The role of intestinal bacteria in the pathogenesis of human inflammatory bowel diseases (IBD), particularly Crohn's disease, is well recognised. These diseases typically occur at sites with the highest concentrations of intestinal bacteria, such as the colon and terminal ileum. Antibiotics and faecal diversion are effective treatment modalities for Crohn's disease whereas re-establishing continuity of the bypassed distal bowel or infusion of intestinal contents into the excluded ileum leads to disease recurrence.

The role of intestinal bacteria in the initiation and perpetuation of chronic intestinal inflammation is most convincingly demonstrated in experimental models of chronic intestinal inflammation in which genetically susceptible hosts develop spontaneous colitis in the presence of commensal intestinal organisms whereas no disease occurs in the germ free (GF) state. HLA-B27 transgenic (TG) rats develop colitis, gastritis, and systemic inflammation in a specific pathogen free (SPF) environment. However, GF TG rats and non-TG rats colonised with SPF bacteria show no evidence of colitis, gastritis, or arthritis. Colitis and gastroduodenal inflammation in TG rats is attenuated by metronidazole therapy and can be prevented and treated with broad spectrum antibiotics. Moreover, caecal bacterial overgrowth within an experimental blind loop exacerbates colitis whereas bypass of the caecum attenuates disease in this model, which correlates with concentrations of luminal bacteroides species. Bacteroides vulgatus preferentially induces colitis in TG rats after monoassociation for four weeks whereas monoassociation with Escherichia coli does not cause disease. These findings indicate that not all bacteria are equal in their capacity to induce colitis.

Probiotics are defined as living commensal microorganisms which are important to the health and well being of the host. Lactobacilli, although not predominant enteric organisms, are present throughout the gastrointestinal tract of healthy humans and rodents. Several lactobacillus strains, such as L plantarum 299v, L acidophilus, L fermentum, and Lactobacillus rhamnosus GG, can colonise the human gastrointestinal tract. Several human clinical trials have shown the efficacy of species of probiotics in viral and bacterial induced infectious colitis as well as in antibiotic associated diarrhoea. Rectal administration of native murine L reuteri prevented colitis in interleukin (IL)-10 deficient mice and a similar effect was shown by the oral probiotic lactulose, which stimulated the growth of endogenous lactobacilli species. Continuous oral treatment of LP 299v in GF IL-10 deficient mice colonised with SPF flora attenuated established colitis. A probiotic preparation including four species of lactobacilli, three bifidobacteria, and one Streptococcus salivarius (VSL#3) maintained remission of refractory pouchitis after transient antibiotic therapy. This probiotic cocktail VSL#3 was also beneficial in the treatment of colitis in IL-10 deficient mice.

The first aim of our study was to investigate if the probiotic species L GG can prevent colitis in B27 TG rats monoassociated...
with the disease inducing *B. vulgatus*. Secondly, we determined if *L. GG* or LP 299v could treat established colitis in SPF B27 TG rats or prevent recurrent disease after broad spectrum antibiotics were stopped. Thirdly, we studied the protective effects of probiotics in the prevention of relapse model.

**MATERIALS AND METHODS**

**Rats**

TG rats of the 33-3-line on an inbred F344 background, bearing several copies of the genes for HLA-B27 and human β2 microglobulin, and their non-transgenic littermates were originally obtained from Dr Joel Taurog, Southwestern Medical School (Dallas, Texas, USA). After caesarean section they were housed in Trexler isolators in GF conditions at the Gnotobiotic Core facility at the College of Veterinary Medicine (Raleigh, North Carolina, USA). SPF TG rats and their non-TG littermates were born and maintained in filter top cages in a pathogen free environment.

