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Oral administration of antigens from intestinal flora anaerobic bacteria reduces the severity of experimental acute colitis in BALB/c mice

E. F. VERDÙ, P. BERCIK*, B. CUKROWSKA, M. A. FARRE-CASTANY, H. BOUZOURENE†, E. SARAGA†, A. L. BLUM‡, I. CORTHÉSY-THEULAZ‡, H. TLASKALOVA-HOGENOVA & P. MICHETTI§ *Institute of Microbiology, Academy of Sciences of the Czech Republic and *Department of Internal Medicine, Postgraduate Institute of Gastroenterology, Central Military Hospital, Prague, Czech Republic, †Pathology Institute and ‡Division of Gastroenterology, CHUV, Lausanne, Switzerland, and §Division of Gastroenterology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA*

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SUMMARY

Homeostasis between indigenous intestinal flora and host response may be broken in inflammatory bowel disease. The present study explores whether repeated oral administration of intestinal flora antigens can protect mice against dextran sodium sulphate (DSS)-induced colitis. Sonicates of Gram-positive, Gram-negative, or anaerobic resident bacteria isolated from mouse intestinal flora were fed to BALB/c mice by gastric gavage, with or without cholera toxin. After four weekly doses of 1 mg of these antigen preparations (or of PBS as control), DSS colitis was induced. One week later colitis was evaluated by clinical scores and histology. Mice fed a pool of the three sonicates had decreased inflammation scores (5 (1–14); median (range)) compared with PBS-fed control animals (15 (7–19); $P < 0{\cdot}05$). Decreased inflammation was observed in mice fed anaerobic bacteria antigens (7 (6–11); $P < 0{\cdot}05$ *versus* control), but not in mice fed a pool of Gram-positive and -negative sonicates (16 (12–16)). Inflammation scores of mice fed antigens with cholera toxin were similar to those of PBS-fed control animals. DSS-induced colitis can be suppressed by oral administration of normal intestinal flora antigens containing anaerobes.

Keywords dextran sulphate colitis intestinal flora

INTRODUCTION

The pathogenesis of inflammatory bowel disease (IBD) involves abnormal interactions between the host (genetic susceptibility, immune system dysregulation) and its environment [1]. There is increasing evidence that IBD results from an abnormal immune response to normal intestinal flora, with a break of tolerance towards non-pathogenic bacteria [2]. This hypothesis is supported by the observation that Crohn's disease (CD) improves following faecal stream diversion and by the fact that infusion of intestinal contents in surgically excluded ileum induces Crohn's lesions [3,4]. In further support of this hypothesis, administration of antibiotics, IL-10, or antibodies to tumour necrosis factor-alpha (TNF-α) was shown to improve intestinal inflammation [5–7]. However, the search for specific pathogenic microorganisms in IBD has remained so far non-conclusive [8–11]. It seems more likely that a large array of bacteria can be involved in the induction or in the maintenance of the chronic inflammation observed in IBD, the role of specific pathogenic bacteria being one of the many possible triggers of the pathological inflammatory reaction [1,12].

Results obtained in animal models further support that tolerance to autologous flora is broken in IBD. This was shown in a murine model of trinitrobenzene sulphonic acid (TNBS) hapten-induced chronic intestinal inflammation, where tolerance could be restored by treatment with IL-10 or antibodies to IL-12 [13]. Moreover, IL-2 and IL-10 knock-out mice develop colitis spontaneously upon colonization with normal intestinal flora, but inflammation does not occur if the animals are kept in germ-free conditions [14–16]. Similarly, colitis is not detected in HLA-B27 transgenic rats kept under germ-free conditions [17]. In this model, colonization with *Bacteroides* spp. was shown to induce intestinal inflammation [18]. Severe colitis can be induced in genetically normal mice by oral administration of dextran sodium sulphate (DSS) [19,20]. Exposed mice develop an acute colitis with bloody diarrhoea, shortening of the colon, weight loss, and neutrophilic infiltration of the left colon within 1 week after administration of 5% DSS in drinking water. In this model, concentrations of *Bacteroides* and *Clostridium* species are increased in the acute phase of the disease [19]. It has been

Correspondence: Pierre Michetti MD, Division of Gastroenterology, Dana 601, East Campus, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215, USA.

