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RESEARCH PAPER

Bovine glycomacropeptide ameliorates experimental rat ileitis by mechanisms involving downregulation of interleukin 17

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Background and purpose: Bovine glycomacropeptide (BGMP) is an inexpensive, non-toxic milk peptide with anti-inflammatory effects in rat experimental colitis but its mechanism of action is unclear. It is also unknown whether BGMP can ameliorate inflammation in proximal regions of the intestine. Our aim was therefore two-fold: first, to determine the anti-inflammatory activity of BGMP in the ileum; second, to characterise its mechanism of action.
Experimental approach: We used a model of ileitis induced by trinitrobenzenesulphonic acid in rats. Rats were treated orally with BGMP and its efficacy compared with that of oral 5-aminosalicylic acid or vehicle, starting 2 days before ileitis induction.
Key results: BGMP pretreatment (500 mg kg^{-1} day^{-1}) resulted in marked reduction of inflammatory injury, as assessed by lower extension of necrosis and damage score, myeloperoxidase, alkaline phosphatase, inducible nitric oxide synthase, interleukin 1β, tumour necrosis factor and interleukin 17. These effects were generally comparable to those of 5-aminosalicylic acid (200 mg kg^{-1} day^{-1}). Neither compound affected the production of interferon γ, tumour necrosis factor and interleukin 2 by mesenteric lymph node cells isolated from animals with ileitis. The expression of Foxp3 was increased in ileitis and not reduced significantly by BGMP or aminosalicylate treatment.
Conclusions and implications: These results demonstrate that BGMP has anti-inflammatory activity in the ileum with similar efficacy to 5-aminosalicylic acid. The mechanism of action may involve Th17 and regulatory T cells and perhaps macrophages but probably not Th1 lymphocytes. Patients with Crohn's ileitis may benefit from treatment with BGMP.
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Keywords: glycomacropeptide; trinitrobenzenesulphonic acid; experimental ileitis; interleukin 17; inflammatory bowel disease; interleukin 1; regulatory T cells

Abbreviations: AP, alkaline phosphatase; IL-, interleukin; IL-1ra, interleukin 1 receptor antagonist; iNOS, inducible nitric oxide synthase; TFF3, trefoil factor 3; TNBS, trinitrobenzenesulphonic acid; TNF-α, tumour-necrosis factor; Treg, regulatory T cells

Introduction

Bovine glycomacropeptide (BGMP) or casein macropeptide is a biologically active peptide derived from the hydrolysis of milk κ-casein (Brody, 2000). This peptide is composed of 64 amino acids and contains varying units of *N*-acetylneuraminic (sialic) acid. BGMP is produced both physiologically as a result of the digestion of κ-casein in the stomach of neonates, and in the industry as a part of the whey produced during the cheese-making process. As a nutrient, BGMP has an excellent safety record and in accordance with this, a recent study has described that it is not immunogenic, because it fails to induce T-cell-specific responses in mice, even when given in polymerised form, in contrast with the parent protein (Mikkelsen *et al*., 2006). As a consequence, this peptide is used in the production of infant formulas and due to its low content on aromatic amino acids, including phenylalanine, BGMP has been proposed to be useful in the making of products for individuals with phenylketonuria

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(Nakai and Modler, 1999). Furthermore, BGMP is included in toothpastes because of its anticariogenic properties. In fact, it has antibacterial activity preventing dental caries and, at the same time, it prevents tooth demineralisation and promotes tooth enamel mineralisation (Aimutis, 2004).

Prebiotic and immunoregulatory effects have been attributed to glycomacropeptide. Thus, BGMP has been shown to promote the growth of bifidobacteria *in vitro* (Idota *et al*., 1994; Yakabe *et al*., 1994), although there is no definitive proof so far *in vivo* because the studies carried out with Rhesus monkeys and human infants were hampered by a high initial level of bifidobacteria (Bruck *et al*., 2003, 2006). In addition, it may combat infection by binding to lectins, viruses and mycoplasma (Brody, 2000). On the other hand, BGMP inhibits mouse splenocyte proliferation induced by lipopolysaccharides (Otani *et al*., 1995), suppresses IL (interleukin)-2 receptor expression in mouse $CD4^+$ T cells (Otani *et al*., 1996) and suppresses serum IgG antibody production by mouse lymphocytes (Monnai *et al*., 1998). Furthermore, it modulates the secretion of the IL-1 family of cytokines in a mouse monocytic cell line (Monnai and Otani, 1997) and enhances the proliferation and phagocytic activities of human macrophage-like cells (Li and Mine, 2004).