**Bacteria**

Freeze-dried cultures of *L. GG* and LP 299v were kindly provided by ConAgra (Omaha, Nebraska, USA). Before administration, lactobacilli strains were grown aerobically overnight at 37°C in MRS broth. The next day bacteria were harvested by centrifugation. After washes, bacteria were resuspended in sterile phosphate buffered saline (PBS) and added to the drinking water. Quantification and purity of bacterial suspensions as bacterial colony forming units (CFU) was performed in serial dilutions of the drinking water on lactobacillus MRS-vancomycin plates. *B. vulgatus* was kindly provided by Dr AB Onderdonk (Harvard University, Cambridge, Massachusetts, USA) and was originally derived from guinea pigs with carrageenan induced colitis. This strain has been used to induce colitis in monoassociated TG rats. *B. vulgatus* was cultured anaerobically in BHI broth before colonisation of gnotobiotic rats.

**Experimental design**

**Prevention study**

In the first part of the study, GF TG rats and their non-TG littermates were divided into three groups of six rats each at the age of two months. The first two groups were monoassociated with either *B. vulgatus* or *L. GG* for four weeks, the third group was selectively colonised with *B. vulgatus* for four weeks but was colonised with *L. GG* for two weeks before co-colonisation with *B. vulgatus*. Colonisation with *B. vulgatus* was performed by oral and rectal swabbing using stool from other *B. vulgatus* monoassociated rats. *L. GG* or LP 299v was administered once in the drinking water at 10^8 CFU/rat/day after overnight culture at 37°C in MRS broth. The amount of bacteria consumed by each rat per day was calculated by measured consumption of 30 ml of water per day times 3.3×10^6 CFU/ml=10^8 CFU. Successful colonisation was assessed after one week by plating stool suspensions on bacteroides bile esculin (BBE) and MRS plates for bacteroides spp and lactobacillus, respectively, for two days of anaerobic culture, as well as by performing Gram stains. No contaminating organisms were discovered. Rats were killed after four weeks of *B. vulgatus* or *L. GG* colonisation. At necropsy caeca and colons were fixed in 10% buffered formalin for histological evaluation. Caecum and colons were collected for histology and snap frozen for MPO and cytokine analysis, as described below. Caecal contents were collected for quantitative microbiological analysis.

**Inhibition of in vitro adherence to IEC-6 cells**

The rat epithelial cell line IEC-6 cells (ATCC # CRL 1592; Rockville, Maryland, USA) were grown to confluency in a 12 well tissue culture plate in antibiotic free Dulbecco’s modified Eagle’s medium containing 5% heat inactivated newborn bovine serum and 10 IU (international units)/ml insulin (Life Technologies, Burlington, Ontario, Canada).

Intestinal epithelial adherence assay was performed according to Mack and colleagues. Briefly, after removal of non-adherent cells, an adherent IEC-6 monolayer was incubated at 37°C with 10^7 CFU *B. vulgatus* in the presence or absence of 10^7, 10^8, or 10^9 CFU *L. GG* per well in 2 ml of antibiotic free complete medium under aerobic conditions. After three hours of incubation, non-adherent bacteria were removed by washing three times with warm PBS, IEC-6 cells plus adherent bacteria were collected by trypsinisation. Collected cells and adherent bacteria were then serially diluted and plated anaerobically on BHI plates for 48 hours. Bacteria were counted by colony identification, confirmed by Gram staining.

**Statistical analysis**

Statistics were performed using the Student’s *t* test or Mann-Whitney test for comparison between groups. Significance was considered to be *p*<0.05. All data are presented as mean (SEM).
RESULTS

Prevention study

Monoassociation of TG rats with \textit{B. vulgatus} resulted in mild caecal and colonic inflammation (histology score for the caecum 1.5 (0.1) and colon 1.2 (0.1)). However, pretreatment of GF TG with \textit{L. GG} followed after two weeks by co-association with \textit{B. vulgatus} did not prevent the development of mild colitis induced by \textit{B. vulgatus} (histology scores for the caecum 1.6 (0.2) and colon 1.1 (0.2)). However, monoassociation of gnotobiotic TG rats with \textit{L. GG} did not induce colitis (caecal and colonic scores both 0.2 (0.1)). Similarly, gnotobiotic non-TG rats colonised with \textit{B. vulgatus}, \textit{L. GG}, or both did not develop colitis (histology score for the caecum or colon 0.2 (0.1)).

