COLIFAGINA, A NOVEL PREPARATION OF 8 LYSED BACTERIA AMELIORATES EXPERIMENTAL COLITIS

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Received September 7, 2007 - Accepted December 11, 2007

Immune reactivity towards the bacterial intestinal flora plays an important part in the pathogenesis of inflammatory bowel disease. Administration of probiotic bacteria has beneficial effects on infectious and inflammatory diseases, principally in bowel disorders. However, little is known about the administration of soluble bacterial antigens in intestinal inflammation. We investigated the therapeutic effects of colifagina in experimental colitis. To assess this effect, C57BL/6 mice with dextran sulphate sodium-induced colitis were treated with colifagina, or with a placebo, for a period of 10 days. The mice were monitored, and inflammation was assessed by disease activity index (DAI). Analysis of fecal IgA concentration and measurement of IgA and inflammatory chemokine production in organ colonic culture was performed by ELISA. Clinically and histologically, bacterial-lysate-treated mice revealed significantly fewer DAI and a reduction of colonic histological inflammation. Treatment of healthy mice with colifagina significantly increased the fecal concentration of IgA and IgA production in organ culture. Colifagina administration in DSS-treated mice significantly increased the fecal concentration of IgA and IgA production in organ culture. MIP-1, MIP-2 and RANTES concentrations in colonic organ culture were significantly lower in colifagina-treated mice than in the placebo group. The use of colifagina is effective in amelioration of murine colitis.

Inflammatory bowel diseases (IBD), including Ulcerative Colitis (UC) and Crohn's Disease (CD), are spontaneously relapsing immunologically mediated disorders of the gastrointestinal tract. Although the etiology of IBD remains unknown, it is commonly believed that disease pathogenesis is multi-factorial involving genetic, environmental and immunologic factors. Homeostasis (tolerance) involves a regulated response of the host to the constant antigenic drive of enteric bacteria while chronic intestinal inflammation represents a malfunction of this tolerance to the comensal microbiota. In the genetically susceptible host, the lack of appropriate mechanisms to terminate mucosal immune responses (loss of immunologic tolerance) and an ineffective mucosal barrier function results in continuous stimulation of the mucosal immune system, excess production of proinflammatory cytokines and chronic inflammation as a consequence

Considering the host microbial environment as the major player in the development of IBD, recently

Key words: colifagina, experimental colitis, IgA, inflammatory bowel disease, probiotics

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0394-6320 (2008)

much attention has been placed on the ability to target the microbiota with the use of probiotics as an option for therapeutic intervention to help restore balance to the intestinal microenvironment and hence reduce the inflammatory response. A probiotic can be described as "a preparation of or a product containing viable defined micro-organisms in sufficient numbers which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects on this host"(1).

The use of probiotics in the treatment of inflammatory diseases is not a new concept, however, their mode of action still remains complex and not completely understood. Many different probiotics have been used in the treatment of several inflammatory conditions including arthritis (2), pouchitis (3), ulcerative colitis (4-5) Crohn s disease (6- 7) and experimental colitis (8). Several mechanisms in which probiotics help improve intestinal function have been proposed which include: 1) the prevention of colonization of pathogenic bacteria to the GI tract by inhibiting their adhesion via competition; 2) reinforcement of the epithelial barrier by controlling cellular permeability (9); 3) alteration of the humoral immune response by elevating the amount of IgA; 4) regulation of proinflammatory cytokine production (10). Interestingly, not all the probiotics used in the IBD field have proven successful, and each probiotic has been shown to act in a specific manner.

In this study the therapeutic potential of the commercially available preparation colifagina was tested for the first time in IBD. Colifagina is a preparation of 8 lysed bacteria containing E. Coli (O1, O2, O55 and O111), Bacillus pumilus, Morganella morgani, Alcaligens faecalis, Shigella fexneri, Enterococcus faecalis, Bacillus subtilis and Proteus vulgaris, which has never been tested in previous reports. The pathogen strains are chosen from those most commonly responsible for gastrointestinal and urogenital infections. The administration of these pathogens is made possible by the complete deactivation of these bacteria through thermal alkaline lysis. Lysed bacteria maintain the same immunogenic properties as the corresponding vital bacteria both qualitatively and quantitatively, with the advantage that the lysed bacteria do not have the capacity to proliferate and invade the host thereby causing infection. The lysed bacteria introduce antigens, which induce an antibody secretory response via the release of immunoglobulins, both locally and systemically.

