Monocolonization with *Bacteroides ovatus* Protects Immunodeficient SCID Mice from Mortality in Chronic Intestinal Inflammation Caused by Long-Lasting Dextran Sodium Sulfate Treatment

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Summary

This study was aimed to evaluate the role of commensal Gram-negative bacterium *Bacteroides ovatus* in murine model of chronic intestinal inflammation. The attempt to induce chronic colitis was done in *Bacteroides ovatus*-monoassociated, germ-free and conventional mice either in immunocompetent (BALB/c) mice or in mice with severe combined immunodeficiency (SCID), using 2.5 % dextran-sodium sulfate (DSS) in drinking water (7 days DSS, 7 days water, 7 days DSS). Conventional mice developed chronic colitis. Some of germ-free BALB/c and the majority of germ-free SCID mice did not survive the long-term treatment with DSS due to massive bleeding into the intestinal lumen. However, monocolonization of germ-free mice of both strains with *Bacteroides ovatus* prior to long-term treatment with DSS protected mice from bleeding, development of intestinal inflammation and precocious death. We observed that though DSS-treated *Bacteroides ovatus*-colonized SCID mice showed minor morphological changes in colon tissue, jejunal brush-border enzyme activities such as γ-glutamyltranspeptidase, lactase and alkaline phosphatase were significantly reduced in comparison with DSS-untreated *Bacteroides ovatus*-colonized mice. This modulation of the enterocyte γ-glutamyltranspeptidase localized to the brush border membrane has been described for the first time. This enzyme is known to reflect an imbalance between pro-oxidant and anti-oxidant mechanisms, which could be involved in protective effects of colonization of germ-free mice with *Bacteroides ovatus* against DSS injury.

Key words

Bacteroides ovatus • Ulcerative colitis • Dextran sulfate sodium colitis • Gnotobiotic mice

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Introduction

Ulcerative colitis and Crohn's disease are relapsing inflammatory disorders of the colon of unknown origin in which genetic, immunological and environmental factors may be involved. Critical role in the etiology of these inflammatory bowel diseases (IBD) is played by intestinal microflora (Tlaskalová-Hogenová *et al.* 2005). Abnormalities have been described in the fecal microbiota of patients with IBD. Genus *Bacteroides* - *B. vulgatus*, *B. fragilis*, *B. merdae distasonis*, *B. ovatus*, *B. thetaiotaomicron* and *B. uniformis* - was found to be increased in colon of patients with ulcerative colitis (Poxton *et al.* 1997, Sokol *et al.* 2006). The higher incidence of population of members of the *Bacteroides* in ulcerative colitis patients suggests that these may have an influence on the pathogenesis of the disease (Lucke *et al.* 2006). Saitoh *et al.* (2002) described that *B. ovatus* is the predominant commensal intestinal microbe causing a systemic antibody response in inflammatory bowel diseases. In experimental animals such as mice the development of the inflammation is influenced by mouse strains used for the experiments, induction agents

and length of treatment responsible for induction of inflammation. Interestingly, after the induction of experimental colitis when BALB/c mice are kept in sterile environments they develop markedly weaker colonic inflammation than their conventional (CV) counterparts (Hudcovic *et al.* 2001). In recent years, monocolonization of germ-free (GF) animals with single bacterial strain enables to demonstrate the influence on immunological and physiological characteristics of gastrointestinal tract. Thus, *B. thetaiotaomicron* as a main representative of mouse colon was used for monocolonization of GF mice and was demonstrated to affect several gene expressions (Hooper *et al.* 2001, Hooper 2004). A widely used experimental model of colitis involving oral consumption of dextran sulfate sodium (DSS) dissolved in drinking water results in an acute distal colitis in rodents, which resembles human ulcerative colitis in both clinical and histopathological findings (Okayasu *et al.* 1990). Histologically the acute phase was characterized by destruction of the colonic epithelial layer and mucosal infiltration of neutrophils, macrophages and lymphocytes (Okayasu *et al.* 1990, Cooper *et al.* 1993); the chronic phase was characterized mainly by mononuclear cell infiltration and significant increases in leukocyte-endothelium interactions and leukocyte extravasations (Farkas *et al.* 2001). Axelsson *et al.* (1996) studied in detail the involvement of immunological mechanisms in DSS-induced colonic inflammation. The authors tested BALB/c mice depleted of $CD4^+$ T cells, athymic mice, T and B cells deficient SCID mice and SCID mice depleted of NK cells. They concluded that the presence of functional T, B and NK cells is not crucial for the induction of DSS colitis in mice and thus confirmed the results of Dieleman *et al.* (1994). Previously we demonstrated that SCID mice are sensitive to DSS treatment depending on microbial colonization (Hudcovic *et al.* 2001). *B. ovatus* known to cause systemic antibody response in patients with IBD (Saitoh *et al.* 2002), has not been tested on development of experimental colitis induced by DSS.

