Effect of *Lactobacillus reuteri* on Intestinal Resistance to *Cryptosporidium parvum* Infection in a Murine Model of Acquired Immunodeficiency Syndrome

John I. B. Alak, Bryan W. Wolf, Emmanuel G. Mdurvwa, Gliceria E. Pimentel-Smith, and Oyewole Adeyemo

Center For Biomedical Research and Department of Microbiology, School of Veterinary Medicine, Tuskegee University, Tuskegee, Alabama; Ross Products Division of Abbott Laboratories, Columbus, Ohio

Efficacy of *Lactobacillus reuteri* as a probiotic for the control of *Cryptosporidium parvum* infection was evaluated in C57BL/6 female mice that were immunosuppressed by intraperitoneal inoculation with the LP-BM5 leukemia virus. Four months after inoculation, mice developed lymphadenopathy, splenomegaly, and susceptibility to *C. parvum* infection. After daily prefeeding with *L. reuteri* (10^8 cfu/day) for 10 days, mice were challenged with 6.5×10^6 *C. parvum* oocysts and fed *L. reuteri* during the entire study. Mice supplemented with *L. reuteri* and challenged with *C. parvum* cleared parasite loads from the gut epithelium. However, unsupplemented animals developed persistent cryptosporidiosis and shed high levels of oocysts in the feces. *L. reuteri* feeding increased its colonization of the intestinal tract, which was inversely related to the fecal shedding of oocysts. These findings suggest that *L. reuteri* may help prevent *C. parvum* infection in immunodeficient subjects.

Cryptosporidium parvum causes diarrhea and mortality in humans and young animals with an undeveloped or suppressed immune system [1]. In the immunocompetent host, cryptosporidiosis is self-limiting; however, *C. parvum* infection of immunocompromised persons, including AIDS patients, results in persistent cryptosporidiosis accompanied by weight loss, diarrhea, and dehydration [2]. Although *C. parvum* has not been identified as a direct cause of death, it may accelerate death by inducing severe malnutrition and dehydration [1, 2]. Treatment consists of fluid and electrolyte replacement. Unfortunately, no clinical therapy has been proven efficacious for the control of cryptosporidiosis.

Beneficial therapy may involve probiotic agents, defined as live microorganism(s) that beneficially affect the host by improving the properties of the indigenous microbiota when consumed [3]. This approach is based on observations that intestinal microbiota provide protection against various diseases. For example, germ-free animals are more susceptible to *C. parvum* infection than are their conventional counterparts with complete gut flora [4]. Positive prophylactic effects of probiotic organisms, including lactic acid bacteria such as lactobacilli, streptococci, and bifidobacteria, have been reported [3]. It is hypothesized that consumption of viable intestinal *Lactobacillus* organisms will hasten the return of the intestinal microbiota to a favorable state. One microorganism of particular interest is *Lactobacillus reuteri,* a normal gastrointestinal inhabitant of humans and animals. Glycerol metabolism by *L. reuteri* enhances microbial excretion of the metabolic intermediate, 3-hydroxypropionaldehyde (reuterin), which has been demonstrated to have antimicrobial activity against various microorganisms [5, 6] and possibly *C. parvum.* This antimicrobial activity may aid in the survival of *L. reuteri* in the gastrointestinal tract of the host.

Murine AIDS (MAIDS) provides a useful animal model for studies of cryptosporidiosis and other opportunistic infections during immunodeficient states. This animal model has several advantages over other animal AIDS models in terms of cost-effectiveness, a relatively short onset of the disease, and clinical conditions that parallel those observed in human AIDS, including susceptibility to opportunistic infections [7–9]. Our study was conducted to determine the ability of *L. reuteri* to prevent *C. parvum* infection in immunosuppressed mice and has potential implications for therapy in human AIDS.

Materials and Methods

Animals. Female C57BL/6 mice (3–4 weeks old) were purchased from Harlan Sprague-Dawley (Indianapolis). Mice were housed (5/cage) in a microisolator unit with an air filtration system at Tuskegee University Central Animal Facility (maintained at 20–22°C, 60%–80% relative humidity, and 12-h light-dark cycle). Animals had ad libitum access to water and mouse chow (Purina, St. Louis).