MATERIALS AND METHODS

Animals

Six to eight-week-old male C57BL/6 mice used in this study, were purchased from Charles River laboratories (Calco, Italy) and maintained under pathogen-free conditions in an animal facility at the Instituto Clinico Humanitas Hospital (Milan, Italy). Experimental procedures involving animals and their care conformed with institutional guidelines in compliance with national and international law after approval by the ethical committee.

Induction of colitis and treatment

The animals were divided into four groups each containing 10 mice. Two groups received 2.5% (wt/vol) Dextran sulfate sodium (DSS) (molecular mass, 40 kDa; MP Biomedicals Irvine, CA), ad libitum in filter-purified drinking water for ten days, as previously reported (11). The DSS alone-treated control group received daily 500 µl of isotonic sterile saline by oral gavage, while the treatment group received daily 500 µl of colifagina (ABC FARMACEUTICI, To, Italy) containing bacterial lysates; Escherichia coli strain 01, 02, 055, 0111 (6 x 10^9), Bacillus Pumilus (4 x 10^8), Morganella morgani, Alcaligenes faecalis, Shigella flexneri, (3 x 10^8) and Enterococcus faecalis, Bacillus subtilis, Proteus vulgaris (8 x 10^7) by oral gavage. The other two groups consisted of healthy untreated mice and healthy mice receiving 500 µl of colifagina daily.

Determination of disease activity

Colitis was scored daily using standard parameters that include body weight, diarrhea, presence of blood in the stools, and rectal prolapse. Disease activity index (DAI) was measured as the combined scores of weight loss, stool consistency and bleeding divided by 3 (11).

Histological grading of colitis

Colonic tissues were fixed in formalin overnight and embedded in paraffin for histological analysis.

Grading of intestinal inflammation was determined, as described previously (11), in a blinded fashion by 3 readers: no inflammation (0); modest numbers of infiltrating cells in the lamina propria (1); infiltration of mononuclear cells leading to separation of crypts and

