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Passive Immunization of Hamsters against Disease Caused by *Clostridium difficile* by Use of Bovine Immunoglobulin G Concentrate

DAVID M. LYERLY,[1]* EILEEN F. BOSTWICK,[2] STEVEN B. BINION,[2] AND TRACY D. WILKINS[1]

Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061,[1] and PROCOR Technologies, Inc., Minneapolis, Minnesota 55440-0116[2]

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Gestating Holstein cows were vaccinated with *Clostridium difficile* toxoid prepared from the culture filtrate of a strain that produces high levels of toxins A and B and other antigens. A bovine immunoglobulin G (IgG) concentrate was prepared from colostrum collected at parturition. The results of our studies showed that hamsters treated prophylactically with the hyperimmune bovine IgG concentrate were protected against *C. difficile* disease. These results suggest that orally administered hyperimmune bovine IgG specific for *C. difficile* culture filtrate may be useful in prophylaxis against *C. difficile* disease.

Clostridium difficile, which causes antibiotic-associated diarrhea and colitis in humans, is a major nosocomial pathogen, especially in elderly debilitated patients (4, 17). The disease develops as a result of the production of two large toxins, toxin A (M_r, 308,000) and toxin B (M_r, 279,000), by the organism in the colon (1, 7, 17). Toxin A is believed to cause most of the gastrointestinal symptoms because of its enterotoxic activity in experimental animals (4, 18, 19). There is some evidence suggesting that the toxins act synergistically during the course of the disease and that the initial tissue damage caused by toxin A allows toxin B to exert its toxic effect (19).

Most patients with *C. difficile* disease are treated effectively with vancomycin or bacitracin. Relapses occur in about 10% of the cases, however, indicating that antibiotic therapy is not always completely effective. As a result, research on alternative types of therapy is continuing. *Saccharomyces boulardii*, a yeast used to treat gastrointestinal illness, has shown promising results as a probiotic for the treatment of the disease in humans and in experimental animals (6, 9, 13, 23, 25). The actual manner in which the yeast confers protection is unclear, although it has been reported that the yeast must be viable in order to provide protection (15). Immunoprophylaxis has also been suggested as a type of treatment. It is known that vaccination against toxins A and B stimulates active immunity against *C. difficile* disease in experimental animals (11, 14). At the present time, however, suitable vaccines against the organism and its toxins have not been developed for individuals at high risk, and it is still unclear whether active immunization is appropriate. Alternatively, treatment by passive immunization has been suggested. In preliminary studies, we found that serum antibodies against a toxigenic isolate of *C. difficile* protected hamsters against *C. difficile* disease when administered orally to the animals (20). Thus, passive immunity may be beneficial as a treatment.

In the following study, we tested the ability of bovine immunoglobulin G (IgG) concentrate (BIC) from the colostrum of cows vaccinated with *C. difficile* toxoid to protect hamsters against experimental antibiotic-associated cecitis. Passive immunization with bovine antibodies has been examined as a possible alternative therapy in the treatment of other infectious diseases of the gastrointestinal tract, including diseases caused by rotavirus, enteropathogenic and enterotoxigenic *Escherichia coli*, *Vibrio cholerae*, and *Cryptosporidium parvum*, and the results indicate that antibodies administered in this manner provide protection (2, 5, 10, 12, 21, 24, 27). Passive immunity from bovine antibodies offers the advantages that most animals and humans tolerate the material given orally and that the predominant antibody species present, immunoglobulin G1 (IgG1), is relatively resistant to proteolysis.

For our study, toxoid was prepared from *C. difficile* VPI 10463 culture filtrate, which contains high levels of toxins A and B. The strain was grown in brain heart infusion dialysis flasks at 37°C for 72 h as previously described (22) for the production of culture filtrate. The culture filtrate was subsequently converted to toxoid by adding 1/100 volume of formalin and incubating the mixture at 37°C for 1 h. Analysis of the toxoid by tissue culture assay and mouse assay demonstrated that it retained <1% of its original cytotoxic and lethal activity. The challenge strain used in the animal model was *C. difficile* VPI 7698, which was isolated from a patient with pseudomembranous colitis and which produces intermediate levels of toxins A and B. Both bacterial strains were obtained from the anaerobe collection housed in the Department of Anaerobic Microbiology at Virginia Polytechnic Institute and State University (Blacksburg, Va.).

We used a total of 14 gestating Holstein cows. Dairy cows were maintained according to generally accepted dairy management practices at the Land O'Lakes Answer Farm (Webster City, Iowa) or the research farm at Virginia Polytechnic Institute and State University. The cows were injected subcutaneously with 5 ml of toxoid (ca. 5 mg protein) emulsified in 5 ml of incomplete Freund adjuvant. Cows received multiple immunizations which began at least 60 days prior to the expected calving date. Test bleed samples were taken at the time of injection.