Inoculation of mice with LP-BM5. Mice were inoculated intraperitoneally with 0.30 mL of LP-BM5 murine leukemia virus

Received 27 February 1996; revised 12 August 1996.

Presented in part: annual meeting of the American Gastroenterological Association and American Association for the Study of Liver Diseases at Digestive Disease Week, San Francisco, May 1996 (Alak JIB, Wolf BW, Mdurvwa EG, Pimentel-Smith GE, Adeyemo O. Effect of *Lactobacillus reuteri* on intestinal resistance to *Cryptosporidium parvum* infection in C57BL/6 female mice rendered immunodeficient by prior infection with LP-BM5, a murine retrovirus [abstract]. Gastroenterology 1996;110:A854).

Mice were maintained according to standard guidelines established by Tuskegee University Animal Care Committee on animal health and welfare.

Grant support: NIH/G12 (RR-03059) and Ross Products Division of Abbott Laboratories (Columbus, OH).

Reprints or correspondence: Dr. John I. B. Alak, Center for Biomedical Research, Tuskegee University, Tuskegee, AL 36088.

The Journal of Infectious Diseases 1997;175:218–21

0022–1899/97/7501–0037$01.00

(MuLV) that had an ecotropic titer of 4.5 $\log_{10}$ pfu/mL in an XC cell line (ATCC CCL 165). The stock of MuLV was a gift of R. R. Watson (University of Arizona School of Medicine, Tucson).

Cryptosporidium inocula. Sterilized oocysts of *C. parvum* inocula, purified from experimentally infected calves as described [10], were a gift of J. A. Harp (National Animal Disease Center, Ames, IA).

Preparation of L. reuteri bacteria. Two mouse isolates of *L. reuteri* from the stomach (strain 4000) and small intestine (strain 4020) were obtained from BioGaia Biologics (Raleigh, NC). The *L. reuteri* strains were grown separately and then equal colony-forming units from each strain were pooled to a final concentration of 5×10^8 cfu/mL. Stock *L. reuteri* was supplied as frozen preparations in 0.1% peptone water for inoculation of mice.

Experimental design. In total, 40 C57BL/6 female mice (10 mice/group) were randomly assigned to one of four treatments (A–D) 4 months after LP-BM5 inoculation. The study was divided into a 10-day priming phase, during which 20 mice (groups C and D) were gavaged daily with *L. reuteri* (10^8 cfu in 0.2 mL) or PBS (groups A and B), and an experimental phase of continued PBS and *L. reuteri* supplementation. Fecal samples (3–4 pellets) were collected on days 0 (baseline), 4, and 7 for total *Lactobacillus* species and *L. reuteri* enumeration as described [11]. On day 10, fecal pellets were collected from all mice for the detection of *C. parvum* oocysts as described [7] and enumeration of total *Lactobacillus* species and *L. reuteri*. The experimental phase was initiated on day 11 of the study, when mice (groups B and D) were challenged with 6.5×10^6 *C. parvum* oocysts in 0.2 mL of sterilized PBS. Fecal samples were collected on days 17 and 25 for *L. reuteri, Lactobacillus* species, and *C. parvum* enumeration.

On day 26, mice were sacrificed by ether inhalation, and the abdominal cavities were surgically opened to expose the gastrointestinal tract. Then 1–2 cm of the proximal stomach, distal ileum, and colon were removed from each mouse and fixed in 10% phosphate-buffered formalin (pH 7.4) for enumeration of *C. parvum* burden on the gut epithelium as described [7]. Ileal infection was reported as number of oocysts per centimeter of intestinal cylinder. Data points for mice that had *L. reuteri* levels below the detection limit (5×10^3 cfu/g of wet feces) were conservatively substituted with 5×10^3 (3.7 $\log_{10}$) cfu/g of wet feces for statistical analysis.

Statistical analysis. Data were analyzed using a nested analyses of variance (ANOVA) model with a main effect of treatment (groups A–D) and a nested effect (cage within treatment). A Shapiro-Wilk test was further used to test the residual data from the nested ANOVA for assumption of normality. Treatment means were considered significant if they were different from each other at the 5% level of probability when compared by Tukey's honestly significant differences.

Results

Feed, water consumption, and body weight. Feed intake was similar among all treatment groups (~3 g/mouse/day). However, animals given supplemental *L. reuteri* had higher water intakes than those receiving PBS (4.85 vs. 3.90 mL/mouse/day). Change in body weight was similar across all treatments (data not shown).