Here we studied whether *B. ovatus* monocolonization of GF mice of two strains – SCID and BALB/c mice – affected the severity of colonic inflammation after long-term DSS treatment. Study was completed by determination of the expression of brush-border enzymes of the small intestinal mucosa where no direct inflammation has been noticed.

Methods

Animals

Two-month-old BALB/c and SCID mice (mice with severe combined immunodeficiency, background BALB/cJHanHsd-SCID) were used for our experiments. Germ-free and *B. ovatus*-associated mice were kept in sterile conditions in separate plastic transparent cages in isolators and fed *ad libitum* sterile (59 kGy irradiated) standard pellet diet (ST1, Inst. Physiol., Acad. Sci. Czech Republic) and sterile water. Conventional mice kept in the CV animal facility were fed the same but not sterile food. The animals were kept in a room with a 12 h light-dark cycle at 22 ^{o}C (Štěpánková 1997). There were 7 to 18 mice in each experimental group.

Experimental design and induction of colitis

Outline of experimental groups is described in Table 1. The group of GF mice was monocolonized with *B. ovatus*, a species of Gram-negative obligate anaerobes found in the human colon (kind gift of Dr. Elena Verdu, University of Lausanne, Switzerland) in concentration 10^7 CFU in 0.2 ml of phosphate buffer solution (PBS) by intragastrical tubing at weaning (21-day-old) and the second generation of mice was used in our experiment. Other groups of mice that remained GF as well as and CV mice served as controls. At the age of two months, the chronic colitis was induced in mice by replacing drinking water with water containing 2.5 % DSS (wt/vol; m.w. 40 kD; ICN Biomedicals, USA) according to the following schedule: 7 days 2.5 % DSS – 7 days water – 7 days 2.5 % DSS, allowing the mice to drink freely. Clinical symptoms such as diarrhea, bleeding and enteric rectal prolapse and changes in body weight were tested. The animals were sacrificed in ether anesthesia by exsanguination. The whole intestine was removed. Jejunum was washed with cold saline and jejunal scrapings were used for brush border membrane isolation. Colon descendens was evaluated histologically. All experiments were approved by the Ethical Committee of the Institute according to the Animal Protection Act.

Histology

The colon tissue was fixed in Carnoy´s fluid for 30 min and transferred into 96 % ethanol overnight, then *via* 100 % ethanol (3 x 1 h) into methylsalicylate (4 h/ overnight/4 h), benzene (3 x 10 min) and benzene paraffin (30 min) to paraffin (2 x overnight). The tissue specimens were sliced into sections 5 μm thick. All

Table 1. Outline of experimental groups involved in the study

Experimental group	Treatment with DSS
Germ-free mice (BALB/c)	7-day-DSS, 7-day-water, 7-day-DSS
Germ-free mice (SCID)	7-day-DSS, 7-day-water, 7-day-DSS
B. ovatus-monocolonized (BALB/c)	7-day-DSS, 7-day-water, 7-day-DSS
B. ovatus-monocolonized (SCID)	7-day-DSS, 7-day-water, 7-day-DSS
B. ovatus-monocolonized (SCID)	untreated
Conventional mice (BALB/c)	7-day-DSS, 7-day-water, 7-day-DSS
Conventional mice (SCID)	7-day-DSS, 7-day-water, 7-day-DSS

Table 2. Effect of chronic ulcerative colitis on mortality, histopathological score in colon descendens and mucin production in *B. ovatus*-monocolonized, GF and CV BALB/c and SCID mice