Caecal IL-1\(\beta\) concentrations from \textit{B. vulgatus} monoassociated TG rats (12 (3) pg/mg caecal wet weight) were not significantly different from those obtained from \textit{B. vulgatus} and \textit{L. GG} co-associated TG rats (11 (2) pg/mg). Caecal IL-1\(\beta\) concentrations from \textit{L. GG} monoassociated TG rats (5 (2) ng/g) were similar to those from gnotobiotic non-TG littermates colonised with \textit{B. vulgatus}, \textit{L. GG}, or both (2 (1) pg/mg).

The concentration of bacteroides spp in the caecal content of TG rats co-associated with \textit{B. vulgatus} and \textit{L. GG} did not differ significantly from \textit{B. vulgatus} monoassociated TG rats (61 (8)\( \times 10^9 \) CFU/g caecal content in \textit{B. vulgatus} and \textit{B. vulgatus} plus \textit{L. GG} associated TG rats, respectively). The concentration of caecal \textit{L. GG} (0.45 (0.04)\( \times 10^9 \) CFU/g) in the \textit{B. vulgatus} plus \textit{L. GG} co-associated rats was 100-fold lower than \textit{B. vulgatus} concentrations but did not differ significantly from the concentration of caecal \textit{L. GG} in \textit{L. GG} monoassociated TG rats.
rats (0.49 (0.10) x 10^9 CFU/g). Gram staining, and aerobic and anaerobic culture of caecal contents from gnotobiotic rats excluded contamination with other organisms.

**Prevention of colitis relapse in SPF B27 TG rats**

Oral administration of broad spectrum antibiotics (oral imipenem/vancomycin) for two weeks to SPF B27 TG rats followed by cessation of this treatment resulted in relapse of colitis after four weeks. Caecal gross gut scores (1.7 (0.2)) as well as total colonic histology scores (1.7 (0.2)) of antibiotic-treated rats four weeks after stopping antibiotics did not differ from untreated control TG animals (caecal gross score 1.7 (0.2), total colonic histology score 1.7 (0.1)) (figs 1–3). Also, LGG administration alone could not effectively treat disease (caecal gross score 1.3 (0.3), total colonic histology score 1.8 (0.2)). However, if antibiotic treatment was followed by daily oral administration of LGG, relapse of colitis could be partially prevented, as shown by a significant decrease in caecal gross score (0.6 (0.1)) and total colonic histological inflammatory score (1.1 (0.3)) of this treatment group (figs 1–3). In contrast with LGG treatment, oral administration of LP 299v alone or after antibiotic treatment had no effect; neither caecal gross scores (data not shown) nor colonic histology scores (fig 3) differed significantly between treatment groups.

Caecal MPO values reflected the gross and histological scores; only TG rats treated with antibiotics followed by LGG showed a significant reduction in caecal MPO (5.6 (1.0) U/g tissue) versus all other treatment groups whereas MPO in caecal homogenates from TG rats treated with antibiotics alone (20.3 (5.6) U/g) or with LGG alone (10.2 (1.4) U/g) did not differ from untreated TG controls (13.3 (3.7) U/g) (fig 4). Caecal MPO from TG rats which had been fed L infantum with or without antibiotic pretreatment did not differ significantly from other treatment groups (data not shown).

**Mucosal cytokines**

Caecal homogenates from SPF TG rats treated with antibiotics and/or LGG contained significantly less IL-1β than the other treatment groups (fig 5A). A similar pattern was shown for caecal TNF, although TNF concentrations were about 10-fold less than caecal IL-1β (fig 5B). Interestingly, IL-10 concentrations were significantly increased in caecal homogenates from antibiotics-LGG treated TG rats versus other treatment groups whereas caecal TGF-β levels were no different among the groups (fig 5B).