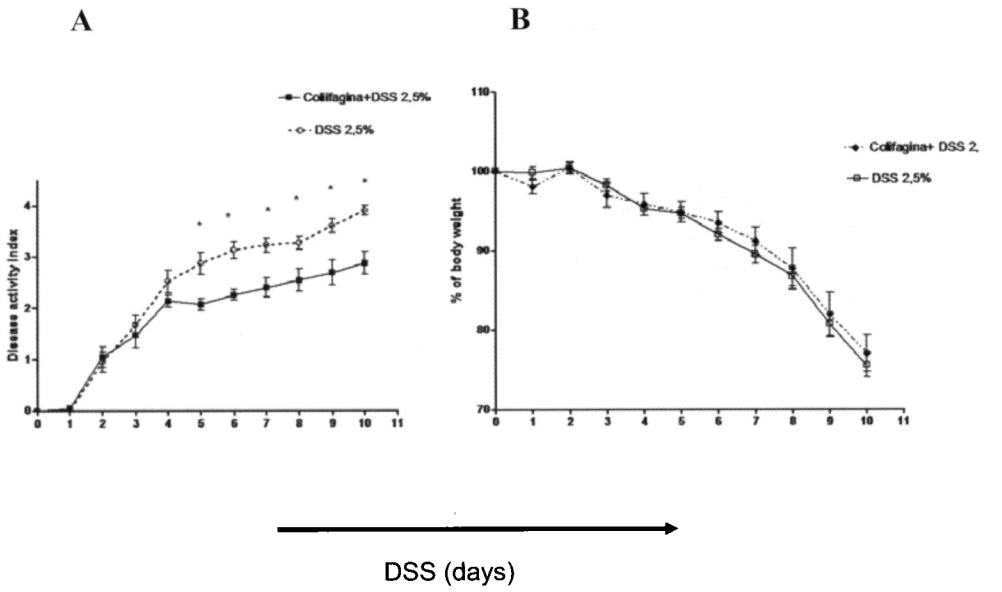

Fig. 1. *Colifagina ameliorates clinical DSS colitis Mice were fed for a period of 10 days with colifagina, or placebo, and monitored daily for disease activity index and weight loss. Colifagina significantly ($p<0.05$) improved disease activity index, but not weight loss. **A** and **B**, *$p<0.05$ *$p<0.05$, $n=10$ per group*

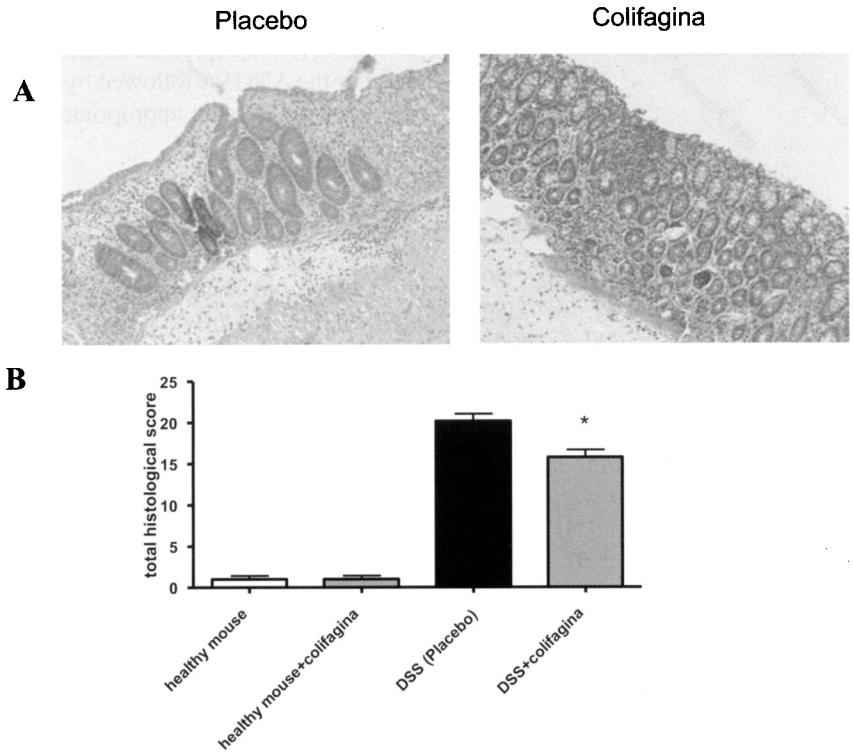

Fig. 2. *Colifagina improves histological inflammation. Mice were fed for a period of 10 days with colifagina, or placebo, and sacrificed after 10 days, and colons assessed for histological inflammation **(A)**. Colifagina treated mice displayed a significant (*$p<0.05$) amelioration of histological inflammation **(B)**. *$p<0.05$, $n=10$ per group*