Experimental groups	Mortality/number	Damage score	Mucin secretion
B. ovatus-monocolonized (BALB/c)	0/7	0 – 1	3 – 4
Germ-free mice (BALB/c)	3/8	1 – 2	2 – 3
Conventional mice (BALB/c)	2/10	4	0
B. ovatus-monocolonized (SCID)	0/18	1 – 2	2 – 3
Germ-free mice (SCID)	7/8	n.d.*	n.d.*
Conventional (SCID)	6/12	4	0

*not done, impossible to evaluate because of high mortality rate

specimens were stained with hematoxylin-eosin, Cresyl Fast Violet (both Vector, USA). The specimens were viewed under Olympus BX 40 microscope equipped with a digital camera Olympus Camedia C-2000. Photographs were taken on proposal of Camedia Master 2.5 and Olympus DP-Soft. The four degrees of damage to the surface epithelium and crypt distortion in individual colon segments were evaluated according to Cooper *et al.* (1993). The secretion of mucin was assessed in four categories in cross sections: 1 – secretion of mucin on 25 % of the colon surface, 2 – secretion of mucin on 50 % of the colon surface, 3 – secretion of mucin on 75 % of the colon surface, and 4 – secretion of mucin on 100 % of the colon surface.

Immunohistochemistry

Colon descendens segments were frozen in liquid nitrogen. Acetone-fixed sections 5 µm thick were used for immunohistochemistry. The membrane marker CD11b was detected directly by fluorescein-labeled monoclonal antibody to CD11b/FITC (MCA 74 F, Serotec, Oxford, UK). The surface antigen CD11b is known to be expressed on the surface of polymorphonuclear leukocytes, monocytes and NK cells.

Preparation of enterocyte brush-border membrane vesicles

Brush border membranes were prepared from jejunal scrapings essentially according to the method of Kessler *et al.* (1978).