E-mail: pmichett@caregroup.harvard.edu

suggested that after induction of mucosal damage by DSS, intestinal bacteria penetrate the injured mucosa and perpetuate mucosal inflammation [20].

Immune and inflammatory responses in the intestine can be modulated in many ways. To avoid chronic immune activation leading to inflammation, in particular in the gut where bacteria and food antigens are in close proximity to a substantial proportion of the body lymphocytes, the immune system must develop unresponsiveness, tolerance, to such antigenic stimuli [21]. Oral and systemic tolerance are induced by repeated oral administration of antigens, in absence of adjuvant, and can suppress autoimmune diseases [22]. Induction of oral tolerance has been previously reported as an approach to improve colitis in experimental mouse models. Colitis induced by the contact-sensitizing agent TNBS has been prevented by feeding TNBS or TNP-conjugated colonic proteins [23,24]. Cholera toxin, in contrast, has been used as a potent mucosal adjuvant for oral immunization and reported to break peripheral tolerance to co-administered antigens [25,26]. Oral immunization with bacterial antigens and cholera toxin can induce antigen-specific T cells and secretory IgA [27,28] which can prevent contact of bacteria with the intestinal mucosa [29]. Recent evidence suggests that the qualitative composition of the intestinal flora also plays a role in preventing intestinal inflammation. Feeding lactobacilli or nutrients able to modify the intestinal flora composition can prevent the development of inflammatory response in the gut [30].

The present study tests the hypothesis that repetitive feeding with normal intestinal flora antigens can prevent the development of DSS colitis. We observed that mice fed anaerobic bacteria antigens had significantly reduced colitis scores following DSS compared with control mice and that this effect was lost if the antigen preparations were co-administered with cholera toxin (CT).

MATERIALS AND METHODS

Preparation of bacterial sonicates

Isolation of facultative and strict anaerobes from the caecal mucosa of BALB/c mice was performed on blood agar, Mac Conkey, vancomycin-nalidixic acid, brilliant green, phenyl-ethyl-alcohol agar, neomycin gelose and Campylobacter media. Bacteria were collected from the cultures and identified. *Enterococcus*, *Staphylococcus, Lactococcus*, and *Bacillus* species were pooled and sonicated under sterile conditions in PBS to yield a Gram-positive bacterial antigen preparation. A Gram-negative preparation was generated by sonication of *Escherichia coli* and a preparation of anaerobes (A) was obtained by sonication of *Bacteroides, Lactobacilli and Clostridia*. Further information on the bacteria used in this study is provided in Table 1. Pools of these antigen preparations were generated by mixing equal amounts of the individual preparations (GG = Gram-positive and Gram-negative bacteria, GGA = Gram-positive, Gram-negative and anaerobes).

Table 1. Bacteria species isolated from BALB/c mice colonic flora

Gram-positive bacteria	Gram-negative bacteria	Anaerobic bacteria
Enterococcus durans	*Escherichia coli*	*Bacteriodes ovatus*†
*Staphylococcus intermedius**		*Lactobacilli* spp.‡
Lactococcus lactis		*Clostridia* spp.‡
Bacillus spp.‡		

**Staphylococcus aureus* family.
†*Bacteriodes fragilis* family.
‡Strain not determined.

Immunization schemes

Eight-week-old BALB/c mice (Velaz, Prague, Czech Republic) were used in this study. Mice were dosed four times by gastric gavage on a weekly schedule with 100 μl of sonicate in PBS, or 100 μl of PBS as a control. Concentrations of sonicates were adjusted so that each mouse received 1 mg of total protein in 100 μl of each oral dose. In addition to the various sonicates and sonicate pools described above, one group of mice was fed GGA supplemented with 10 μg of CT. Two weeks after the last oral dose of sonicate, DSS (5% in distilled sterile water) was administered in drinking water for 7 days. A proportion of the animals in each group were fed with water.

Evaluation of colitis

Immediately after the end of DSS feeding, mice were killed. The presence of rectal prolapse, rectal or colonic bleeding and weight loss was recorded. A symptom score (range 0–4) was constructed attributing one point to each of the following events: rectal prolapse, rectal bleeding, colonic bleeding and death. The length of the colon was measured and sigmoid samples were obtained for histology. Two pathologists, blinded to the treatment schedule of the animals, reviewed the slides. The severity of the colitis was scored, attributing between zero and three points to each of the following parameters: (i) polymorphonuclear infiltrate; (ii) mononuclear infiltrate; (iii) oedema; (iv) erosions and ulcerations; (v) crypt abscess; (vi) crypt destruction; and (vii) distribution of the inflammation (mucosa = 1, mucosa and submucosa = 2, transmural inflammation = 3). Based on the total score (range from 0 to 21), the inflammation was graded as mild (1–5 points), moderate (6–10 points) or severe (> 10 points).

