Bovine Glycomacropeptide Has Intestinal Antiinflammatory Effects in Rats with Dextran Sulfate-Induced Colitis1–3

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Abstract

Milk κ-casein-derived bovine glycomacropeptide (GMP) has immunomodulatory and bacterial toxin-binding effects, and it has been shown to exert intestinal antiinflammatory activity in the trinitrobenzenesulfonic acid-induced model of colitis. However, its mechanism of action is not well characterized, and it is not known whether GMP is effective in other experimental models. The intestinal antiinflammatory activity of GMP was assessed in the dextran sulfate sodium (DSS)-induced model of rat colitis. DSS was applied at a starting concentration of 5% (wt:v) in drinking water and adjusted when the disease activity index (DAI) increased substantially for 10 d. There were 3 experimental groups: control (no inflammation), DSS, and GMP (GMP-treated rats with DSS-induced colitis). GMP pretreatment (500 mg · kg−1 · d−1, starting 2 d before DSS treatment) reduced the DAI by 60% and lowered the colonic damage score by 44% (P < 0.05). GMP fully normalized the colonic expression of interleukin (IL) 1β, IL17, IL23, IL6, transforming growth factor β, IL10, and Foxp3 as assessed by quantitative RT-PCR. The production of interferon-γ by mesenteric lymph node cells ex vivo was also normalized by GMP treatment. In contrast, GMP did not change colonic thickening, myeloperoxidase, cyclooxygenase 2, or alkaline phosphatase. Histology analysis showed better preservation of the epithelium and attenuated infiltration and submucosal thickening in rats treated with GMP. We conclude that GMP exerts intestinal antiinflammatory activity in this model, which may be primarily related to actions on Th1 and Th17 lymphocytes and perhaps macrophages. J. Nutr. 140: 2014–2019, 2010.

Introduction

Inflammatory bowel disease (IBD), comprising ulcerative colitis and Crohn’s disease, is a chronic relapsing condition of the intestine that causes a marked deterioration of the quality of life of patients and which has a substantial (and increasing) prevalence (1,2). Despite an intense investigative effort, the etiology of IBD remains unknown, but it is thought to involve an interplay of genetic, environmental, microbial, and immunological factors. Because intestinal inflammation generally cannot be elicited in vivo in experimental animals reared in germfree conditions (but it develops normally in specific pathogen free conditions), IBD probably represents an uncontrolled and exacerbated response to luminal antigens that are innocuous for the normal population. Thus, intestinal inflammation would be the culmination of a cascade of events and processes initiated by antigens due to inadequate handling by the host’s immune system. These processes have been long thought to be related to augmented adaptive immunity responses, but it has also been proposed that a defect in innate immunity may paradoxically underlie the etiology of IBD (3–6). The reasoning in the latter scenario is that if the initial response to a bacterial challenge fails to contain bacterial infiltration of the mucosa, a subsequent, more robust reaction must come into play, giving rise to inflammation. Whatever the exact mechanism, IBD is regularly managed pharmacologically with drugs that downregulate the immune system such as corticoids, infliximab, aminosalicylates,
or azathioprine. All of these agents have a plethora of serious adverse effects that limit their application and they are not effective in all patients. Hence, the search for new treatments with a low intensity of adverse effects is much warranted (7).

Bovine glycomacropeptide (GMP), also referred to as casein macropeptide, is a 64-amino acid peptide that contains varying amounts (0–5 units) of N-acetylenearminic (sialic) acid. This peptide results from the enzymatic hydrolysis of milk κ-casein in the bovine stomach due to the action of chymosin (pepsin in humans) (8). In addition, GMP is present at 10–15% in milk whey as a result of the action of the same enzyme during the cheese-making process. Therefore, there is a substantial natural exposure to this peptide. GMP has nutritional interest, because its amino acid profile is high in branched-chain amino acids and lacks aromatic amino acids, therefore being one of the few naturally occurring proteins safe for individuals with phenylketonuria and perhaps useful in the management of some liver diseases (9,10). A number of biological activities have been also ascribed to GMP. Thus, there is some evidence that GMP may modulate colonic microflora (11,12). Some authors have proposed that GMP may combat infection by binding to lectins, viruses, and mycoplasma (8). In particular, we (13) and others (14) have noted the stimulatory effect of GMP on macrophages in terms of cytokine production and phagocytic activity. GMP may interfere with interleukin (IL) 1β receptor binding (15). However, GMP has been described to inhibit the proliferation of splenocytes and Peyer’s patch cells (16). We have recently observed that GMP inhibits interferon (IFN)-γ release by rat splenocytes by blocking STAT4 activation. Conversely, the production of IgG by mouse B-lymphocytes seems to be increased by GMP (17). Taken together, these data suggest that GMP may constitute an excellent agent to treat IBD, in that it appears to boost innate immunity but block T cell-driven adaptive immunity. Indeed, we have established the intestinal anti-inflammatory activity of GMP in trinitrobenzenesulfonic acid (TNBS)-induced colitis and ileitis (18,19). We performed this study to obtain confirmatory evidence in a separate model of colonic inflammation and characterize further the mechanism of action of GMP.