In a study carried out in our laboratory (Daddaoua *et al*., 2005), BGMP was found to be anti-inflammatory in a rat model of colitis induced by the administration of trinitrobenzenesulphonic acid (TNBS). This effect was comparable to that of sulfasalazine, a drug widely used to treat inflammatory bowel disease (Baumgart and Sandborn, 2007). This study indicated that BGMP could be beneficial in inflammatory bowel disease, a chronic and relapsing disease that significantly diminishes the quality of life of patients. Inflammatory bowel disease is comprised of two different but closely related conditions, namely ulcerative colitis and Crohn's disease (Sands, 2007). Ulcerative colitis affects the large intestine at the mucosal level, whereas Crohn's disease is characterised by transmural inflammation and may involve any segment of the gastrointestinal tract from the mouth to the colon, especially the ileum and colon (Baumgart and Carding, 2007).

The immune response in Crohn's disease has long been considered to be dominated by Th1 cells, based mainly on studies of colonic disease in humans and animal models. There is some evidence that both Th1 and Th2 cells may contribute to Crohn's ileitis (Desreumaux *et al*., 1997). In SAMP1/Yit mice, which develop spontaneous ileitis, inflammation appears to be initiated by Th1 cells but Th2 cells also play a role in later stages (Bamias *et al*., 2005). Interestingly, luminal bacteria are not required for the development of the disease, whereas experimental colitis is highly dependent on the presence of viable flora (Bamias *et al*., 2007). Furthermore, in models of adoptive transfer of naive ($CD4^+CD45RB^{high}$) T cells into immunodeficient recipient SCID or RAG KO mice, where naive T cells interact with antigen-presenting cells to become activated to a Th1 disease-producing phenotype, colitis with none or very mild ileal inflammation has been reported (Izcue *et al*., 2006). On the other hand, Dohi *et al*. (2003) transferred non interferon-producing Th cells (Th2) cells into T deficient mice and found that ileitis and not colitis was produced. Taken together, these results suggest that the immune cell responses involved in the inflammatory processes in ileitis and colitis may be different. Recently, the role of a novel T-cell subtype, Th17 cells, in a number of inflammatory diseases has been unravelled. At this point, the significance of Th17 cells in inflammatory bowel disease is suspected but unproven (Neurath, 2007).

Ileitis is not only observed in Crohn's disease but can also result from intestinal manifestations of several diseases or from ileal bacterial or nematode infections (Sands, 2004; Navarro-Llavat *et al*., 2007). In addition, ileitis is a frequent complication of the ileal pouch-anal anastomosis interventions practiced to treat ulcerative colitis (Alexander, 2007). The pharmacological therapy of intestinal inflammation is influenced by the segmental localisation of the lesion, which determines the use of particular galenic release forms or different drugs altogether. For instance, sulfasalazine is efficacious in the large intestine, whereas its active moiety 5-aminosalicylic acid (5-ASA) is used as such in case of small intestinal involvement (Baumgart and Sandborn, 2007). In the present study, we have used a model of ileitis induced by the administration of TNBS in rats (Sanchez de Medina *et al*., 2004) to test the hypothesis that BGMP could be useful in the treatment of ileitis. Our objective was twofold: first, to elucidate whether the intestinal anti-inflammatory effects of BGMP are maintained in the ileum. Second, to further explore the mechanism of action of this peptide. The results validate the intestinal anti-inflammatory activity of BGMP at the ileal level and suggest that it may work by down-regulating Th17 cells, maintaining regulatory T cells (Tregs) and probably inhibiting macrophages, although additional mechanisms may be operative, such as prebiotic or systemic actions.

Methods

Animals

All animal procedures in this study were 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). Female Wistar rats (175–225 g) obtained from the Laboratory Animal Service of the University of Granada were used, housed individually in makrolon cages and maintained in our laboratory in air-conditioned animal quarters with a 12-h light–dark cycle. Animals were provided with free access to tap water and food (Panlab A04, Panlab, Barcelona, Spain).

Induction of ileitis

Animals were fasted for 24 h and anaesthetised with halothane. Under these conditions, ileitis was induced, after a middle abdominal laparatomy, by the injection of TNBS in the ileal lumen, approximately 3 cm proximal to the caecum (Sanchez de Medina *et al*., 2004). Each ileitic rat received 10 mg of TNBS dissolved in 0.25 mL of 50% ethanol (v/v). Sham-operated rats received an equal volume of phosphate-buffered saline. Injection of ethanol by itself was shown to elicit only a short-lived inflammatory reaction in pilot

experiments, as described for TNBS colitis (Morris *et al*., 1989) and was not explored further (data not shown).