**Bacteriology of caecal contents**

No differences were found in total bacterial counts of caecal contents between the antibiotic and/or LGG treatment groups in SPF TG rats. Also, concentrations of caecal anaerobic and aerobic bacteria, as determined by culture on Brucella and...
refractory pouchitis with an oral probiotic cocktail VSL\#3, which includes four strains of lactobacilli, three bifidobacteria species, and *Streptococcus salivarius* subsp *thermophilus* after the broad spectrum antibiotic rifaximin was stopped. Similarly, prospective treatment with rifaximin followed by VSL\#3 could also prevent endoscopic postoperative recurrence of Crohn’s disease.$^{27}$

Another major finding of our study was the specificity of prevention of relapsing colitis by various lactobacillus species, as demonstrated by the lack of efficacy of LP 299v in preventing colitis relapse in SPF TG rats after antibiotic treatment. However, this result is in contrast with our previous reports that oral LP 299v attenuated colitis in IL-10 KO mice, whereas *L. GG* had no effect.$^{18}$ A preventive effect of LP 299v was also shown in methotrexate induced enterocolitis in rats.$^{11}$ These contrasting responses suggest that different lactobacilli may have variable host specificity or different efficacy in various inflammatory conditions, indicating that not all probiotics are equally protective in chronic experimental colitis. The concept of selective dependency of beneficial effects of a probiotic bacterial species on the species and genetic background of the host is similar to that of disease inducing bacteria.$^{11,14,25}$

Other studies have reported primary beneficial effects of probiotics in the treatment or prevention of human or experimental intestinal inflammation. VSL\#3 was able to treat established colitis in IL-10 KO mice.$^{7}$ The same cocktail has also shown efficacy in an open labelled study on maintenance therapy of ulcerative colitis.$^{9}$ *L. GG* was previously shown to inhibit *Clostridium difficile* diarrhoea,$^{19}$ traveller’s diarrhoea$^{24}$ and rotavirus infections in children.$^{39}$ The non-pathogenic *E. coli* strain Nissle was shown to be as effective as low dose mesalamine in maintaining remission of ulcerative colitis.$^{40}$

The protective mechanisms of probiotic bacteria are still relatively unknown, with postulated effects on luminal microbiology, mucosal barrier function, and immunoregulation. In our study there was no decrease in the number of luminal bacteroides spp, raising the possibility that some of the protective effects of *LGG* could be explained by immunomodulation. Not only does this therapy result in decreased caecal proinflammatory cytokines IL-1\beta and TNF, but it also increased mucosal IL-10. Lack of stimulation of mucosal IL-10 by *L. GG* or antibiotics alone indicate that neither treatment is responsible for induction of IL-10. It is possible that a combination of antibiotics and probiotics alters the balance of enteric bacterial components, leading to altered mucosal cytokine profiles. IL-10 is an immunoregulatory cytokine, mainly produced by macrophages, dendritic cells, and T regulatory 1 lymphocytes. However, we recently reported that in caecal bacterial lysate stimulated MLN cells of SPF TG rats as well as their non-T-cultures, IL-10 is primarily produced by B cells.$^{33}$ In vitro, lysates of VSL\#3 stimulate IL-10 production and diminish TNF secretion by rat splenocytes stimulated with caecal bacteria.$^{46}$ In preliminary data, it was demonstrated that DNA extracts of VSL\#3 induce IL-10 and exert protective immune responses.$^{47}$ IL-10 can inhibit antigen specific proliferation and cytokine secretion by Th1 lymphocytes and has downregulatory effects on macrophages and dendritic cells, such as suppression of activation and IL-12 production.$^{48,49}$ IL-10 can also prevent interferon \(\gamma\) induced disruption of colonic epithelial barriers.$^{50}$ Defective in vivo production of IL-10 results in spontaneous colitis in IL-10 KO mice in the presence of normal intestinal bacteria.$^{51}$ Furthermore, oral administration of *Lactobacillus acidophilus*, engineered to produce high colonic IL-10 levels, prevents the onset of colitis in IL-10 KO mice as well as in dextran sodium sulphate induced colitis.$^{52}$ Thus stimulation of colonic IL-10 production by the combination of antibiotic use and *L. GG* colonisation could have a variety of beneficial effects on mucosal immunoregulation and barrier function.