Materials and Methods

Reagents. Except where indicated, all reagents and primers were obtained from Sigma. Retrotranscription was achieved with the iScriptTM cDNA Synthesis kit and iTm Sybr Green Supermix was used for amplification (Bio-Rad). Antibodies were purchased from Cayman Technologies and Sigma. GMP (BioPURE-GMP) was the kind gift of Davisco Foods International. According to the manufactures, the GMP content was 93% and fat and lactose contents accounted for 0.2% and <1%, respectively.

Rats. Female Wistar rats (175–225 g) from Harlan were used, housed in makrolon cages (up to 7 rats/cage), and maintained in our laboratory in air-conditioned animal quarters with a 12-h-light/-dark cycle. Rats were provided with free access to tap water and food (Panlab A04, Panlab) (20). This 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 and experimental design. Colitis was induced by adding dextran sulfate sodium (DSS), obtained from ICN Biomedicals, to drinking water for 10 d (21,22). We selected the conditions to achieve a mild to moderate degree of colitis by starting with 5% (wt/v) of DSS and lowering the concentration to 4 or 3% whenever the disease activity index (DAI) increased substantially to prevent death. The status of the rats was monitored by general examination and specifically by means of the DAI, a combined score for weight loss, diarrhea, and hematochezia, which are 3 main signs of pathology in this model (23).

Rats were randomly assigned to 3 different groups. The control group (n = 5) did not receive DSS and was administered 1% methylcellulose daily by means of a gastroesophageal catheter. The remaining rats drank DSS-supplemented water and received by gavage either 500 mg · kg⁻¹ · d⁻¹ of GMP (GMP group, n = 7) or vehicle (1% methylcellulose, DSS group, n = 7). Treatment started 2 d before DSS supplementation and was maintained until rats were killed by cervical dislocation 10 d after DSS was started. A small blood sample was drawn from the tail vein before rats were killed under halothane anesthesia for subsequent determination of the leukocyte formula (Giemsa staining).

Assessment of colonic damage. The status of the large intestine was evaluated as previously described (24,25). Myeloperoxidase and alkaline phosphatase (AP) activities were measured spectrophotometrically as described (22) and expressed as U · g⁻¹ and mU · mg protein⁻¹, respectively. In addition, the sensitivity to the AP inhibitor levamisole was assessed. The colonic levels of cyclooxygenase 2 (COX2) were determined by immunoblotting as described (20).

Analysis of gene expression by RT-PCR analysis. Total RNA was obtained by the Trizol method (Invitrogen) and 1 μg retrotranscribed and specific RNA sequences amplified with a Stratagene MX3005P real-time PCR device using the following primers: AAT GAC GTG TTC TTT GAG GCT G / CGA GAT GCT GCT GTG AGA TTT (IL1α), TAC TGA ACT TCG GGG TGA TTG / CAG CCT TGG CCC TIG AAG AGA [tumor necrosis factor-α (TNFa)], CTG CTT GGC TTC TAG TGC TGA CGA GAA CCC AAG AAA AAC AGC AGC AAC CTG (Foxp3), AAT GAG CCA GAC CCA CAT G / TGC ACC GCC TGT TTG (IL8), TGG ACT CTG AGC CGC AAT GAG G / GAC GCA TGG CGG ACA ACG GAG (IL17), GCA CAC TAG CCT GGA GTG CA / AGA TGT CCG AGT AGC GCA GC (IL23 p19), CCT GTC GTC TTC AGG TCG TCC G / TGG TAT GGT CTT GCT CCT TAG CC (IL6), ACT GGC GAG CTT TAG TTT G / CGT GGC TTC TAG TGC TGA CG [transforming growth factor-β (TGFβ)], and CCA TTT GAG GAG AAC TCT GGT G / CGG CGG TCC AAG AAT TCT ACC (18S). Results are expressed as 2⁻ΔΔCT using 18S as reference gene.