Experimental design
Rats were randomly assigned to four different groups ($n=$ 6–8): the control group (C), which did not receive the TNBS challenge and three more groups to which ileitis was induced (groups TNBS, 5-ASA and BGMP). The BGMP group received BGMP ($500\,mg\,kg^{-1}\,day^{-1}$ in 1% methylcellulose p.o.) starting 2 days before the TNBS challenge. The 5-ASA group received 5-ASA ($200\,mg\,kg^{-1}\,day^{-1}$ in 1% methylcellulose p.o.) in the same conditions, whereas group TNBS together with group C received the vehicle every day. The dose of 5-ASA is equivalent to $2.24\,g\,day^{-1}$ for humans on a body surface basis, in the high end of normal dosing. Animals were killed 7 days after the induction of ileitis.

Assessment of inflammation
The distal ileum (approximately 10 cm) was removed and placed on an ice-cold plate, 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 ileum was longitudinally opened and scored for macroscopically visible damage by an observer unaware of the treatment, according to the criterion previously proposed by us (Daddaoua *et al*., 2005), but slightly modified. The score was assigned as follows: adhesions (0–3), obstruction (0–2), hyperaemia (0–3), fibrosis (rigidity and deformation, 0–5), necrosis (0–5) and other features (proximal dilatation, fragility, scarring, 0–4). The ileum was subsequently divided in several longitudinal pieces for biochemical determinations. The fragments were immediately frozen in liquid nitrogen and kept at $-80\,^{\circ}C$ until used.

Biochemical determinations
Alkaline phosphatase (AP) activity was measured spectrophotometrically, using disodium *p*-nitrophenylphosphate (5.5 mM) as substrate in 50 mM glycine buffer with 0.5 mM $MgCl_2$ (pH = 10.5) Results are expressed as mU per mg of protein. Sensitivity to the AP inhibitor, levamisole (1 mM), was measured and results are expressed as per cent inhibition of AP activity (Sanchez de Medina *et al*., 2004). Myeloperoxidase (MPO) activity was measured according to the technique described by Krawisz *et al*. (1984), using 0.5% hexadecyltrimethylammonium bromide in phosphate-buffered saline (pH = 6.0) for tissue homogenisation and *o*-dianisidine dihydrochloride (0.5 mM) as chromogen. The results are expressed as MPO units ($\mu mol\,min^{-1}$) per gram of wet tissue.

Analysis of gene expression by reverse transcriptase-PCR analysis
The expression of IL-1β, IL-1 receptor antagonist (IL-1ra), tumour-necrosis factor (TNF), IL-17 and trefoil factor 3 (TFF3) was examined by reverse transcriptase RT-PCR. For RT-PCR analysis, total RNA was extracted with Trizol (Invitrogen, Barcelona, Spain). Five micrograms of RNA per sample were subjected to reverse transcription using the First-strand cDNA synthesis kit (GE Healthcare, Madrid, Spain). PCR amplification was performed using 2 μl of cDNA for a final PCR reaction volume of 25 μl. TAq polymerase was purchased from GE Healthcare. The expression of the ribosomal 18S unit was examined as a standard of loading. The primers of the amplified fragments were: IL-1β (sense 5′-AATGACCTGTTCTTTGAGGCTG-3′; antisense 5′-CGAGAT GCTGCTGTGAGATTTGAAG-3′); IL-1ra (sense 5′-GAGTCA GCTGGCCACCCTG-3′; antisense 5′-CAGACTTGACACAAG ACAGGCA-3′); tnf (sense 5′-TACTGAACTTCGGGGTGATTGG TCC-3′; antisense 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′); IL-17 (sense 5′-TTCTCCAGAACGTGAAGGTC-3′; antisense 5′-GGACAATAGAGGAAACGCAG-3′); TFF3 (sense 5′-ATGGA GACCAGAGCCTTCTG-3′; antisense 5′-ACAGCCTTGTGCTG ACTGTA-3′); ribosomal 18S unit (sense 5′-CCATTGGAGGG CAAGTCTGGTG-3′; antisense 5′-CGCCGGTCCAAGAATTT CACC-3′). To set up the PCR conditions, different amounts of colonic RNA from a pool of samples and different number of cycles were assayed (data not shown). The cycle numbers and hybridisation temperatures for each PCR were as follows: for IL-1β (32 cycles and 57 °C), IL-1ra (40 cycles and 57 °C), for TNF (32 cycles and 61 °C), for IL-17 (35 cycles and 51 °C), for TFF3 (25 cycles and 59 °C) and for ribosomal 18S unit (15 cycles and 60 °C). After the PCR amplification 10 μl of each reaction were resolved in 2.5% (w/v) agarose gels. Bands were quantitated with NIH software (Scion Image).