Colostrum was collected from the first six milkings of hyperimmunized cows and frozen within 2 to 3 h of collection. Colostrum was stored frozen until ready for processing. Briefly, the colostrum from all 14 cows was thawed and pooled for further processing. The colostral proteins were

* Corresponding author.

TABLE 1. Neutralizing titers of treatments evaluated for protection against *C. difficile* disease

Treatment[a]	Neutralization titer against[b]:	
	Toxin A	Toxin B
Enfamil	<20	<20
Nonimmune BIC	<20	<20
Nonimmune BIC and Enfamil	<20	<20
Hyperimmune BIC	160	2,560
Hyperimmune BIC and Enfamil	80	2,560

[a] Nonimmune BIC was prepared by using colostrum from nonvaccinated cows. Hyperimmune BIC was prepared by using colostrum from cows vaccinated with *C. difficile* VPI 10463 toxoid.

[b] For the assay, samples (0.05 ml) of serial twofold dilutions of each treatment were mixed with samples (0.05 ml) of PBS containing 20 100% tissue cell infective doses of either toxin A or toxin B for 30 min at room temperature and assayed for residual cytotoxic activity. Assays were done in duplicate. The various treatments were prepared as described in the text.

then concentrated, by using a multistep procedure developed by PROCOR Technologies, Inc., to produce an IgG concentrate. The resulting IgG concentrate was dried, giving a shelf-stable powder. A nonimmune IgG concentrate from the colostrum of nonvaccinated cows was prepared in a similar fashion.

Tissue culture assays for the quantitation of neutralizing antibodies against toxins A and B were done as previously described (8). Briefly, serial twofold dilutions of BIC were prepared in phosphate-buffered saline (PBS). Samples (0.05 ml) of each dilution were mixed with an equal volume of PBS containing ca. 20 100% tissue culture infective doses of either toxin A or toxin B. The mixtures were incubated at room temperature for 1 h and assayed for residual cytotoxic activity against Chinese hamster ovary K-1 tissue culture cells. Neutralization titers were expressed as the reciprocal of the highest dilution of antibody that inhibited cytotoxic activity.

For the protection studies, female golden Syrian hamsters (outbred LVG weighing ca. 100 g; Charles River Laboratories, St. Constant, Canada) were administered BIC intragastrically at 8-h intervals three times daily by using a gavage needle. Treatments were begun 48 h prior to the intragastric administration of 3 mg of clindamycin-HCl (Sigma Chemical Co.) in deionized water. The clindamycin served to predispose the hamsters to infection with *C. difficile*. Twenty-four hours after the administration of clindamycin-HCl, each hamster was challenged with 1 ml of PBS containing ca. 10^8 cells of *C. difficile* VPI 7698. The challenge dose was prepared from brain heart infusion broth cultures grown anaerobically at 37°C overnight. Treatments were continued every 8 h through a 10-day period following challenge with the organism. The following six treatments were used for the study: (i) no treatment, (ii) 1 ml of Enfamil low iron infant feeding formula (Mead Johnson Nutritionals, Evansville, Ind.), (iii) 1 ml of nonimmune BIC (0.3 g per ml of deionized water), (iv) 1 ml of nonimmune BIC (0.3 g per ml of deionized water) mixed with 1 ml of Enfamil infant feeding formula, (v) 1 ml of hyperimmune BIC (0.3 g per ml of deionized water), and (vi) 1 ml of hyperimmune BIC (0.3 g per ml of deionized water) mixed with 1 ml of Enfamil infant feeding formula. The incorporation of Enfamil infant feeding formula, which has been used in studies on passive immunity against other bacterial toxins (2), enhanced the protection seen with the hyperimmune BIC in our preliminary studies.