Mesenteric lymph node cells. Mesenteric lymph nodes (MLN) were extracted from the rats using sterile techniques and dissected mechanically. The cells were incubated in RPMI-1640 medium supplemented with 0.05 mmol · L⁻¹ mercaptoethanol, 10% v·v⁻¹ fetal bovine serum (Boehringer Mannheim), 100 mg · L⁻¹ streptomycin, 100 κU · L⁻¹ penicillin, and 2.5 mg · L⁻¹ amphotericin B. The cells were cultured at 10⁶ cells · L⁻¹ and stimulated with concanavalin A (ConA). Cell culture medium was collected after 48 h and assayed for cytokine content by commercial ELISA (Biosource Europe and BD Biosciences).

Statistical analysis. The results are expressed as mean ± SEM. Data were analyzed by 1-way ANOVA and post hoc significant difference tests. All the analyses were carried out with the SigmaStat program (Jandel). Statistical significance was set at P < 0.05.

Results

The supplementation of drinking water with DSS caused significant colonic damage, as reflected in the increased DAI, beginning at d 5 after DSS introduction (Fig. 1A) and the augmented colonic damage score and weight:length ratio (Table 1). Pretreatment of rats with 500 mg · kg⁻¹ · d⁻¹ of GMP daily partially prevented DSS-induced colitis, as evidenced by a significantly lower DAI from d 7 onward (Fig. 1A). Body weight was reduced by colitis and was not affected by GMP (Fig. 1B). In addition, compared with controls, DSS-treated rats exhibited anorexia, particularly after d 5 (9.1 ± 0.4 vs. 17.3 ± 0.5 g · d⁻¹; P < 0.05). This was slightly but significantly improved in the GMP group (11.7 ± 0.4 g · d⁻¹), which differed from both other

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groups \((P < 0.05)\). Water intake did not differ among the groups (data not shown).

Neutrophil recruitment to the inflammatory site was apparent in histological sections of the DSS group (Supplemental Fig. 1), but it did not increase colon MPO activity, which did not differ from controls (Fig. 2A). Histological analysis of DSS-treated rats also revealed a marked deterioration of the epithelial layer, with moderate submucosal thickening (Supplemental Fig. 1A–C). Samples from the GMP group had a lower damage score (Table 1), derived mainly from less marked fibrotic features (adhesions, deformation). The colonic weight:length ratio was intermediate between those of the control and DSS groups, so they did not significantly differ from either (Table 1). GMP-treated rats had a better preserved epithelium and a less prominent infiltrate and submucosal enlargement (Supplemental Fig. 1D,E).

Colon AP activity, a marker of intestinal inflammation and epithelial stress, was significantly greater in the DSS group than in the control group (Fig. 2B). In addition, its sensitivity to levamisole was higher than in the controls, consistent with the isoform shift previously described in enterocytes (22). Colonic AP activity tended to be lower in the GMP group than in the DSS group (Fig. 2B; \(P = 0.09\)), but sensitivity to levamisole was unaffected (Fig. 2C).

This model of colitis is associated with a Th1 immune response, and, accordingly, ConA-stimulated mesenteric node cells of the DSS group had higher IFN\(\gamma\) and IL2 secretions than

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**TABLE 1** Morphological indicators of inflammation in control rats and in those with DSS-induced colitis treated with 500 mg \(\times\) kg \(^{-1}\) d \(^{-1}\) of GMP or vehicle.