Western blots
The colonic levels of inducible oxide nitric synthase (iNOS), COX-2 and Foxp3 were determined by immunoblotting. Colonic samples were homogenised in lysis buffer (0.1% w/v SDS, 0.1% w/v sodium deoxycholate, 1% v/v Triton X-100 in phosphate-buffered saline) with protease inhibitors (1 mM 1,10-phenanthroline, 1 mM phenylmethylsulphonyl fluoride and $18\,mg\,l^{-1}$ 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 (10%), electroblotted to nitrocellulose membranes and probed with the corresponding antibodies overnight at 4 °C. The bands were detected by enhanced chemiluminescence (PerkinElmer) and quantitated with NIH software (Scion Image). After the transference of the samples to nitrocellulose membranes, equal loading was checked routinely by reversible Ponceau staining. The composition of the Laemmli buffer (5 ×) was: 312 mM SDS, 50% v/v glycerol, 1% v/v 2-mercaptoethanol, 22.5 mM EDTA trisodium salt, 220 mM Tris and traces of bromophenol blue (pH = 6.8).

Mesenteric lymph node cells
Mesenteric lymph nodes were extracted from the rats in the study using sterile techniques and dissected mechanically. The cells were incubated in Dulbecco's modified Eagle's medium supplemented with 10% v/v fetal bovine serum (Boëhringer Mannheim, Barcelona, Spain), $100\,mg\,l^{-1}$ streptomycin, $100\,000\,U\,l^{-1}$ penicillin and $2.5\,mg\,l^{-1}$ amphotericin B. The cells were cultured at 10^6 cells per mL and stimulated with concanavalin A. Cell culture medium was

collected after 24 h and assayed for cytokine content by commercial ELISAs (Biosource Europe, Nivelles, Belgium and BD Biosciences, Erembodegem, Belgium).

Statistical analysis

The results are expressed as mean ± s.e.m. Differences among means were tested for statistical significance by one-way analysis of variance and a posteriori least significance tests on preselected pairs. Nonparametric data (ileum damage score) were expressed as median (25–75% quartiles) and analysed by one-way analysis of variance on ranks followed by Dunn's tests. All the analyses were carried out with the SigmaStat program (Jandel Corporation, San Rafael, CA, USA). Statistical significance was set at $P<0.05$.

Materials

Except where indicated, all reagents and primers were obtained from Sigma (Barcelona, Spain). BGMP (BioPURE-GMP) was a kind gift from Davisco Foods International, Inc. (Eden Prairie, MN, USA). Product certificates of analysis indicated that BGMP content was 93% whereas fat and lactose contents were 0.5% and less than 1% respectively. The BGMP product also contained small amounts of β-lactoglobulin and α-lactalbumin (0–4 and 1–2% of total protein, respectively) and 4.0% minerals.

Results

As expected, the induction of ileitis produced anorexia and a significant body weight loss in the TNBS group (Table 1). Furthermore, a high damage score was assigned to the ileal specimens owing mainly to the presence of epithelial necrosis, ileal adherences and fibrosis, producing rigidity and obstruction. Intestinal fibrosis resulted in an elevated weight to length ratio, which did not reach statistical significance, possibly because the segment of ileum taken for analysis was too long. The administration of either BGMP or 5-ASA failed to prevent the anorexia produced by ileitis (data not shown). Body weight loss in the rats treated with either BGMP or 5-ASA was intermediate between that of the control and TNBS groups, without reaching statistical significance (Table 1). The benefits of BGMP administration were appreciated when macroscopic damage parameters were studied. Thus, the administration of BGMP reduced significantly the extent of necrosis and the intestinal damage score (Table 1). The latter was attributable to a favourable effect on adherences, necrosis, obstruction and fibrosis (data not shown). The ileal weight to length ratio was not modified significantly. These effects were comparable to those of 5-ASA, although the damage score in this group was still significantly different from that of the control, unlike BGMP.

The biochemical characterisation of the ileal inflammatory response confirmed the expected features of increased AP (leukocyte infiltration and epithelial cell stress), MPO (neutrophil infiltration), COX-2 and iNOS (Figures 1 and 2). In accordance with their anti-inflammatory effect, the administration of either BGMP or 5-ASA resulted in a normalisation of AP and MPO activities and a marked reduction in the expression of COX-2 and iNOS (the effect of BGMP on COX-2 did not reach statistical significance). We also studied the effect on the expression of TFF3, a peptide with epithelial-regenerating properties. TFF3 was upregulated in ileitis and BGMP treatment normalised its expression fully, whereas 5-ASA did not affect this parameter significantly (Figure 3). Thus TFF3 is unlikely to play a role in the mechanism of action of BGMP.