RESULTS

Clinical signs of colitis

A significant decrease in median symptom score was observed in the mice fed with the pool of Gram-positive, Gram-negative and anaerobic bacteria antigens, compared with PBS-fed control mice (Fig. 1a). When isolated sonicates were given to mice, colitis was reduced only in mice fed with anaerobic bacteria antigens, but not in animals fed a pool of Gram-positive and Gram-negative bacteria antigens. Animals given pooled antigens of Gram-positive, Gram-negative and anaerobic bacteria with CT had symptom scores that were comparable to PBS-fed controls, suggesting that addition of CT abolished the protective affect of antigen feeding.

Grading of colonic inflammation

In parallel with improved symptom scores, administration of GGA decreased the severity of DSS-induced inflammation scores compared with mice treated with PBS prior to the induction of colitis (Fig. 1b). While severe inflammation, crypt destruction, and ulceration of the mucosa were observed in the PBS-fed group (Fig. 2a,b), minimal inflammation was observed in the GGA-fed group (Fig. 2c,d). Compared with PBS controls, median inflammation scores were also reduced by the administration of A but

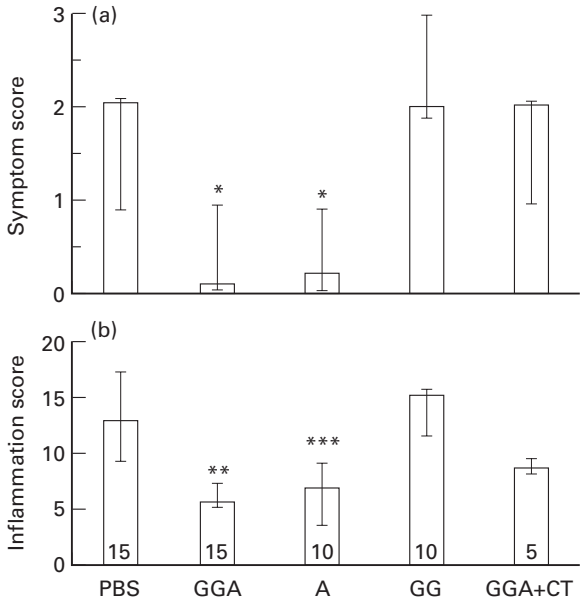

Fig. 1. (a) Clinical signs of dextran sodium sulphate (DSS)-induced colitis following oral feeding with bacteria antigen preparations. PBS, Control mice fed PBS instead of bacteria antigens; GGA, mice fed a pool of Gram-positive, Gram-negative, and anaerobic bacteria antigens; A, mice fed anaerobic bacteria antigens; GG, mice fed a pool of Gram-positive and Gram-negative bacteria antigens; CT, cholera toxin. Median symptom scores were calculated attributing one point to each of the following events: rectal prolapse, rectal bleeding, colonic bleeding, mortality, and divided by the number of mice in each treatment group. Results are shown as median symptom scores with 95% confidence interval (CI). $*P < 0{\cdot}05$ *versus* PBS, GG or GGA + CT. (b) Inflammation scores in sigmoid biopsies following DSS administration to antigen-fed mice. Inflammation was maximal in control mice (PBS). Administration of GGA and A decreased the severity of DSS-induced inflammation ($**P < 0{\cdot}05$ *versus* PBS, GG or GGA + CT; $***P < 0{\cdot}05$ *versus* PBS or GG). Co-administration of CT reversed the protective effect of GGA. Results are shown as medians with 95% CI. Numbers in bars indicate number of animals per group.

not by the administration of GG (Fig. 1b). In addition, protection was also associated with decreased splenocyte production of interferon-gamma (IFN-γ) (data not shown). Mice exposed to GGA + CT had an inflammation score that was not statistically different from PBS controls. Mice fed with water instead of DSS had no colonic inflammation, regardless of the antigen they had previously received, indicating that the administration of the bacterial sonicates had no intrinsic harmful effect on the colonic mucosa (data not shown).