<table>
<thead>
<tr>
<th></th>
<th>Colonic damage score</th>
<th>Colon weight:length ratio</th>
<th>Spleen weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>arbitrary units</td>
<td>mg cm (^{-1})</td>
<td>mg</td>
</tr>
<tr>
<td>Control</td>
<td>0 ± 0(^a)</td>
<td>73.4 ± 5.7(^a)</td>
<td>539.8 ± 34.5(^a)</td>
</tr>
<tr>
<td>DSS</td>
<td>5.9 ± 0.8(^b)</td>
<td>105.9 ± 7.1(^b)</td>
<td>807.7 ± 85.0(^b)</td>
</tr>
<tr>
<td>GMP</td>
<td>3.3 ± 1.0(^ab)</td>
<td>92.5 ± 5.0(^ab)</td>
<td>680.0 ± 53.9(^ab)</td>
</tr>
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\(^a\) Values are means ± SE, \(n = 7\) or 5 (control). Means without a common letter differ, \(P < 0.05\).
the controls. Secretions of TNFα and IL4 were not significantly affected (Fig. 3; data not shown). Mesenteric node cells from GMP-treated rats had a fully normalized IFNγ secretory response to ConA, and IL2 secretion also did not differ from controls (Fig. 3). The spleen of DSS-treated rats was significantly enlarged compared with the controls and those of the GMP group were of intermediate size (Table 1). DSS-treated rats did not differ from controls in peripheral blood counts (data not shown) or in colonic COX2 expression (Fig. 4). The blood cell formula and the colonic levels of COX2 in the GMP group were similar to those of the DSS group (data not shown; Fig. 4). In the DSS group, colonic mRNA levels of IL1β, IL23, IL6, TGFβ, IL10, Foxp3, and especially IL17 were all significantly greater than in the controls (Fig. 5). In contrast, TNFα did not differ (not shown). GMP had a major effect on the colonic expression of a variety of inflammatory markers, including IL1β, IL6, IL23, and especially IL17 (Fig. 5). The Treg marker Foxp3 and the antiinflammatory cytokines IL10 and TGFβ also were fully normalized, indicating a therapeutic response.

Discussion

Our data demonstrate that GMP pretreatment reduces the severity of DSS colitis in rats at the dose of 500 mg·kg⁻¹·d⁻¹. This dose is roughly equivalent to 5 g for an adult human (on a body surface basis), an amount that cannot be easily achieved by milk consumption but that is easily attainable as a functional food or a drug. We previously established the intestinal anti-inflammatory activity of this peptide in the TNBS-induced model of colitis (19) and also in TNBS ileitis (18). In these experiments, GMP was more effective as a pretreatment than as a post-treatment and its effect was associated with a lower expression of IL1β and inducible nitric oxide synthase as well as of MPO and AP activities. In experimental ileitis, GMP did not have any clear effect on cytokine production by MLN cells ex vivo, but it did decrease IL17 and TNFα, suggesting that GMP may act via modulation of Th17 but not Th1 cells. Thus, the mechanism of action of GMP needs further clarification. On the other hand, the therapeutic effect of this peptide, combined with its very low toxicity, makes it a good candidate for clinical application, but testing in humans requires extensive preclinical evidence of activity. Thus, we set out to test GMP in other models of IBD. Because there is no ideal model of IBD (26), establishing therapeutic efficacy in different types of experimental intestinal inflammation represents a good approach to this problem. Furthermore, because there are substantial differences in the mechanism of colitis induction and in the pathology of the different models, relevant conclusions as to the mode of action of the agent tested may be reached. For instance, the flavonoid luteolin has completely different effects in DSS-induced colitis and IL10⁻/⁻ colitic mice, being deleterious in the former and...
protective in the latter (27). These disparate results might be explained by the inhibitory action of the flavonoid on the nuclear factor (NF)-κB pathway in the epithelium, which may compromise the defense of the mucosa toward epithelial disruption by DSS. Indeed, DSS is thought to elicit intestinal inflammation by slowly altering the epithelial integrity, augmenting permeability, and ultimately resulting in an immune reaction against luminal antigens (28). In contrast, TNBS acts as a hapten by a delayed hypersensitivity mechanism, with added oxidative stress (29,30). Therefore, the mechanisms are quite different in both models and the epithelium may play a more important role in DSS-induced than in TNBS-induced colitis.

In this study, we selected the conditions to produce a mild to moderate colitis compared with the extensive damage (including necrosis) associated with TNBS-induced colitis (19,24,29). This in turn resulted in a somewhat high variability in the magnitude of colitis. This is evident in the dispersion in MPO and COX2, as well as colonic cytokines, in the DSS group. The advantage of using a mild to moderate colitis is that it is more amenable to treatment, but the downside is that variability may make it more difficult to detect significant differences. Nevertheless, GMP had a dramatic effect on most inflammatory markers, including the DAI, colonic damage score, IL17, IL23, IL1β, IL6, TGFβ, IL10, and Foxp3, most of which were fully normalized by the treatment. The effect on AP was much less pronounced. AP is increased in the inflamed intestine as a result both of leukocyte infiltration and of an increase in epithelial enzyme expression, which is also associated with a change of isoform that can be detected by a higher sensitivity to inhibition by levamisole (22). COX2, however, is expressed by various cell types, one of the most important of which is enterocytes (31,32). Because the primary effect of DSS is on the epithelium, as discussed above, these results are consistent with a mode of action of GMP that does not involve enterocytes, because AP and COX2 are downregulated in the TNBS models (18,19). Indeed, preliminary data obtained with intestinal epithelial cell lines support this hypothesis.