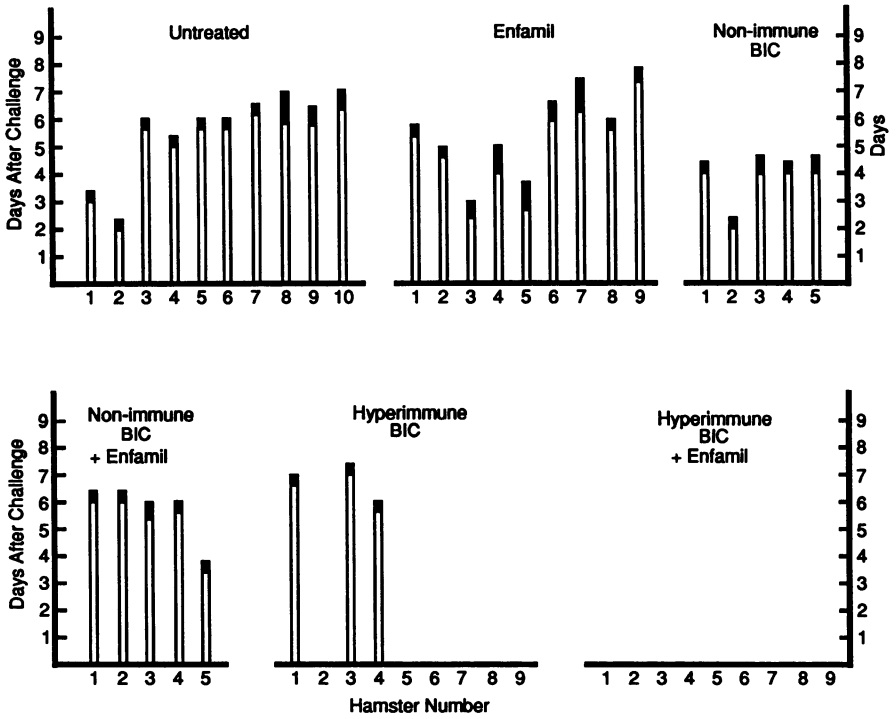

FIG. 1. Protection against *C. difficile* disease in hamsters treated with BIC against *C. difficile* VPI 10463 toxoid. Nonimmune BIC was prepared by using colostrum from nonvaccinated cows. Hyperimmune BIC was prepared by using colostrum from cows vaccinated with *C. difficile* VPI 10463 toxoid. Open bars denote the onset of diarrhea and solid bars denote death.

The neutralization titers of the various treatments are shown in Table 1. Treatments containing hyperimmune BIC were the only ones that neutralized the cytotoxic activity of the toxins. Previous studies from our laboratory showed that hamsters treated with clindamycin and challenged with *C. difficile* VPI 7698 consistently developed diarrhea and died from *C. difficile* disease (16). However, protection was observed when animals were administered hyperimmune BIC (Fig. 1). Untreated hamsters as well as hamsters treated only with infant feeding formula, nonimmune BIC, or a mixture of nonimmune BIC and infant feeding formula developed diarrhea within 2 to 7 days and died within 24 h after the onset of diarrhea, demonstrating a lack of protection against the disease. Hamsters receiving only hyperimmune BIC were partially protected. Six of nine hamsters in this group were completely protected throughout the 10-day treatment period. Hamsters that received a mixture of hyperimmune BIC and infant feeding formula were completely protected from the disease during the 10-day treatment period. Following termination of treatment, the surviving hamsters in the hyperimmune BIC-treated group and the group receiving hyperimmune BIC and infant feeding formula developed diarrhea and died within 72 h. We attempted to treat the hamsters by administering the hyperimmune BIC and infant feeding formula once diarrhea developed, but the treatments were not protective.

Our results show that hamsters treated prophylactically with hyperimmune BIC containing antibodies against *C. difficile* toxoid are passively immunized against the disease if they are treated before the onset of diarrhea. The incorporation of infant feeding formula appeared to enhance the protection, possibly by stabilizing the immunoglobulin, since treatment with infant feeding formula alone had no protective effect. When the treatments were stopped, the animals developed typical *C. difficile* disease, indicating that the protection most likely resulted from the neutralization of the toxins rather than elimination of the organism. Results from previous studies indicate that colonization with nontoxigenic strains of *C. difficile* or normal gut flora protect hamsters against *C. difficile* disease (3, 26). Therefore, oral administration of hyperimmune BIC in conjunction with some other treatment such as the reestablishment of the normal bacterial flora may give complete protection in the hamster model. We were unable to effectively treat the hamsters with the hyperimmune BIC once they developed diarrhea. This may be because hamsters are extremely sensitive to the action of the toxins, and once diarrhea occurs, there is significant tissue damage to the gut mucosa. At this stage of the disease, there probably is systemic intoxication, and neutralizing antibodies in the gastrointestinal tract from orally administered hyperimmune BIC are not sufficient to prevent the death of the animal.

Our findings suggest that orally administered bovine antibodies against toxigenic *C. difficile* may be suitable for persons at high risk. Outbreaks of the disease are continuing to occur, especially in large hospitals and medical centers containing large numbers of elderly patients. In addition, passive immunization may be beneficial for treating immunocompromised patients who chronically relapse with the disease.

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