From an immunological point of view, TNBS ileitis was characterised by an increase in the expression of the proinflammatory cytokines IL-1β and TNF-α, as well as of the anti-inflammatory IL-1ra, a natural antagonist that opposes both IL-1α and IL-1β activities. BGMP and 5-ASA reduced the IL-1β, TNF -α and IL-1ra mRNA levels to the levels found for control rats (Figure 3). In an attempt to elucidate whether the effect of BGMP is mediated by actions on lymphocytes, we examined the behaviour of mononuclear cells isolated from mesenteric lymph nodes. We focused on Th1 signature cytokines, namely TNF -α, IL-2 and interferon-γ. As expected, the mesenteric lymph node cells isolated from rats affected by ileitis produced higher levels of proinflammatory cytokines when stimulated *in vitro*; however, neither BGMP nor 5-ASA showed any effect at this level, suggesting that the mechanism of action in both cases does not involve Th1 cells (data not shown).

Next we addressed the role of other T-cell subtypes, namely Tregs and Th17 cells. To this end, we measured Foxp3 levels by western blot and IL17 mRNA levels by RT-PCR in whole ileal tissue (Figures 2 and 3). The expression of Foxp3, a transcription factor that drives Treg phenotypic differentiation, was markedly increased in rats with ileitis, compared to control rats, indicating that Treg number or

Table 1 Effect of BGMP on body weight gain and intestinal macroscopic parameters

	Body weight gain (%)	*Damage score*	*Extent of necrosis (cm)*	*Ileal weight/length ratio (mg cm^{-1})*
C	−0.9 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	64.8 ± 3.4
TNBS	−10.5 ± 3.7^{+}	7.0 (3.5–9.5)$^{\dagger,+}$	1.6 ± 0.5$^{+,\dagger}$	112.2 ± 20.3
5-ASA	−5.0 ± 1.5	3.5 (2.0–6.0)$^{+,\dagger,*}$	0.3 ± 0.1*	101.1 ± 9.8
BGMP	−5.6 ± 1.8	4.0 (1.3–6.3)$^{\dagger,+,*}$	0.6 ± 0.4*	106.1 ± 27.9

Abbreviations: 5-ASA, 5-aminosalicylic acid; BGMP, bovine glycomacropeptide; C, control; TNBS, trinitrobenzenesulphonic acid.
The doses of 5-aminosalicylic acid and glycomacropeptide were 200 and 500 mg kg^{-1} day^{-1}, respectively. Body weight gain is expressed as per cent change from the start of the experiment. Values are means ± s.e.m., $n=6$–8.
$^{\dagger,+}P<0.05$ vs control (C) group; $^{*}P<0.05$ vs TNBS group.

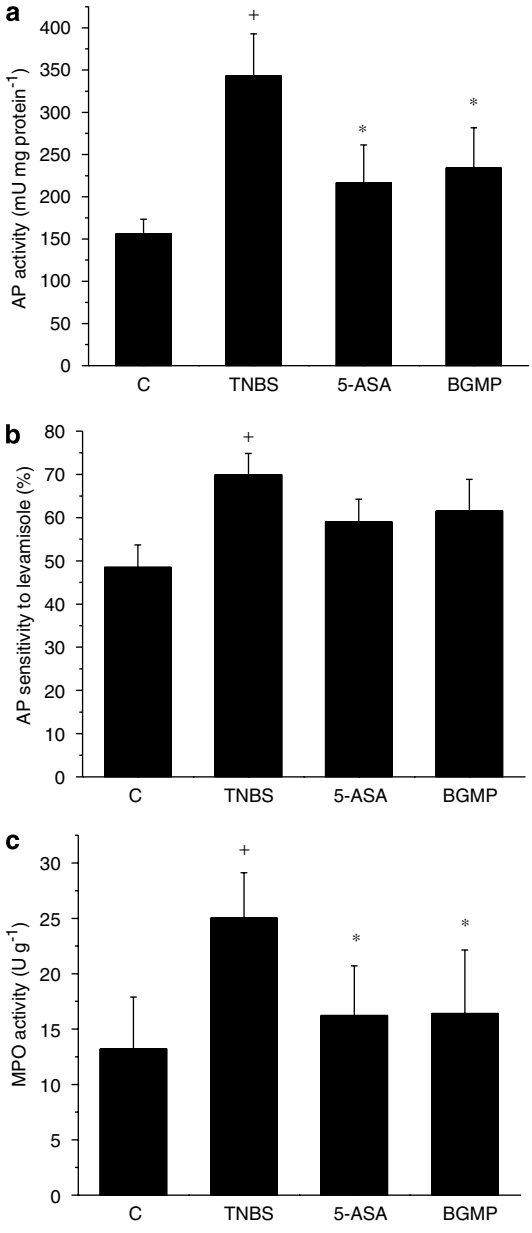

Figure 1 Effect of BGMP (bovine glycomacropeptide) on ileal AP (alkaline phosphatase) and myeloperoxidase (MPO) activity. (**a**) Ileal AP activity. (**b**) AP sensitivity to the inhibitor levamisole (1 mM). (**c**) Ileal MPO activity. Values are means ± s.e.m, $n = 6$–8. $^{+}P < 0.05$ vs control (C) group; $^{*}P < 0.05$ vs trinitrobenzenesulphonic acid group.