Table 1. Effects of feeding *L. reuteri* on fecal shedding of *C. parvum* oocysts and colonization of the distal ileal epithelium of mice immunosuppressed by prior inoculation with LP-BM5 and challenged or not challenged with *C. parvum*.

Group*	Fecal shedding† (no. of oocysts × 10^3/g ± SE) Day 0	Day 7	Day 14	Ileal colonization (no. of oocysts × 10^3/cm of intestine ± SE)
A	0.00	0.00 ± 0.00‡	0.00 ± 0.00‖	0.00 ± 0.00‡
B	0.00	1.58 ± 0.24§	9.19 ± 4.29§	4.00 ± 1.13§
C	0.00	0.00 ± 0.00‡	0.00 ± 0.00‖	0.00 ± 0.00‡
D	0.00	1.34 ± 0.33§	0.46 ± 0.13‡	0.00 ± 0.00‡

* 10 mice/group (5 mice/cage). Groups C and D were supplemented with *L. reuteri;* groups B and D were challenged with *C. parvum.*

† Days after *C. parvum* challenge.

‡,§,‖ Values within same column with unlike superscript symbols differ ($P < .05$). Data were analyzed by analysis of variance, Tukey's honestly significant differences test.

Shedding of C. parvum. No *C. parvum* oocysts were detected in feces of mice not challenged with *C. parvum* (groups A and C). However, mice challenged with the parasite (groups B and D) developed persistent cryptosporidiosis (table 1). Infection with *C. parvum* without *L. reuteri* supplementation (group B) increased ($P < .05$) shedding of oocysts at 7 and 14 days after infection. While there was no difference ($P > .05$) in oocyst shedding between groups B and D at 7 days after infection, shedding was reduced ($P < .05$) at 14 days after challenge in mice fed supplemental *L. reuteri* (group D). Furthermore, *Cryptosporidium* parasite loads were cleared from the intestinal epithelium (specifically the distal ileum) of group D mice. In addition, no parasites were detected in the intestinal villi of uninfected mice (groups A and C). However, significant ($P < .05$) parasite burdens were detected in the intestines of mice (group B) infected with *C. parvum* alone (table 1). Contrary to the colonization of the distal ileum, no *C. parvum* parasites were observed in stomach or colon tissues of challenged mice.

Lactobacillus colonization. Fecal levels of total *Lactobacillus* species were similar across all treatments for the duration of the study (table 2). Only on day 17 were statistically different levels found (group B > A). The level of *L. reuteri* in the feces was similar ($P > .05$) across all treatments at day 0 (baseline). On day 4, treatment groups C and D had higher ($P < .05$) levels of *L. reuteri* and tended to have higher levels on day 7. However, on days 10, 17, and 25, all mice except group B consistently shed high levels of *L. reuteri* in feces.

Discussion

Infection of mice with LP-BM5 induced MAIDS with progressive splenomegaly, lymphadenopathy, and increased susceptibility to *C. parvum* similar to human AIDS, as reviewed

Table 2. Effects of feeding *L. reuteri* on fecal level of total *Lactobacillus* species and *L. reuteri* in C57BL/6 female mice immunosuppressed by prior inoculation with LP-BM5 and challenged or not challenged with *C. parvum*.

Group*	Day after *L. reuteri* feeding					
	0	4	7	10	17	25
	Total *Lactobacillus* species					
A	8.90 ± 0.11	9.11 ± 0.12	9.07 ± 0.12	9.25 ± 0.06	8.49 ± 0.17†	9.15 ± 0.10
B	8.83 ± 0.11	8.92 ± 0.06	9.04 ± 0.07	9.00 ± 0.08	9.18 ± 0.10‡	9.07 ± 0.16
C	8.71 ± 0.11	9.77 ± 0.12	9.33 ± 0.08	9.22 ± 0.15	9.01 ± 0.10†‡	9.07 ± 0.08
D	8.60 ± 0.08	9.70 ± 0.11	9.35 ± 0.15	9.12 ± 0.20	8.93 ± 0.13†‡	8.98 ± 0.08
	L. reuteri					
A	4.85 ± 0.48	4.67 ± 0.51†	5.29 ± 0.60†‡	6.12 ± 0.68	6.88 ± 0.24	7.15 ± 0.41
B	4.25 ± 0.28	4.65 ± 0.39†	5.17 ± 0.47†	5.29 ± 0.65	5.76 ± 0.69	5.01 ± 0.63
C	4.33 ± 0.34	6.79 ± 0.60‡	6.75 ± 0.39†‡	8.20 ± 0.19	6.66 ± 0.51	7.64 ± 0.14
D	5.13 ± 0.39	7.48 ± 0.45‡	6.88 ± 0.49‡	7.97 ± 0.23	7.26 ± 0.18	8.16 ± 0.07