Determination of enzyme activities

Lactase of the lactase-phlorizin hydrolase complex (EC 3.2.1.23/62/108), sucrase of the sucrase-isomaltase complex (EC 3.2.1.48/10) and glucoamylase of the maltase-glucoamylase complex (EC 3.2.1.20/3) activities were determined with 50 mM lactose (Kraml *et al.* 1972), 50 mM sucrose and 12 g/l of starch as substrates, respectively (Kolínská and Kraml 1972). The liberated glucose was measured with the Tris-glucose oxidase-peroxidase reagent (Dahlqvist 1964). Alkaline phosphatase (EC 3.1.3.1) activity was determined in BBMV with 0.1 mM p-nitrophenylphosphate (Fluka, Buchs, Switzerland) as substrate in Tris-HCl buffer (pH 8.5). The liberated p-nitrophenol was measured at A_{405} using the Test Kit (Lachema, Brno, Czech Republic). The activity of γ-
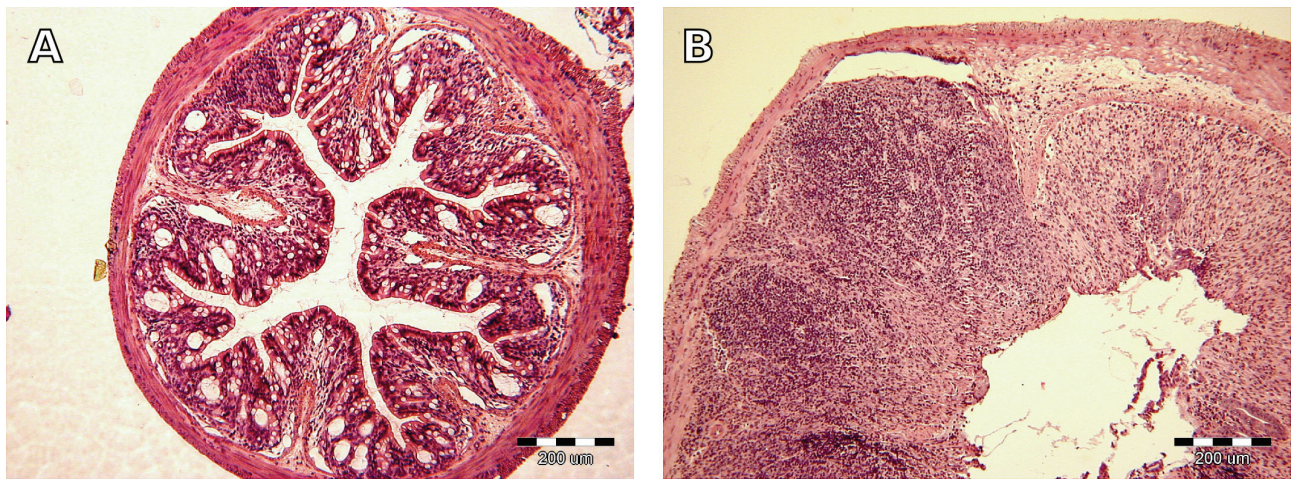
Fig. 1. Representative cross-sectional views of hematoxylin and eosin stained colon descendens of (**A**) GF BALB/c mice (damage grade 1 - 2, mucin secretion 2 - 3) compared with damaged colon descendens of (**B**) CV BALB/c mice (damage grade 4, mucin secretion 0) in a chronic model of ulcerative colitis.
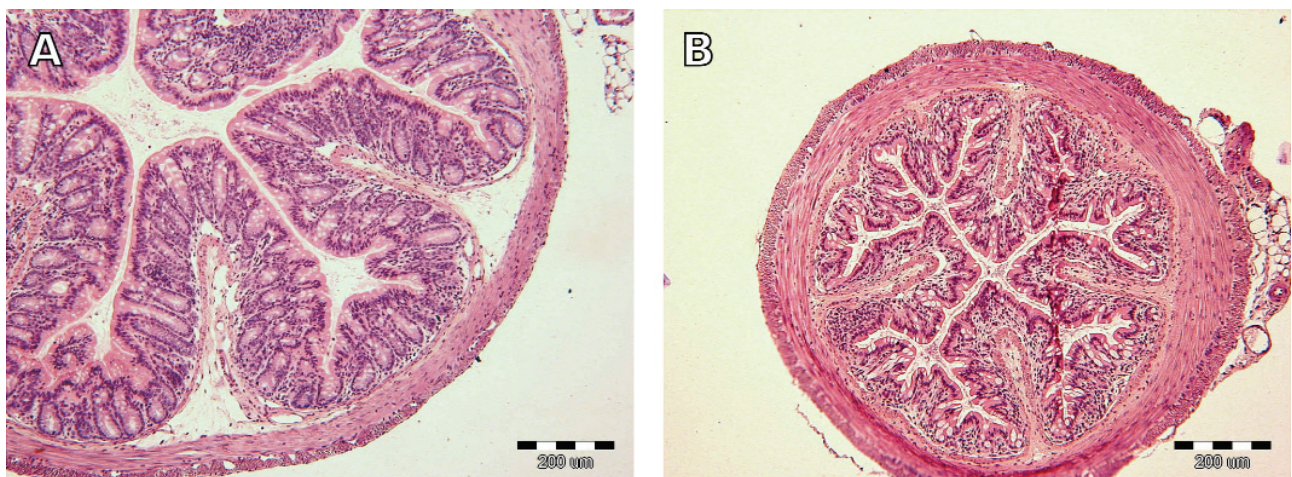
Fig. 2. Representative cross-sectional views of hematoxylin and eosin stained colon descendens in a chronic model of ulcerative colitis of (**A**) *B. ovatus*-monoassociated BALB/c mice (damage grade 0 - 1, mucin secretion 3 - 4) and mild changes in colonic submucosa in (**B**) *B. ovatus*-monoassociated SCID mice (damage grade 1 - 2, mucin secretion 2 - 3).

glutamyltranspeptidase (GGT; EC 2.3.2.2) was determined with 7 mM 5-L-glutamic acid-4-nitroanilide (Fluka, Buchs, Switzerland) as donor substrate, 0.1 mM glycylglycine (pH 8.2; Fluka, Buchs, Switzerland) as acceptor substrate and 0.1 mM NaCl as activator. Released 4-nitroaniline was measured at A_{405} (Thompson *et al.* 1976). The activity of dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) was determined with 1.4 mM glycyl-L-proline-4-nitroanilide (Sigma-Aldrich, Diesenhofen, Germany) in 66 mM Tris-HCl buffer, pH 8.0. The reaction was stopped with 1 mM Na-acetate buffer (pH 4.2) and the released 4-nitroaniline was measured at A_{405} (Nagatsu *et al.* 1976). Enzyme activities were expressed as nkat/mg protein, one nanokatal being the amount of the enzyme that converts 1 nanomole of substrate per second under the given conditions. Protein in BBMV was determined by the method of Lowry *et al.* (1951) using bovine serum albumin, fraction V (Serva, Heidelberg, Germany) as standard.