Increased mucosal IL-10 production and decreased levels of the proinflammatory cytokines IL-1\beta and TNF by the
combination of antibiotics and L GG in our study are in agreement with the findings of Ulisse et al who showed increased IL-10 as well as decreased TNF and IFN-γ in pouch biopsies of refractory pouchitis patients treated with VSL#3 after antibiotic pretreatment. Similar to our findings, these authors found that tissue IL-10 levels were unchanged in inflamed pouches and were not affected by prior antibiotic treatment. However, in IL-10 KO mice the probiotic cocktail VSL#3 treated colitis with a concomitant decrease of mucosal IFN-γ and TNF secretion, indicating that the beneficial effects of probiotics in colitis can be mediated by factors other than IL-10 induction.

Several non-immune protective mechanisms have been described for probiotic bacterial species. These organisms can exert growth inhibitory and bactericidal activities on enterotoxigenic E coli. However, in our study caecal concentrations of the disease inducing bacteroides spp were not significantly decreased. Another potential protective non-immune mechanism involves inhibition of epithelial adherence of disease inducing organisms. Intestinal bacteria compete with other organisms for a limited number of epithelial receptors. Increased amounts of colonic mucosal associated bacteria were found in IL-10 KO mice as well as in mucosal biopsies from IBD patients. Madsen et al also reported a relative lack of adherent lactobacilli in IL-10 KO mice and restoration to normal levels after probiotic treatment. Our in vitro coculture studies demonstrated that L GG decreased adherence of B vulgatus to rat IEC-6 cells only at high concentrations of 10⁶ CFU/ml, with no effects at lower doses. This result is in agreement with in vitro studies by Madsen and colleagues and Mack and colleagues in which probiotic bacteria prevented the epithelial adherence of Salmonella dublin or enteropathogenic E coli. However, in our study, in vivo co-association of B vulgatus and L GG did not prevent colitis in gnotobiotic TG rats. This lack of effect by L GG in the dual association model could be explained by the overwhelming colonisation capacity of the disease inducing B vulgatus versus L GG, which were 100-fold more frequent than L GG. It is possible that at this relatively low in vivo luminal concentration L GG would not be able to mediate protection by inhibiting epithelial adherence of B vulgatus, as equal concentrations of L GG were required to interfere with B vulgatus epithelial adherence. Based on these findings we postulate that protective effects by probiotics in SPF TG rats could only be achieved by pretreatment of rats with vancomycin/imipenem, which would result in a significant decrease in B vulgatus before L GG treatment. However, despite the prevention of colitis relapse by the combination treatment of vancomycin/imipenem followed by L GG, this treatment did not significantly reduce luminal bacteroides spp, even though L GG was found in caecal luminal content in caecal culture. Taken together, reduction of luminal bacteroides spp could not explain the protective effects of broad spectrum antibiotics followed by L GG. Prevention of colitis relapse as well as immunomodulatory effects were shown only with the combination treatment of vancomycin/imipenem followed by L GG. It is possible that the combination treatment promoted synergistic interaction of L GG with other endogenous probiotic species, resulting in disease prevention. The absence of these potential beneficial interactions with other intestinal protective bacteria in gnotobiotic TG rats co-associated with L GG and B vulgatus could also explain the lack of efficacy of L GG in the prevention of colitis. Alternatively, lack of exposure to lactic acid bacteria early in life in these gnotobiotic rats may prevent stimulation of protective responses by L GG at an older age. In summary, oral L GG can prevent relapse of colitis in SPF B27 TG rats after antibiotics are stopped whereas oral administration of L plantarum did not have any effect, demonstrating the selective protective effects of two lactobacillus species. The beneficial in vivo effects of L GG following antibiotics are possibly the result of enhanced IL-10 production. This is the first study in experimental colitis in which antibiotics and probiotics display synergistic in vivo effects. Restoring the microbial balance between detrimental and protective luminal bacteria by combining antibiotic and probiotic approaches may be the most physiological approach to treat IBD and may alter the natural history of these chronic relapsing diseases.

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