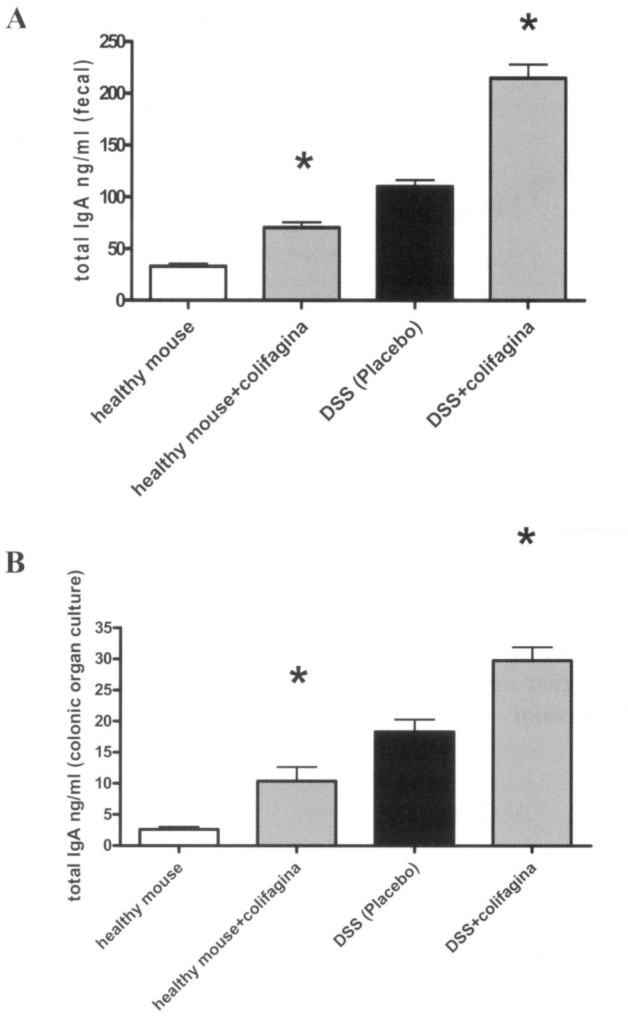

Fig. 3. *Colifagina enhances fecal and colonic secretion of IgA Mice were fed for a period of 10 days with colifagina, or placebo, and sacrificed after 10 days, and a colonic organ culture was established. Total IgA were measured in the supernatants, as well as in the stools of the mice at day 10. Colifagina treated mice displayed a higher IgA content in their stools (p=0.04 for healthy intestine compared to healthy intestine treated with colifagina, and p=0.02 for DSS versus DSS+colifagina, Fig. 3* ***A)*** *as well as in the organ culture (both p=0.03, Fig. 3* ***B)****. (n=10 per group).*

mild mucosal hyperplasia (2); massive infiltration with inflammatory cells accompanied by disrupted mucosal architecture, loss of goblet cells, and marked mucosal hyperplasia (3); all of the above plus crypt abscesses or ulceration (4), with a histological score from 0 to 15.

Colon organ culture

Colons from all mice were excised, opened and cut longitudinally in three parts. One of all three parts was washed in cold PBS supplemented with penicillin, streptomycin and amphoteracin B (BioWhittaker Cambrex, Milan) and incubated in serum free RPMI 1640 medium supplemented with 0.1% FBS, penicillin, streptomycin and amphoteracin B, at 37° in 5% CO2. After 24 hours, supernatant fluid was collected, centrifuged and stored at -20°. Supernatant was analyzed for IL-8, MIP-1, MIP-2, and RANTES by ELISA as reported (12).

Fecal *extracts*

Fecal extracts were prepared as follows. Two fecal pellets were collected from each mouse (housed in separate cages) weighed and then were mixed with 0.5 ml of extraction buffer (30 mM disodium EDTA, pH 7.6, 100 µg/ml soybean trypsin inhibitor, and 10 mg/ml bovine serum albumin in PBS). Pellets were homogenized and centrifuged at 4°C, and supernatants were stored at −20°C until immunoglobulin A (IgA) measurements were performed.

Statistical analysis

Data were analyzed by Graphpad software (San Diego, CA) and expressed as mean ± SEM. The Student's t test or the ANOVA followed by the appropriate post hoc test were used when appropriate. Statistical significance was set at $p<0.05$.

RESULTS

Colifagina ameliorates clinical and histological experimental colitis

In our experiments we used mice undergoing 2.5% DSS treatment in drinking water. In our animal facility, during a period of 10 days, the mice showed typical signs of disease such as a decrease in body weight, loose stools and presence of blood in stools.