DISCUSSION

In the present study we explore the role of oral administration of normal intestinal flora in the development of DSS-induced colitis. First, we show that oral administration of normal intestinal flora antigens induces resistance to DSS-induced colitis in normal mice. Second, we show that this resistance against of DSS colitis is critically dependent on feeding of anaerobic bacteria antigen preparations. Third, we show that this resistance is abrogated when the antigen preparation is given with CT. This last observation suggests that the decreased inflammation did not result from the development of an immune response to the bacterial antigen preparations, but the mechanisms by which the protective effect occurred remain unclear. One possibility is that oral administration of antigens induced immune tolerance, with decreased immune response to the intestinal flora upon DSS challenge. Administration of CT has been shown to increase gut permeability [31], which in our system could facilitate bacterial antigenic exposure, increasing the severity of DSS colitis. Alternatively, administration of anaerobic bacteria antigens may have altered the intestinal flora homeostasis in a way that rendered mice less susceptible to DSS challenge.

The mechanism by which DSS induces colitis is not well defined, but seems to result from an alteration of colonic epithelial cells, and not from an alteration of T nor B cell responses, because DSS colitis develops in immunodeficient mice [32,33]. This suggests that DSS modifies either the nature or the sampling of luminal antigens [34]. Antibiotics and live bacteria (probiotics) have been used to prevent and ameliorate experimental colitis by changing the equilibrium of commensal bacteria in the intestinal flora [30,35]. In our study, bacteria were washed twice in PBS before sonication, making very unlikely the possibility that the protective effect was due to antibiotics contaminating the final antigen preparation. It is also improbable that live bacteria contained in the sonicates would have modified the intestinal flora of the mice. Sonicates were frozen and thawed before each dosing, a procedure that would have impaired the survival of any bacteria. We cannot rule out however that the administration of bacterial components changed the equilibrium of the intestinal flora, either by providing excess free bacterial antigens to the mucosal immune system, or by competing with live bacteria for intestinal epithelial binding sites.

Tolerance induction has been demonstrated to be an efficacious mechanism of protection in hapten-induced colitis [23,24]. In that model, tolerance induced either to the hapten in solution or to haptenized colonic proteins abrogates hapten-induced colitis, but the antigens used for tolerance induction are unknown to the immune system of the animals before the experiment. In that model, tolerance is thus induced *de novo* to a foreign antigen. In our model in contrast, the mice should already be tolerant to their endogenous flora, as the antigens used for oral administration were derived from bacteria already present in the lumen. Indeed, in mice physiological tolerance to the intestinal flora establishes during the first weeks of life [36,37]. If immune tolerance participates in the protection observed in this study, then our results suggest that the physiological tolerance to intestinal bacteria can be raised or modified to prevent the development of DSS-related inflammatory response. Oral tolerance can be induced either by clonal deletion or anergy of Th1 cells, or by induction of suppressive Th2 and Th3 clones secreting transforming growth factor-beta (TGF-β), IL-4, and IL-10 [38–40]. In our study, CT abrogated the protective effect of antigenic anaerobic feeding, which would argue against deletion of antigen-specific T cells. Further studies in this model and in T lymphocyte-driven models of colitis will be needed to determine if tolerance to intestinal bacteria antigens takes place in this model and the precise mechanism by which it is induced.

Anaerobic bacteria, especially *Bacteriodes* spp., have been implicated in the pathogenesis of IBD, and recent evidence suggests that anaerobic bacteria play a role in the initiation of DSS colitis in mice [33]. Our results are compatible with these studies in showing that anaerobic antigens play a crucial role in inducing protection. Whether oral administration of anaerobic antigens led

