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Survival of Anti-*Clostridium difficile* Bovine Immunoglobulin Concentrate in the Human Gastrointestinal Tract

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To be therapeutically active, oral hyperimmune bovine immunoglobulin concentrate (BIC) must survive its passage through the intestinal tract. This led us to study the gastrointestinal stability of orally administered BIC directed against *Clostridium difficile* toxins (BIC-*C. difficile*). BIC-*C. difficile* was stable at neutral pH in vitro but was degraded at low pH, particularly in the presence of pepsin. Healthy volunteers ($n = 6$) took BIC-*C. difficile* (45 or 8 g) as a single oral dose. Total bovine immunoglobulin G (IgG) and specific anti-*C. difficile* IgG were measured in the stool. BIC was given under the following conditions: in the fasting state, in the fed state, with antacid, during omeprazole therapy, or in enteric capsules (released at pH > 6). The mean fecal bovine IgG content of 3-day stool collections was similar in the fasting (536 mg; 3.8% of the ingested dose of BIC), fed (221 mg; 1.6%), and antacid (381 mg; 2.7%) groups. Omeprazole therapy was associated with increased fecal bovine IgG levels (1253 mg; 8.8%), but this difference did not reach statistical significance ($P = 0.07$). Administration of 8 g of BIC-*C. difficile* in enteric capsules resulted in substantially higher fecal bovine IgG levels (1,124 mg; 32.7% of the oral dose) than those obtained after administration of nonencapsulated BIC (22 mg; 0.6%; $P = 0.004$). An inverse relationship was noted between intestinal transit time and fecal bovine IgG content ($R = 0.83$; $P = 0.04$ [data from omeprazole group]). Filtrates of stool samples collected after oral administration of BIC-*C. difficile* neutralized the cytotoxicity of *C. difficile* toxins A and B, whereas control stool filtrates did not. Bovine colostral IgG undergoes partial degradation in the intestinal tract. Exposure to acidic gastric secretions and prolonged colonic transit may both contribute to IgG degradation. Nonetheless, humans taking BIC-*C. difficile* orally have neutralizing antitoxin activity in their stool.

Clostridium difficile is the most commonly diagnosed cause of infectious diarrhea in hospitalized patients (15). Therapy with broad-spectrum antibiotics disrupts the normal bacterial flora of the colon, allowing *C. difficile* to colonize the colon (3, 23). Pathogenic strains of *C. difficile* release two large protein exotoxins: toxin A (308 kDa) and toxin B (270 kDa) (2, 3, 9, 15, 23, 34). These toxins injure the colonic mucosa, causing diarrhea and colitis (16, 22, 29, 33). *C. difficile* antibiotic-associated diarrhea and colitis are treated with further antimicrobial therapy, specifically, metronidazole or vancomycin (14, 15). These agents are very effective in the short term, but approximately 20% of patients have relapses when therapy is discontinued. Some patients suffer multiple relapses, and this complication may be extremely difficult to resolve (14, 15). It appears likely that therapy with metronidazole or vancomycin predisposes patients to relapsing diarrhea by causing a further disruption of the colonic microflora. Thus, there is a need for an effective treatment for *C. difficile* colitis that also facilitates the return of the normal colonic bacterial flora.

We previously reported that a hyperimmune bovine colostral antibody preparation is capable of neutralizing the biological effects of *C. difficile* toxins A and B (17, 24). Bovine colostrum is rich in antibodies and contains 50 to 120 mg of immunoglobulin per ml (26). Unlike human colostrum, which is immunoglobulin A (IgA) enriched, bovine colostrum contains predominately IgG-class immunoglobulins (approximately 92%), with lesser amounts of IgA (7.5%) and IgM (0.5%). Colostral IgG levels against targeted antigens can be increased substantially by immunization. We prepared bovine immunoglobulin concentrate (BIC) directed against *C. difficile* (BIC-*C. difficile*) from the colostral milk of Holstein cows by immunization with *C. difficile* toxoids during the animals' late gestation period. BIC-*C. difficile* contains high levels of bovine IgG directed against *C. difficile* toxins A and B, neutralizes the cytotoxic effects of these toxins in vitro, and inhibits their enterotoxic effects in animal models of *C. difficile* enterocolitis (17, 24). The presumed mechanism of action of BIC-*C. difficile* is antibody-mediated blocking of toxin binding to its intestinal receptors (17). These findings suggest that BIC-*C. difficile* may be a useful nonantibiotic agent for the treatment or prevention of *C. difficile* diarrhea.