We have recently reported that GMP stimulates macrophage function via mitogen-activated protein kinase and NF-κB (13). Although the immediate consequence of this action would be to increase proinflammatory cytokines in the mucosal milieu, the pathology of IBD is more complex than that. It has been shown, for instance, that conditional suppression of NF-κB signaling in epithelial cells spontaneously produces severe colonic inflammation (3). Similarly, granulocyte-macrophage colony-stimulating factor appears to dampen rather than stimulate inflammation (4), and the absence of monocytes and dendritic cells aggravates rather than ameliorates experimental colitis (5,6). Thus, recent evidence suggests that a defective response to proinflammatory stimuli may actually aggravate the outcome, possibly because it impairs a prompt resolution, triggering a more robust reaction to a normally trivial challenge. Hence, macrophages may constitute a relevant target of GMP in its therapeutic effect.

Lymphocytes are also affected by GMP (16,17). Our unpublished results (F. Sánchez de Medina, A. Daddaoua, R. González, P. Requena, A. Zarzuelo, M.D. Suárez, O. Martínez-Augustin) suggest that GMP may directly inhibit IFNγ production by rat splenocytes. In the present study, GMP treatment was associated with a dramatic effect on IFNγ release by MLN cells, again supporting the hypothesis that GMP may act on Th1 cells. We could not measure IL17 in these cells because of a lack of antibody, but its mRNA levels, along with those of IL23, were also comparable to those of the control group in the colonic tissue, consistent with our previous observations (18). Of note, MLN cells also exhibited a lower IL2 output, such that it was not significantly different from that of noncolitic rats, which would be expected if our hypothesis is correct. The fact that IL10 and TGFβ, cytokines associated with Treg immunosuppression, were greatly diminished by GMP treatment may simply represent the result of overall tissue improvement. Thus, in a genomic study by our group of TNBS-induced colitis in rats genes with established or presumed antiinflammatory or immunosuppressive function were generally downregulated as colitis resolved (25). Although this does not rule out a role of Treg cells in the GMP effect, this mechanism is certainly not supported by the data from this study.

It is quite clear, however, that TNFα was not upregulated in this experiment. Because this cytokine has been reported by other authors to be increased in this model and considering the relatively low increase of IFNγ in MLN cells, it is likely that the mild degree of inflammation accounts for this discrepancy (33,34). However, our data argue against a major role of TNFα in DSS-induced colitis. In fact, TNFα might even be protective in this model, because DSS-induced colitis has been reported to be aggravated in TNFα−/− mice (35).

We have also performed a microarray study on the mechanism of action of GMP in the TNBS model, which suggests that the IL6 pathway is a major target of GMP in the inflamed intestine (F. Sánchez de Medina, A. Daddaoua, R. González, P. Requena, A. Zarzuelo, M.D. Suárez, O. Martínez-Augustin, unpublished data). In fact, because Th17 cell development is elicited by the combination of IL6 and TGFβ, both of which are dramatically lowered by the peptide, the modulation of Th17 by GMP may be indirect. Consistent with this, IL6 was completely normalized in the present study and has been unequivocally confirmed to be involved in DSS pathology (36).

Because it has been suggested that GMP modulates white blood cell populations (11), we examined the percentages of leukocytes in all groups. However, there was no effect of either DSS-induced colitis or GMP on this variable, as there were no significant differences among groups. Thus, this mechanism can be ruled out by the present experiment.

In conclusion, GMP has substantial intestinal antiinflammatory effects in DSS-induced colitic mice. The mechanism is probably related to actions on macrophages and Th1/Th17 lymphocytes but not epithelial cells. More experiments are required to fully delineate the mechanistic aspects of GMP action and to introduce this nontoxic agent in clinical practice.

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Literature Cited