To test the capacity of colifagina to ameliorate experimental colitis, the mice were fed for a period of 10 days with colifagina or placebo, and monitored daily for disease activity index and weight loss. Compared to the placebo group, the mice treated with colifagina developed less severe disease resulting in significantly ($p< 0.05$) improved disease activity index. Bacterial-lysate-treated mice revealed significantly fewer signs of colitis than placebo treated mice, as assessed by a 30%

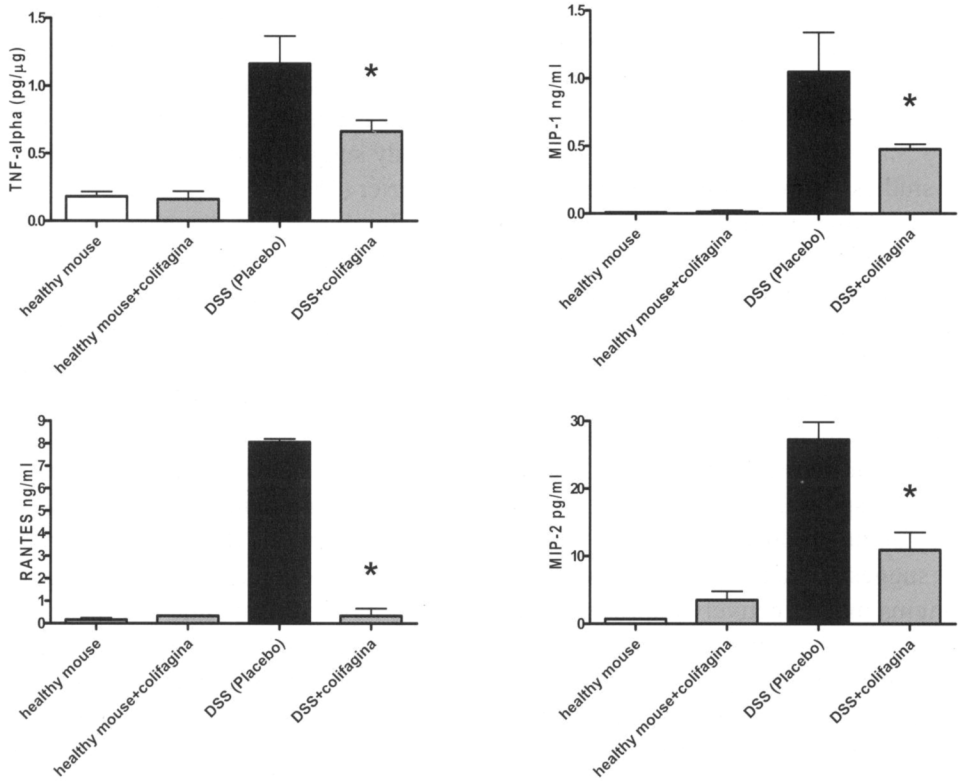

Fig. 4. *Colifagina inhibits inflammatory chemokine secretion Mice were fed for a period of 10 days with colifagina, or placebo, and sacrificed after 10 days, and a colonic organ culture was established. Several chemokines were measured by ELISA. Colifagina treatment significantly inhibited MIP-1 (p=0.04), MIP-2 (p=0.04), TNF-α (p=0.03) and RANTES (p=0.02) secretion (*p<0.05, n=10 per group).*

reduction in DAI ($p<0.05$) (Fig. 1A). Surprisingly colifagina did not have any significant effect on body weight loss (Fig. 1B).

After 10 days of colifagina treatment, the mice were sacrificed and the colons were assessed for histological inflammation. Histological examination of the distal part of the colon of placebo treated mice showed the typical pathological changes of colitis, while mice treated concurrently with colifagina showed significant ($p<0.05$) amelioration of histological inflammation (Fig. 2). No colitis induction was observed in healthy mice treated with colifagina alone. (Fig. 2).