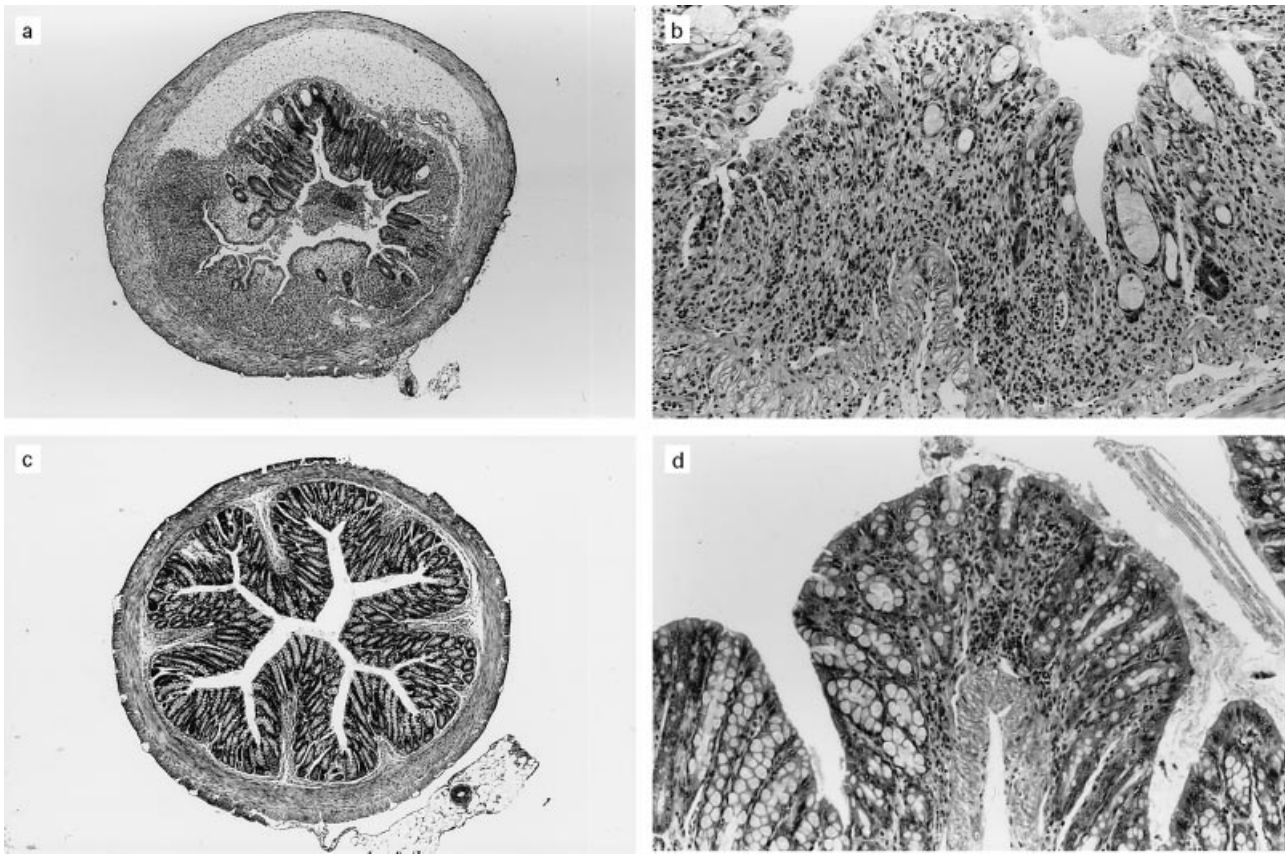

Fig. 2. (a,b) Severe inflammation observed in a mouse fed PBS before induction of dextran sodium sulphate (DSS) colitis. (a) Low-power view of a transversal cut section through the sigmoid colon showing oedema of the submucosa and extensive inflammation and ulceration of the mucosal layer. (b) High-power view of the sigmoid mucosa showing severe colitis with subtotal destruction of the crypts and massive inflammatory infiltrate in the lamina propria. (c,d) Minimal inflammation in the colon of a mouse fed Gram-positive, Gram-negative, and anaerobic bacteria antigens (GGA) before DSS colitis induction. (c) Low-power view of a transversal cut section through the sigmoid colon showing no visible alteration of the mucosa. (d) Focal inflammation of the sigmoid mucosa without ulceration or destruction of the crypts at high-power view.

to induction of oral tolerance or to increased resistance of the mucosal barrier remains to be determined. The oral antigen feeding technique described here may represent a novel approach to identify the bacteria and their components involved in the pathogenesis of IBD. Further studies will be required to determine if other intestinal bacteria antigens are able to confer this type of protection.

In conclusion, our data extend the evidence that non-pathogenic luminal bacteria participate in the pathogenesis of chronic intestinal inflammation. Investigations into the mechanisms participating in this protection are in progress. Administration of intestinal flora components may be relevant to the development of novel therapeutic strategies in the management of IBD patients.

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