In order to be therapeutically active against *C. difficile* diarrhea, a portion of orally administered BIC-*C. difficile* must survive digestion and reach the colon intact. Thus, anti-*C. difficile* bovine IgG must resist degradation by gastric acid, pepsin, and pancreatic and small intestinal digestive enzymes as well as proteolytic enzymes released by the colonic microflora. Our initial in vitro studies indicated that acid-pepsin digestion is an important factor in bovine IgG degradation. We then went on to examine the gastrointestinal stability of BIC-*C. difficile* taken orally by healthy human volunteers. In this study we determined the amount of BIC-*C. difficile* which survives degradation in the gastrointestinal tract by measuring total and specific (anti-*C. difficile*) bovine IgG in the feces. We also studied BIC-*C. difficile* survival when gastric acid was neutral-

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ized with an antacid, when acid secretion was blocked with a proton pump inhibitor, or when BIC was shielded from the effects of gastric acid by enteric encapsulation. We find that although BIC-*C. difficile* is partially degraded in the gastrointestinal tract (particularly by exposure to acidic gastric secretions), bovine IgG is present in human feces after BIC-*C. difficile* ingestion, where it retains its ability to neutralize *C. difficile* toxins A and B.

MATERIALS AND METHODS

Preparation of BICs. BICs were prepared by GalaGen Inc. from the colostral milk of Holstein cows as described previously (17, 24). Two different BIC preparations were used in these studies: (i) *C. difficile* filtrate BIC from cows immunized with a toxoid of filter-sterilized conditioned medium from a culture of toxigenic *C. difficile* containing both toxin A and toxin B and (ii) *C. difficile* toxin A BIC from cows immunized with a toxoid of purified *C. difficile* toxin A. Total bovine IgG levels in the BIC preparations were measured by single radial immunodiffusion (RID) assay (ICN ImmunoBiologicals, Cosa Mesa, Calif.) (10, 17). The anti-*C. difficile* filtrate BIC used in these clinical studies contained 31.6 mg of IgG per 100 mg of powdered concentrate, and the anti-*C. difficile* toxin A BIC contained 43.0 mg of IgG per 100 mg of powdered concentrate.

Antitoxin activity of BIC-*C. difficile*. The levels of bovine IgG to *C. difficile* antigens were measured by enzyme-linked immunosorbent assay (ELISA) as described previously (13, 17, 20). The BIC prepared against *C. difficile* culture filtrate showed high levels of bovine IgG against both *C. difficile* toxin A and toxin B. The BIC prepared against *C. difficile* toxin A contained even higher levels of bovine IgG against *C. difficile* toxin A but had little anti-toxin B IgG (17).

Cytotoxicity was determined by rounding of fibroblasts (R9AB; American Type Culture Collection, Rockville, Md.) in monolayer culture after exposure to purified *C. difficile* toxin A or B (17). The minimum 50% cytotoxic dose for each toxin preparation was defined as the minimum dose resulting in 50% cell rounding at 24 h (2 to 20 ng/ml for toxin A and 0.06 to 0.002 ng/ml for toxin B in these experiments). Inhibition of cytotoxicity was quantified by adding serial twofold dilutions of BIC-*C. difficile* to twice the minimum 50% cytotoxic dose of each toxin preparation. After 15 min the BIC-toxin mixture was added to the fibroblast monolayer cultures, and cell rounding was assessed after 24 h. As described previously, *C. difficile* filtrate BIC inhibited the cytotoxic effects of both toxin A and toxin B. *C. difficile* toxin A BIC inhibited the cytotoxicity of toxin A but had little neutralizing activity against toxin B (17).

Degradation of BIC-*C. difficile* by acid and pepsin in vitro. *C. difficile* filtrate BIC was dissolved in phosphate-buffered saline (PBS) with various concentrations of hydrochloric acid to achieve a final pH of 1, 2, 4, or 7. These mixtures were then incubated at 37°C for 4 h. Aliquots were collected at intervals and were immediately titrated to pH 7.0 by using 1 M sodium hydroxide. Additional PBS was then added to equalize the final sample volumes. In a second series of experiments pepsin (40 μg/ml; Sigma, St. Louis, Mo.) was added to the solutions prior to incubation at 37°C (5, 8, 27). A third series of experiments used human gastric secretions obtained from four fasting patients who underwent clinically indicated nasogastric aspiration. The pHs of aliquots of these human gastric aspirates were again adjusted to 1, 2, 4, or 7 prior to incubation with BIC. Acid-exposed BIC preparations were assayed for total bovine IgG by RID assay and for specific bovine IgG against *C. difficile* culture filtrate by ELISA (17).

