inhibitory effect on this enzyme activity after fiber treatment may be explained in basis of the predominant cell types in the leukocyte infiltrate in this experimental model of rat colitis, which, given its chronic nature, consists largely of monocytes, lymphocytes and plasma cells, and small number of neutrophils and eosinophils (6, 8).

Different mechanisms can collaborate in the intestinal anti-inflammatory activity showed by dietary fiber supplementation in this model of rat colitis. During the last decade, it has become increasingly clear that chronic colonic inflammation is associated with enhanced NO production, mainly via inducible NOS (iNOS) activity, in both humans and experimental models, including the spontaneous intestinal inflammation in HLA-B27 transgenic rats (6, 33–35). Thus, inhibition of NO overproduction has been reported to be beneficial in IBD (7, 34, 36). The present study confirms these observations since the intestinal anti-inflammatory effect exerted by dietary fiber was associated with a significant inhibition of colonic NOS activity when compared to transgenic control rats. This could prevent, at least partially, the deleterious activity ascribed to NO when it is produced in high amounts by iNOS in these intestinal conditions. Other mechanisms involved in the anti-inflammatory effect observed after dietary fiber supplementation could be the reduction in colonic leukocyte infiltration, as evidenced histologically. Margination and extravasation of circulating monocytes markedly contribute to chronic injury in this model of inflammatory bowel disease. Different mediators participate in the recruitment and activation of these cells, including LTB_4 (37), and the inhibition in the synthesis and/or release of this mediator can result in a lower leukocyte infiltrate. The results obtained in the present study revealed that fiber treatment of colitic rats was associated with a decrease in colonic LTB_4 levels. As a consequence, an inhibitory effect on the synthesis and/or release of chemotactic substances can be considered as a mechanism involved in the intestinal anti-inflammatory effect of dietary fiber. Finally, the intestinal anti-inflammatory activity exerted by dietary fiber was characterized by a downregulation of TNFα, an important pro-inflammatory mediator that have been proposed to play a key role in colonic inflammation (38). In fact, different drugs capable of interfering with the activity of this mediator has been successfully developed for IBD therapy (39, 40).

Different studies have reported an impairment of colonic SCFA production, especially butyrate, in both human and experimental colitis (14, 15, 30). Actually, the results obtained in the present study revealed that intestinal inflammation in HLA-B27 transgenic rats is associated with a lower concentration of butyrate than in non-colitic animals. This may be the result of an alteration in the autologous luminal bacterial composition and load in these rats (41, 42), which may impair the metabolization of the fiber and the subsequent production of the different SCFA. When dietary fiber was administered to colitic rats, there was a significant increase in fecal butyrate and propionate levels, thus providing the energetic substrates to the colonocyte and resulting in prevention from inflammation. These results are in accordance with previous studies, both of humans suffering from ulcerative colitis and those using experimental models of rat colitis, which showed that the intestinal anti-inflammatory effect of dietary fiber supplementation was associated with an increase in SCFA production (18, 30, 43). In addition, it has been previously reported that butyrate decreases TNFα production by intestinal biopsies and by isolated lamina propria mononuclear cells via inhibition of nuclear factor-κB (NF-κB) activation and IκBα degradation (44). The inhibitory effect of butyrate on NF-κB activation has also been reported in HT-29 cells, probably derived from the ability to inhibit histone deacetylase activity via histone hyperacetylation, an activity also described for propionate (45–47). This may be of interest since NF-κB is a transcription factor that has been described to be activated in human IBD (48) and, in combination with others, plays a central role in regulating the expression of genes encoding iNOS as well as numerous cytokines in immune and inflammatory responses (49). The results obtained in the present study support the role of SCFA in the intestinal anti-inflammatory activity in this model of spontaneous colitis. First, in vivo experiments have shown the inhibitory effect of dietary fiber supplementation on NOS activity and TNFα production, together with an increased production of butyrate and propionate. Second, in vitro assays have confirmed the ability of both SCFA, butyrate and propionate, to inhibit the stimulated TNFα production in THP-1 cells. Similarly to previous reports with other cytokines, butyrate showed a higher potency and efficacy than propionate in the downregulation of cytokine production (46). In addition, we have also analyzed the interactions between butyrate and propionate using mixtures of different range of proportions of both SFCA. Using an isobolographic analysis, we found that most of the points of the butyrate/propionate mixtures were significantly different from the line of additivity when the analysis were performed at the level of 25% of inhibition for TNFα production, which indicated that there were interactions between both SCFA, showing the synergism of this SFCA combination. Furthermore, the combination showed a higher efficacy in inhibiting TNFα production than those obtained when both SCFA were assayed separately, and this may be of interest given the key role attributed to this cytokine as commented before.

In conclusion, dietary fiber supplementation prevents the development of inflammation in this model of

spontaneous colitis, an effect associated with an amelioration in the production of some of the mediators involved in the inflammatory response of the intestine, such as cytokines, including TNFα, LTB_4 and NO. This beneficial effect could be ascribed to the enhanced production of propionate and butyrate, which may act through the combination of different mechanisms. First, both are considered energetic substrates for the colonocyte, thus facilitating the restoration of the intestinal barrier and avoiding the entry of luminal agents that contribute to the maintaining of the exacerbated immune response. Second, these SCFA are able to synergistically modulate the immune response through downregulation of different mediators such as pro-inflammatory cytokines.

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