NOTE. Data are $\log_{10}$ cfu/g ± SE.

* 10 mice/group (5 mice/cage). Groups C and D were supplemented with *L. reuteri;* groups B and D were challenged with *C. parvum.*

†‡‖ Values within same column with unlike superscript symbols differ ($P < .05$). Data were analyzed by analysis of variance, Tukey's honestly significant differences test.

by Watson [9]. Unlike human AIDS, which is associated with weight loss and sometimes life-threatening diarrhea, MAIDS in this study did not manifest these conditions.

Fecal levels of total *Lactobacillus* organisms were similar across all experimental treatments over all time points. Maximum colonization (as measured in feces) by *Lactobacillus* species (10^9 cfu/g) suggests possible mechanisms controlling the microbiota populations in the intestinal tract. Similar results have been noted by others in studies with supplemental lactobacilli feeding [11, 12]. However, it is difficult to know if the levels enumerated in fecal samples mimic the levels within the distal ileum, where lactobacilli are most dominant.

L. reuteri levels were similar among treatment groups at baseline, although *L. reuteri* was enumerated from several animals prior to their receiving it as supplementation. This result is not surprising, because *L. reuteri* is a ubiquitous organism of the small intestine of animals and humans [13]. Fecal levels tended to rise in mice supplemented with *L. reuteri* throughout the study. In addition, *L. reuteri* levels tended to rise in group A mice (PBS-supplemented, no *C. parvum* challenge). The progressive immunodeficiency that developed in these animals after LP-BM5 infection might have facilitated the intestinal growth of indigenous *L. reuteri* in all animals.

There was an inverse relationship between *L. reuteri* numbers and clearance of *C. parvum* parasites from the intestinal tract in treatment groups B and D. Thus, mice infected with *C. parvum* and concomitantly fed *L. reuteri* (group D) cleared parasite loads from the intestinal tract. However, mice infected with *C. parvum* alone (group B) shed oocysts persistently but were less colonized by *L. reuteri* (tables 1, 2).

The mechanisms by which *L. reuteri* inhibits the growth of *C. parvum* are not known. It is speculated that *L. reuteri* may inhibit the growth of other bacteria and parasites in the gut microbiota by the secretion of inhibitory products, including reuterin, which has antimicrobial activity against potential pathogens such as *Salmonella, Listeria, Clostridium,* and *Escherichia* species [5, 6]. The inhibitory effects of this by-product of *L. reuteri* may adversely affect the survival of several intestinal parasites, including *C. parvum,* in this MAIDS model.

In addition, *L. reuteri* may compete for binding sites on the gut epithelium, inhibiting *C. parvum* attachment and proliferation. *L. reuteri* strains isolated from mice were utilized in this study because it has been shown that *L. reuteri* strains are species-specific. For example, Molin et al. [14] fed 1 human isolate and 1 rat isolate to rats and found that the rat isolate colonized the intestinal mucosa while the human isolate did not. This finding supports the hypothesis that the mucosal colonization ability of lactobacilli is host-specific [15]. Furthermore, it has been suggested that the normal intestinal flora mediate a nonspecific immune response, enhancing resistance to *C. parvum* infection [4]. In conclusion, this study provides evidence that *L. reuteri* may be beneficial for the prophylactic treatment of cryptosporidiosis in immunocompromised subjects.

Acknowledgments

We thank Sreevani Kolavala and Maurice Curtis for expert technical assistance in data collection and assay procedures, Walter J. Sapp and Keith A. Garleb for editing the manuscript, Ivan A. Casas for coordinating the enumeration of lactobacilli from the mouse samples, and Debra Gonyon Ataya for assistance in the statistical analysis of data.

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