function is augmented as part of the inflammatory response. Foxp3 expression was not reduced significantly by treatment with either BGMP or 5-ASA and remained elevated compared to the control group, although the levels were intermediate between those of the C and TNBS rats. On the other hand, IL-17 mRNA, which is characteristic of Th17 cells, was markedly increased in the inflamed ileum, suggesting an involvement of this cell type in TNBS ileitis. Interestingly, the administration of either 5-ASA or BGMP normalised these parameters, suggesting that Th17 cells may be a target of both therapies.

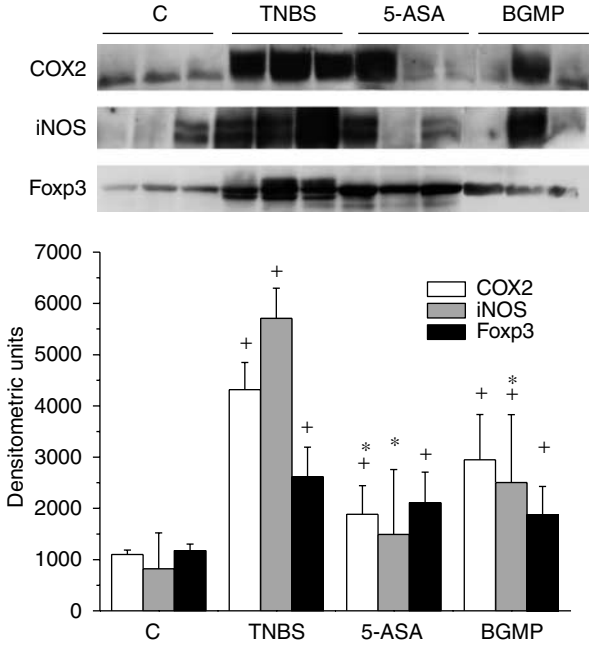

Figure 2 Effect of bovine glycomacropeptide on COX2, inducible nitric oxide synthase and Foxp3 protein levels. Ileal samples were analysed by western blot and quantitated with Scion Image software. Representative blots are shown. Values are means ± s.e.m., $n = 6$–8. $^{+}P < 0.05$ vs control (C) group; $^{*}P < 0.05$ vs trinitrobenzenesulphonic acid group.

Discussion

Bovine glycomacropeptide has been described as an inexpensive, nontoxic natural product with a number of biological properties. We have previously established the intestinal anti-inflammatory activity of BGMP in an experimental model of colitis (Daddaoua *et al.*, 2005). The beneficial effect of BGMP was found to be maximal when administered as a pretreatment at the dose of 500 mg kg^{-1}, that is, the same condition used in the present study and was comparable to the protection afforded by oral sulfasalazine. BGMP had a significant impact on the upregulation of IL-1β and iNOS brought about by colonic inflammation, but its mechanism of action was not characterised further. Thus, we designed the present study to confirm that BGMP can act as an intestinal anti-inflammatory treatment at the ileal, rather than the colonic, segment and to explore its mechanism of action further. It should be noted that the BGMP product assayed was not completely pure but included some minor components, namely β-lactoglobulin, α-lactalbumin and unspecified fatty substances. β-Lactoglobulin has no clearly identified biological function, although it seems to protect hydrophobic substances from gastric secretions (Madureira *et al.*, 2007). α-Lactalbumin appears to have some effects on cell division, which are concentration-dependent, and there is some evidence that it may influence B-cell response (Bounous and Kongshavn, 1985; Madureira *et al.*, 2007). Because their relative concentration is rather low (equivalent to that in milk, actually), it is unlikely that they play a role in the anti-inflammatory effect observed, but it certainly cannot be ruled out based on existing evidence. On the