Colifagina enhances fecal and colonic secretion of IgA

After colifagina treatment, the mice were sacrificed at day 10, intestines removed and a colonic organ culture was established. Total IgA were measured in both the colonic organ culture supernatants and in the stools of the mice. Mice treated with 2.5% DSS and colifagina displayed higher IgA levels both in their stools and in colonic organ culture compared with mice receiving placebo and 2.5% DSS ($p<0.05$, Fig. 3A and B). In addition, healthy mice receiving colifagina showed a significant ($p<0.05$) increase in the production of IgA in both stools and colon organ culture with respect to untreated healthy mice (Fig. 3A and B).

Colifagina inhibits inflammatory chemokine secretion

In the same organ culture, besides IgA production, inflammatory cytokine and chemokine levels were also measured by Elisa.

In healthy, untreated mice little to no production of TNF-α, MIP-1, MIP-2 and RANTES was detected, comparable to healthy mice receiving colifagina (Fig. 4). As expected, expression levels of the pro-inflammatory mediators significantly increased in DSS treated mice with respect to healthy mice. Interestingly, DSS colitic mice treated

with colifagina displayed a significant inhibition ($p<0.05$) of TNF-α, MIP-1, MIP-2, and RANTES secretion.

DISCUSSION

Previous studies have demonstrated that probiotics have inhibitory effects on pathogenic bacteria of the gastro-intestinal tract. Several mechanisms by which probiotics enhance protection against pathogens have been suggested, including prevention of colonization of pathogenic bacteria to the GI tract by inhibiting their adhesion via competition, reinforcement of the epithelial barrier by controlling cellular permeability, alteration of the humoral immune response, and regulation of pro-inflammatory cytokine production.

Our data suggests that treatment with the probiotic colifagina may effectively improve clinical experimental colitis. The protection obtained by the use of colifagina correlates well with enhanced mucosal immune responses evidenced by an increase in both fecal and colonic IgA secretion and a relevant decrease in the expression of pro-inflammatory cytokine and chemokines such as TNF-α, MIP-1, MIP-2, and RANTES.

One finding of our study was that administration of colifagina significantly altered the severity of clinical and histological colitis, even though it had no effect on percentage body weight loss.

Notably, colifagina induced a strong production of both fecal and colonic IgA, both in healthy and colitic mice, acting as an enterovaccine. These observations are in line with previous studies that show a significant increase in intestinal antibacterial IgA levels in probiotic fed mice (13-14). Our results therefore strongly suggest that one of the mechanisms through which the probiotic colifagina ameliorates colitis is by improving the intestine's immunological barrier and by enhancing local IgA production. Secretory IgA released from the intestinal mucosal surface is resistant to proteolysis and does not activate complement or inflammatory responses (15). For this very reason, IgA has a central role in the mucosal immune system by preventing the adherence of pathogenic enteric bacteria to the epithelial cells and neutralizing biologically active antigens like bacterial toxins or viruses (16-17). In humans, Lactobacillus GG bacteria, (LGG) have been reported to promote IgA production in children affected by CD. In adult CD patients oral application of L. Casei strain for 10 days led to a significant increase in mucosal IgA levels. The results from this study suggest an improvement of the immunological barrier (18).

In our study we also observed that colifagina administration has the capacity to inhibit the pro-inflammatory cytokines and chemokines TNF-α, MIP-1, MIP-2 and RANTES, thus displaying anti-inflammatory effects. Similarly, other probiotics such as *Lactobacillus* GG Bacteria (LGG) have been largely used in clinical trials and experimental models and have been shown to be able to alter the expression of several pro-inflammatory cytokines including TNF-α. (19) interleukins IL-1 beta, IL-8 and IFN-γ (8, 20).

Taken together, our results suggest that colifagina may have a therapeutic role in ameliorating intestinal inflammation by enhancing gut immune defense by stimulating IgA production, and by down-regulating the secretion of several pro-inflammatory cytokines. In conclusion, our results suggest that lysed bacteria like colifagina may serve as a promising new therapeutic option for the treatment of IBD, but before this data can be translated into clinical trials the findings need to be confirmed by independent groups and other experimental models of colitis, such as the TNBS model, and controlled clinical trials in human patients are also necessary.

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