Human study protocol. A single-site, open, crossover, phase I study was performed with healthy volunteers to examine the safety and bioavailability of a single dose of BIC-*C. difficile*. The study was performed under an Investigational New Drug application (BB-IND 3852) submitted to the U.S. Food and Drug Administration. The study protocol was approved by the Human Studies Committee of the Trustees of Health and Hospitals of the City of Boston. The clinical portion of the study was performed at the General Clinical Research Center, Boston City Hospital. An outline of the study plan is presented in Fig. 1.

(i) Study aims. The objectives of the study were (i) to investigate the survival of intact bovine IgG and of specific anti-*C. difficile* bovine IgG after passage through the normal human gastrointestinal tract and (ii) to determine the effect, if any, of oral administration of BIC-*C. difficile* with food, with antacid (to temporarily neutralize gastric acid), during acid antisecretory therapy with the proton pump inhibitor omeprazole, and in enteric-coated capsules on the subsequent survival of BIC passage through the normal human gastrointestinal tract.

(ii) Study subjects. Healthy volunteers ages 18 to 65 years were enrolled in the study. Exclusion criteria were a history of gastrointestinal disease or surgery which might be expected to affect normal gastrointestinal function, a history of clinically significant cow's milk protein allergy, allergy to milk products, lactose intolerance or milk intolerance, evidence of ongoing systemic or infectious disease, or use of medication that might be expected to affect normal gastrointestinal function. Volunteers received monetary compensation for study participation.

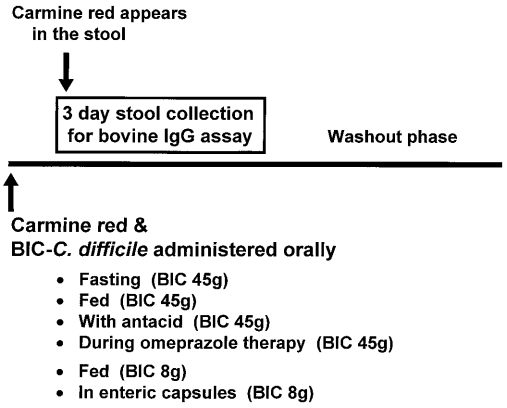

FIG. 1. Outline of study protocol.

(iii) Study groups. There were six study groups, as follows: fasting (45 g), fed (45 g), antacid (45 g), omeprazole (45 g), fasting (8 g), and enteric capsules (8 g) (Fig. 1). For each volunteer a 14-day interval was observed between crossing over to another study group and further BIC-*C. difficile* ingestion. During the study periods, which extended for 5 to 8 days after BIC-*C. difficile* ingestion depending on gastrointestinal transit time, volunteers ate a standardized diet.

BIC-*C. difficile* was administered orally at 9 a.m. after an overnight fast. *C. difficile* filtrate BIC (45 g; containing 14.22 g of bovine IgG) was used for the following study groups: fasting (45 g), fed, antacid, and omeprazole (Fig. 1). Volunteers took 45 g of BIC orally mixed in 360 ml of water. The fasting group had their first meal at 12 p.m., 3 h after BIC ingestion. The fed group took a standard meal at 8 a.m., 1 h before BIC ingestion. The antacid group received 40 ml of Maalox TC, to temporarily neutralize gastric acid, 5 min prior to BIC ingestion (28). The omeprazole group took the proton pump inhibitor omeprazole (Prilosec) at 20 mg twice daily for 5 days prior to BIC ingestion (28).

A lower, 8-g dose of *C. difficile* toxin A BIC (containing 3.44 g of bovine IgG) was used for two study groups: fasting (8 g) and enteric capsules (8 g) (Fig. 1). This lower dose was used to reduce the number of capsules administered and because we anticipated, on the basis of our in vitro data, that enteric coating would substantially increase the amount of bovine IgG delivered intact to the colon. The fasting group took *C. difficile* toxin A BIC (8 g) mixed in 360 ml of water. The enteric capsule group took 21 size 00 capsules each containing 383 ± 12 mg of BIC. These capsules were coated with two layers of cellulose acetate phytate (mean cellulose acetate phytate content per capsule, 196.1 mg). Capsule preparation was performed by the Division of Pharmaceutical Service, College of Pharmacy, University of Iowa. These capsules remained intact for at least 2 h at pH 1 to 2 but released their contents within 30 min at pH ≥ 6.

Stool collection and processing. A capsule containing carmine red was taken by study subjects 1 h before BIC-*C. difficile* ingestion. All stools were collected beginning at the time of BIC ingestion until 96 h after the carmine red was first detected in the stool (Fig. 1). The stool samples were labeled and stored at −20°C.