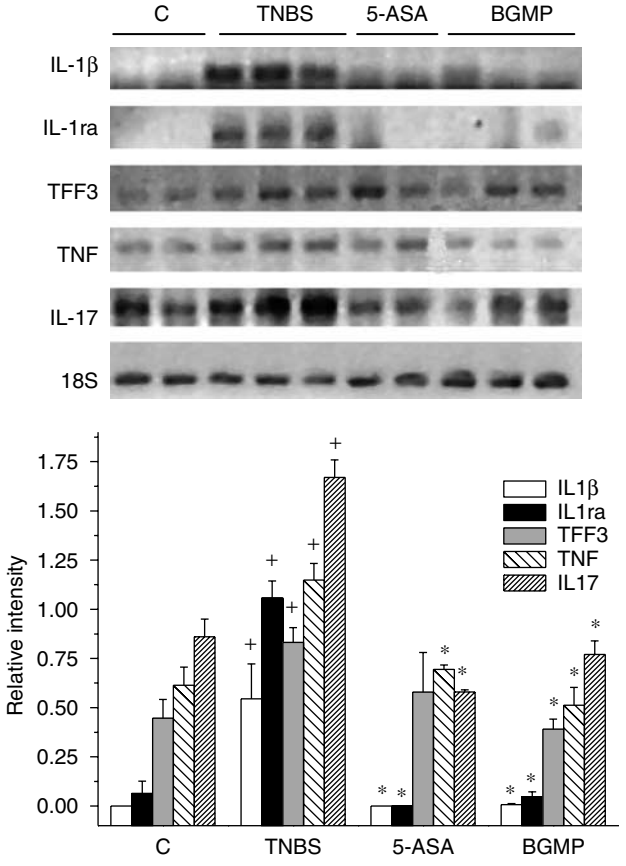

Figure 3 Effect of bovine glycomacropeptide on the ileal mRNA levels of IL (interleukin)-1β, IL-1ra, trefoil factor 3, tumour-necrosis factor and IL-17. mRNA levels were measured by semiquantitative RT-PCR and densitometric analysis with Scion Image software. 18S mRNA was used as reference. Densitometric analysis represents the ratio over the 18S signal. Representative blots are shown. Values are means ± s.e.m., $n = 6$–8. $^{+}P < 0.05$ vs control (C) group; $^{*}P < 0.05$ vs trinitrobenzenesulphonic acid group.

other hand, while fat content is uncharacterised, it should be noted that after purification, the product is maintained at 4 °C, conditions under which any active eicoisanoid products are extremely unlikely to survive.

Our data demonstrate that BGMP does have a significant therapeutic effect in the TNBS model of ileitis in the rat, as shown by the reduction in the extension of necrosis, damage score, MPO, AP and iNOS. We could not detect significant changes in the ileal weight to length ratio, possibly because we examined relatively long segments (~10 cm), which contained substantial amounts of normal tissue, thus 'diluting' the impact of inflammation. The anti-inflammatory effect of BGMP was generally comparable to that of 5-ASA, a drug used profusely in the treatment of ileal Crohn's disease. Interestingly, BGMP failed to combat anorexia and only counteracted body weight loss to a limited extent, while it did have beneficial effects in the colitis model. The reason for this discrepancy is unknown, although it might be related to the fact that TNBS colitis, but not ileitis, is accompanied by diarrhoea. Thus, it is possible that BGMP improves food intake/weight gain in colitis by reducing the deleterious effects of diarrhoea.

The effect of BGMP is linked to a marked reduction in TNF-α and IL-1β. These proinflammatory cytokines have a wide and largely overlapping array of biological effects, such as endothelial activation, monocyte stimulation, induction of acute phase proteins, etc. In particular, TNF-α is a known molecular target in human inflammatory bowel disease, although it is unclear whether the drugs that act by binding TNF–α work by cytokine neutralisation or by inducing apoptosis or targeted cell lysis in the cells expressing membrane-bound TNF-α (Gottlieb, 2007; Nesbitt *et al.*, 2007). One of the main sources of both TNF-α and IL-1β are monocytes/macrophages, suggesting that this cell type may be affected directly or indirectly by BGMP. BGMP has been reported to stimulate the proliferation and phagocytic activity of U937 monocytic cells, but no cytokines were measured (Li and Mine, 2004). We have preliminary evidence that BGMP evokes cytokine secretion by THP-1 cells, another human monocyte cell line, but the effect may differ in primary monocytes depending on tissue of origin (data not shown). Inasmuch as BGMP appears to have a stimulatory effect on monocytes/macrophages, the inhibition of TNF-α and IL-1β observed *in vivo* is most likely to be indirect. Of note, BGMP has been reported to increase IL-1ra (Monnai and Otani, 1997), an action that has potential anti-inflammatory impact. However, our data do not support that this is an important mechanism in this case, as IL-1ra levels are drastically reduced rather than increased.

Because BGMP has been also reported to exert modulatory effects on T cells (Otani *et al.*, 1995, 1996; Monnai *et al.*, 1998), we examined the expression of cytokines by mesenteric node cells isolated from the different experimental groups. We focused on Th1 signature cytokines, that is, interferon-γ, TNF-α and IL-2. Although there was a significant increase in the release of all three cytokines by concanavalin A stimulated lymphocytes from ileitis animals, neither BGMP not 5-ASA had any effect. Hence it is unlikely that any of these compounds modulates ileal inflammation through an action on Th1 cells.