Statistical analysis

The results were expressed as means ± S.D. Statistical analyses were performed using SigmaStat (Jandel Corporation). Multiple comparison procedures were made by one way analysis of variance (ANOVA), Student-Newman-Keuls method. Values of p<0.05 were considered significant.

Results

The majority of GF SCID mice did not survive
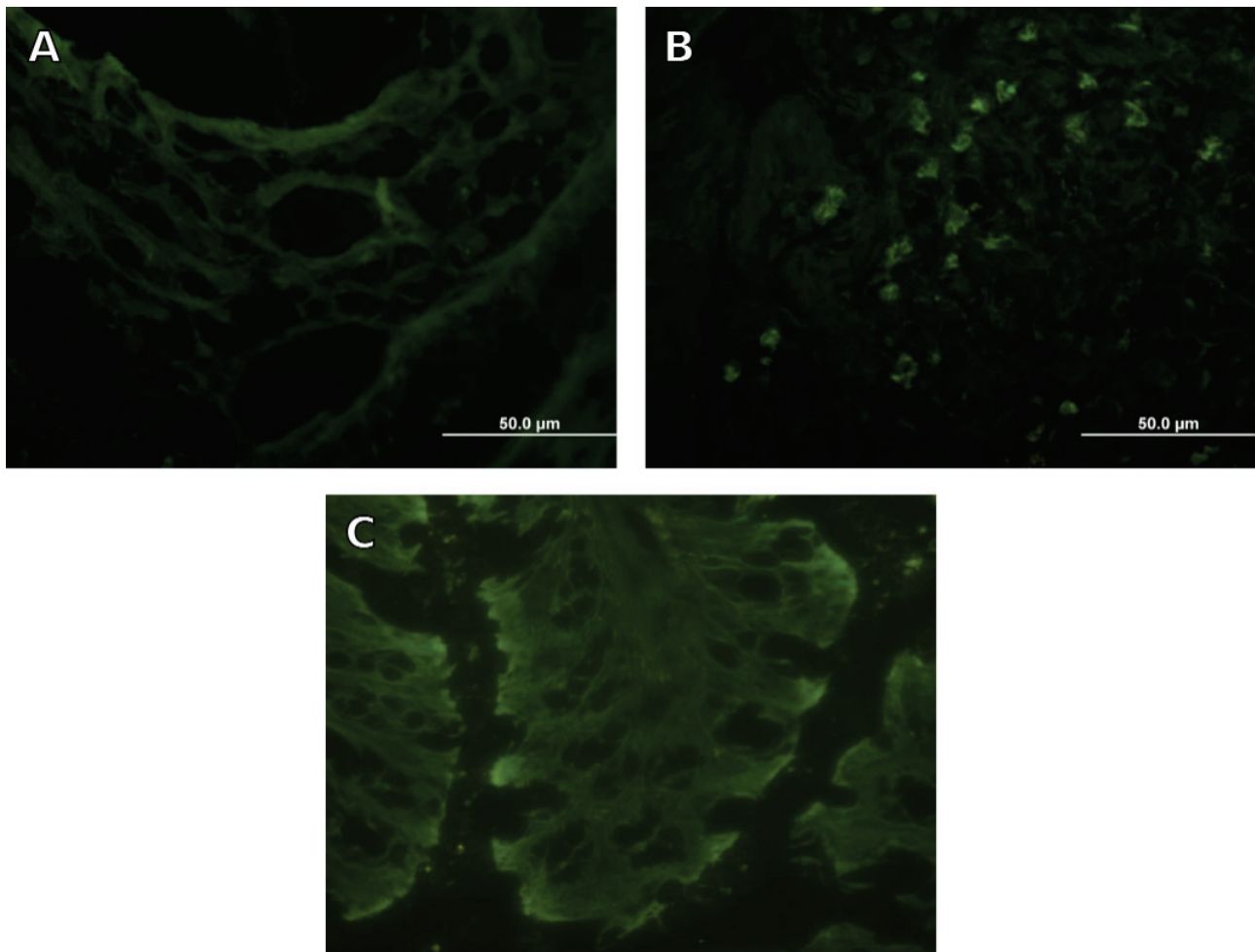
Fig. 3. Immunohistochemical detection of inflammation-induced infiltrated cells using FITC labeled anti-CD11/b in chronic DSS colitis. (**A**) *B. ovatus*-monoassociated BALB/c mice (no infiltration of inflammatory cells), (**B**) conventional BALB/c mice (massive infiltration of cells in lamina propria), (**C**) *B. ovatus*-monoassociated SCID mice (no infiltration of inflammatory cells).

the long-term treatment with DSS (7 days DSS, 7 days water, 7 days DSS) and therefore the values of damage score and mucin production for this group are not given in Table 2. Germ-free BALB/c mice showed mild histological changes in colon mucosa (damage score 1-2, Fig. 1A and Table 2). According to Cooper *et al.* (1993) degree 1 of damage stands with crypt shortening by 1/3, with epithelial dilatation and thinning, with 25 % damage of colon mucosa. Damage is accompanied with appearance of a hyaline layer between crypt base and muscularis mucosae indicating early colon damage, thinning of the pericryptal collagen sheath, mild increase in collagen content between crypts and mucin production in 100 % colon surface. Degree 2 of damage corresponds to 26-50 % damage to colon mucosa, crypt shortening by 2/3 and mucin production in 75 % colon surface. On the other hand, CV BALB/c mice exhibited a gross destruction of the colon mucosa, disappearance of crypts

followed by infiltration of inflammatory cells, damage score 4, no mucin secretion – grade 0 (Fig. 1B and Table 2). This finding corresponds to our previous studies (Hudcovic *et al.* 2001). According to Cooper *et al.* (1993) degree 4 stands with early epithelial hyperplasia in less than 2/3 of the crypts with the loss of mucin, increased mitotic activity, large inflammation in mucosa and submucosa and mucin production in 25 % colon surface.

To study the influence of *B. ovatus* on the development of intestinal inflammation, GF BALB/c and SCID mice were monoassociated with *B. ovatus* and were treated in a chronic model of ulcerative colitis by two cycles with 2.5 % DSS in drinking water (7 days DSS, 7 days water, 7 days DSS). We found that *B. ovatus* protected both BALB/c and SCID mice from bleeding and precocious death observed after long-lasting DSS treatment in their GF counterparts and from severe forms of intestinal inflammation. The values of damage score

Table 3. Brush border enzyme activities (nkat/ mg protein) in mouse jejunum after monoassociation with *B. ovatus* in chronic model of ulcerative colitis in SCID mice

Enzymes	Untreated controls GF	*B. ovatus*-monoassociated, controls	*B. ovatus*-monoassociated, DSS treated
Lactase	3.9 ± 0.84	5.20 ± 0.34	2.78 ± 1.33*
Sucrase	12.42 ± 2.82**	25.49 ± 0.69	27.49 ± 1.06
Glucoamylase	10.33 ± 1.54	8.85 ± 0.24	8.82 ± 0.79
DPP IV	5.93 ± 0.72	6.38 ± 0.12	7.24 ± 0.13
GGT	9.10 ± 1.46	10.12 ± 0.40	6.50 ± 0.29*
Alkaline Phosphatase	8.19 ± 0.62	7.32 ± 0.25	5.28 ± 0.47*

Values are expressed as the means ± S.D. *P<0.05, **P<0.01 significantly different from *B. ovatus*-monoassociated controls.

and mucin production were in *B. ovatus*-colonized BALB/c mice of grade 0-1 and in *B. ovatus*-colonized SCID mice of grade 1-2 (Fig. 2A, 2B and Table 2).