Finally, we focused on other T-cell types, which may play a role in the pathophysiology of intestinal inflammation, namely Tregs and Th17 cells. Tregs can develop intrathymically or in peripheral tissues and are believed to be an essential safeguard to limit excessive inflammatory/immune responses (Izcue *et al.*, 2006). In fact, the transcription factor which drives the phenotypic conversion of naive T cells to Tregs, Foxp3, was originally identified as a defective gene in the so-called scurfy mice, which succumb early in life to dramatic autoimmune disease manifestations (Ochs *et al.*, 2007). The role of Tregs in inflammatory bowel disease has been insufficiently investigated to date, but the evidence available from animal models suggests that an imbalance in T helper/Treg cell function is sufficient to cause chronic intestinal inflammation (Fantini *et al.*, 2007). We have observed that TNBS ileitis is associated with a significant increase in Foxp3 immunoreactivity, suggesting that the inflammatory reaction is accompanied by augmented Treg cell function and/or number. Interestingly, Foxp3 levels remained significantly elevated after successful treatment with both BGMP and 5-ASA, that is, Treg cells are similarly present in treated and untreated rats despite the fact that

intestinal inflammation is actually tempered. Thus, Tregs may be induced by BGMP and 5-ASA and participate in their therapeutic effect. Alternatively, it is possible that Tregs are induced early in the inflammatory process and are not affected promptly by amelioration of the disease. Further studies are required to clarify this issue.

On the other hand, Th17 cells are emerging as a novel and important T-helper subtype that may be relevant to tissue damage in diseases formerly thought to depend on Th1 cells, like encephalomyelitis, rheumatoid arthritis and allergic airway hypersensitivity (Weaver *et al*., 2007). Th17 cells may be developed extrathymically by the exposure of T helper naive cells to IL-6/transforming growth factor-β (Bettelli *et al*., 2007). Mature Th17 cells are characterised by the production of IL-17, which in turn can activate signalling pathways that result in activation of NF-κB, induction of iNOS, COX-2, etc. Although IL-23 (involved in Th17 development/maintenance) and IL-17 are both increased in inflammatory bowel disease and IL23R appears to be a susceptibility gene, interventional studies that confirm its relevance are limited so far to animal studies (Neurath, 2007). Our data confirmed that IL-17 mRNA was increased in TNBS ileitis, consistent with a role of Th17 cells in this model. Interestingly, both BGMP and 5-ASA reduced IL-17 expression to control levels, indicating that the mechanism of action may involve interference with Th17 cells. Further experiments are required to test this hypothesis specifically.

In addition to the aforementioned direct mechanisms, BGMP may ameliorate ileal inflammation by prebiotic effects, as suggested by other investigators (Idota *et al*., 1994; Yakabe *et al*., 1994; Brody, 2000). We could not obtain suitable samples for bacterial measurements because of the localisation of intestinal inflammation in this particular study, but we plan to assess this hypothesis in future experiments using faecal samples in a colitis model. Previous animal and human studies *in vivo* have failed to show a substantial impact of BGMP on intestinal flora, but they were hampered by high bifidobacterial counts before dietary intervention (Bruck *et al*., 2003, 2006). Interestingly, in one of these studies, BGMP was shown to augment circulating neutrophils and to partially prevent diarrhoea induced by enteropathogenic *Escherichia coli* (Bruck *et al*., 2003). This observation is consistent with Zimecki *et al*. (2006), who recently reported that parenteral BGMP has protective effects against experimental bacteremia in mice, which were associated to an increase in circulating granulocytes. Thus it is possible that the anti-inflammatory effect of BGMP involves systemic actions. Little is known about the pharmacokinetics of BGMP, but two studies have previously shown that it reaches the bloodstream in significant amounts ($\sim 1\,\mu g\,mL^{-1}$) and the concentration remains relatively stable for at least 8 h, in addition to being detected in the duodenum shortly after oral intake (Chabance *et al*., 1995, 1998). Therefore, the intestinal anti-inflammatory effect observed may be due to luminal and/or systemic actions.

In summary, we have demonstrated that BGMP is an intestinal anti-inflammatory agent in the ileal segment with an efficacy similar to that of 5-ASA, using a preclinical model. The mechanism of action is related to inhibition of IL-1β and TNF and possibly a downregulation of Th17 cells. Treg cells may be also involved. Additionally, BGMP may exert prebiotic and/or systemic actions. BGMP may be a valuable tool in the therapy of Crohn's ileitis.

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Conflict of interest

The authors state no conflict of interest.

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