Infiltration by inflammatory cells of the intestine is a marker of the inflammatory processes. Using monoclonal antibody to CD11/b, a membrane marker of polymorphonuclear leukocytes, monocytes and natural killer cells, we found that the colon of BALB/c monocolonized with *B. ovatus* and treated with DSS did not exhibit infiltration by these cells (Fig. 3A). On the other hand, massive infiltration of pro-inflammatory cells in lamina propria occurred in conventional BALB/c mice treated with DSS (Fig. 3B). SCID mice monocolonized with *B. ovatus* and treated with DSS did not exhibit infiltration by these cells (Fig. 3C).

The enterocyte brush-border enzymes – lactase, sucrase, glucoamylase, alkaline phosphatase, γ-glutamyl-transpeptidase (GGT) and dipeptidyl peptidase IV – were determined in jejunum of GF and *B. ovatus*-monoassociated SCID mice. This part of the intestine has not shown histological changes in experimental colitis. Specific activities of enterocyte enzymes of *B. ovatus*-monoassociated mice treated with DSS were compared with enzyme activities detected in brush borders from *B. ovatus*-monoassociated DSS-untreated mice. Though we did observe only mild morphological changes in colonic mucosa (Table 2), some enterocyte brush-border enzyme activities were reduced as a consequence of DSS treatment (Table 3). In DSS-treated mice monocolonized with *B. ovatus,* specific activity of GGT, which is very sensitive to oxidative stress, was markedly lower compared to controls. Similarly, lactase and alkaline phosphatase activities in DSS-treated mice were significantly reduced compared with controls. Enzyme activities of untreated GF controls are given for

comparison (Table 3). Lower expression of sucrase activity in untreated GF control than in *B. ovatus*-monoassociated control is due to delayed maturation in GF animals.

We studied also brush-border enzyme activities in chronic model of colitis under conventional conditions. However, for high mortality rate in this group of animals, we had to shorten the duration of DSS treatment to one week (acute form of colitis). In this acute colitis (*B. ovatus* and DSS-treated group) we obtained again a decrease of some enzyme activities compared with conventionally reared DSS-untreated mice. Activities of GGT, glucoamylase and alkaline phosphatase decreased from 5.0±0.8 to 2.4±0.2, 24.0±2.8 to 6.7±0.3 and 9.6±1.3 to 5.2±0.4 nkat/mg protein, respectively.

Discussion

The composition of gut microflora plays the main role in maintaining physiological and immune homeostasis as well as in regulating host inflammatory responses. Animal models of intestinal inflammation are essentially associated with the presence of commensal intestinal microbiota. This was demonstrated by development of severe colitis in rodents raised under CV conditions, and by the lack of colitis in GF animals (Ehrhardt *et al.* 1997, Schultz *et al.* 2004). Our previous and present results, however, concerning chronic forms of DSS-induced inflammation in GF SCID mice contradict these studies (Hudcovic *et al.* 2001). When chronic DSS-colitis was induced in GF SCID mice, they did not survive the long-term treatment with DSS. Similarly, in contrast to BALB/c mice increased mortality was observed in sensitive mouse strain IQI/Jic after oral administration of 5 % DSS (Kitajima *et al.* 2001). It was

suggested that DSS is impairing the intestinal barrier (Okayasu *et al.* 1990, Kitajima *et al.* 2002). Direct cytotoxic effect of DSS leading to destruction of mucosal architecture, rather than infiltration by inflammatory cells could be the explanation of precocious death in susceptible mouse strains (Schultz *et al.* 2004).

Our main finding, i.e. that a single bacterial strain – *B. ovatus* administered intragastrically to GF BALB/c and SCID mice prior to DSS treatment – protected mice against the development of chronic colitis indicates that the intestine of GF mice can be easily colonized with *B. ovatus* and that mucosal barrier integrity was not altered. Similar results were described for colonization with *B. vulgatus* (Sydora *et al.* 2005). The mechanisms of protective activity of *B. ovatus* colonization are not fully elucidated. It can be speculated that colonization with *B. ovatus* increased the mucosal barrier integrity and thus the resistance to DSS treatment. Recently it has been hypothesized whether the inflammatory effects participate in tight junction alteration during infection. The authors suggest that only direct bacterial contact with epithelial layer significantly affects tight junction structure (Guttman *et al.* 2006). Protection of GF mice from DSS injury was also observed after monocolonization with probiotic *E. coli* Nissle 1917 but not with pathogenic *E. coli* O6K13 (Hudcovic *et al.* 2007). Protection against DSS treatment was demonstrated only in originally GF mice after monocolonization with *B. ovatus*. Intragastric administration of *B. ovatus* to conventional mice prior to and during DSS treatment led to more pronounced reduction of body weight and even to precocious death of animals with severe enterocolitis (results not shown). Interaction of component of microflora with *B. ovatus* is probably crucial in worsening of the experimental intestinal inflammation. In conventional environment the association of *B. ovatus* with other bacteria of higher luminal concentration may add to alterations of epithelial integrity and potentiate developing inflammation as was the case of conventionally raised SCID mice in our studies. Similarly, other bacterial strains were considered to play important role in mediating inflammation in which *B. vulgatus* plays a key role (Rath *et al.* 1999).

The effects of colonization of GF healthy animals on brush border enterocyte enzyme activities indicate the impact of microbiota on physiological digestive processes (Kozáková *et al.* 1998, 2001, 2006). In this study the levels of jejunal brush-border enzymes lactase, GGT and alkaline phosphatase in *B. ovatus*

monoassociated SCID mice under the effect of DSS were significantly reduced compared to controls monoassociated with *B. ovatus* without DSS treatment. Interestingly, decreased activity of GGT in isolated brush-border membrane of jejunum was observed, even if intestinal inflammation did not develop in either *B. ovatus*-monocolonized GF SCID or BALB/c mice treated with DSS. Similarly, decreased mucosal activity of GGT was found not only in inflamed ileum of patients with Crohn's disease but also to a lesser extent in non-inflamed ileum (Sido *et al.* 1998). Thus, in terms of decreased activity of GGT in our study and increased IL-18 expression (Siegmund *et al.* 2001, Sivakumar *et al.* 2002), inflammation in DSS-induced colitis mimics human Crohn's disease. The decreased activity of GGT was recently localized to lamina propria T-cells in IBD mucosa in an active state of inflammation (Reyes *et al.* 2005). Our observation localizes for the first time the impaired GGT to jejunal brush-border membrane of SCID mice lacking T-cells, and supports the notion that the treatment with DSS led to structural changes in small intestinal mucosa where no direct inflammation was produced. In patients with IBD infiltrating macrophages and neutrophils are abundantly present in inflamed gut and participate in substantial oxidative stress by producing reactive oxygen radicals that lead to oxidative damage to colon protein and impaired glutathione synthesis (Sido *et al.* 1998). Among other enzymes, GGT is involved in the synthesis and homeostasis of glutathione, an antioxidant defense system, effective in reducing reactive oxygen species (ROS). In experimental ulcerative colitis the antioxidant defense system is impaired (Nieto *et al.* 2000). An imbalance between pro-oxidant and anti-oxidant mechanisms increases the susceptibility to oxidative tissue injury.

Similarly alkaline phosphatase, a dephospho-rylating enzyme may play an important role in host defense system against pathological stress such as inflammation and infection (Harada *et al.* 2003). Reduced jejunal lactase and alkaline phosphatase in patients with ulcerative colitis was described by Arvanitakis (1979). Reduced glucoamylase in acute experimental colitis, which in healthy animals is most abundant on the tips of villi as seen from villus-crypt gradient (Kolínská *et al.* 1990), might be associated with some injury in the small intestine just on the villi tips. Interestingly, the villus-crypt gradient of sucrase showed a broad maximum activity down to the middle of the villus and thus no decrease in sucrase activity was noticed in experimental colitis.

In summary, these observations provide data on the protective property of *B. ovatus* to prevent death as a consequence of long-term DSS treatment in GF SCID and BALB/c mice. Though DSS-treated *B. ovatus*-monocolonized SCID mice showed minor morphological changes in the colon, DSS treatment caused a decrease in three activities of the small intestinal brush-border enzymes (GGT, alkaline phosphatase, lactase) as compared to DSS-untreated GF or *B. ovatus*-monoasssociated mice. Especially the localization of altered GGT activity to enterocyte brush-border membrane represents a new finding. Until now lamina propria T cells have been considered as the main locus of GGT changes in an active state of inflammation.

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