Processing of stool samples was performed at 4°C. Samples were thawed to 4°C, diluted 1:3 by volume with PBS, and homogenized in PBS (pH 7.0). The final volume of the resulting slurry was recorded. Three aliquots were obtained, and protease inhibitors were added to two of these three aliquots. The final concentrations of protease inhibitors were as follows: aprotinin, 10 μg/ml; leupeptin, 10 μg/ml; phenylmethylsulfonyl fluoride, 10 μg/ml; *N*-*p*-tosyl-L-lysine chloromethyl ketone, 10 μg/ml; and L-1-tosylamido-2-phenylethyl chloromethyl ketone, 10 μg/ml. One aliquot was stored at −20°C. The second aliquot was mixed with the others from the same individual to provide a combined sample representing a 3-day stool collection beginning at the time of appearance of the carmine red marker. The aliquots were mixed pro rata depending on the total volume of each slurry to constitute a representative sample. The stool slurry was centrifuged at ~6,000 × *g* for 30 min and then microcentrifuged (13,000 × *g*) for a further 12 min to remove solid particles prior to performing ELISA or RID assays. The third aliquot, which did not contain protease inhibitors, was filter sterilized and was used for cytotoxicity neutralization experiments.

Special laboratory studies. Total bovine IgG levels in the fecal homogenates were measured by single RID assay (bovine IgG, low level; ICN ImmunoBiologicals). Gels were stained with Coomassie blue to facilitate reading. The resulting immunoprecipitation rings varied in their staining intensities between different fecal samples. Some samples yielded a single ring, whereas others showed two or three concentric rings, in which case the outermost ring was used. The variations in the character of the immunoprecipitation rings formed are likely to reflect partial degradation products of bovine IgG in the fecal samples. Fecal anti-*C. difficile* bovine IgG levels were measured by ELISA as described previously (17).

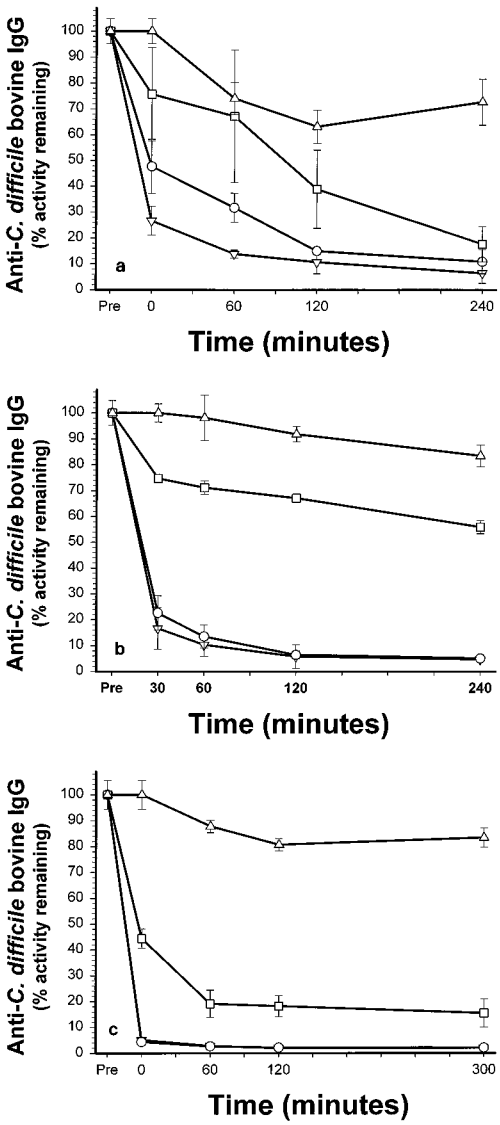

FIG. 2. Degradation of BIC-*C. difficile* by acid and pepsin. Bovine IgG degradation was determined by measuring specific anti-*C. difficile* culture filtrate bovine IgG activity by an ELISA. The amount of activity remaining after incubation of BIC-*C. difficile* in solutions containing hydrochloric acid at various pHs (a), solutions containing hydrochloric acid and pepsin (40 μg/ml) at various pHs (b), and solutions containing human gastric secretions at various pHs are illustrated. Data are presented as means ± standard errors ($n = 3$). △, pH 7; □, pH 4; ○, pH 2; ▽, pH 1.

Statistical analyses. Statistical analyses were performed by using SigmaStat for Windows (version 1.00; Jandel Scientific Software, San Rafael, Calif.). Unless stated otherwise, analysis of variance, in some instances followed by protected *t* tests, were used for intergroup comparisons.

RESULTS

Degradation of BIC-*C. difficile* by acid and pepsin in vitro. As shown in Fig. 2a, anti-*C. difficile* bovine IgG levels fell quickly when BIC-*C. difficile* was exposed to acidic solutions. At pH 1 there was an immediate fall in antibody activity, as measured by ELISA. Acid degradation continued with the passage of time, most noticeably at midrange acidic pH (pH 2 and 4). BIC-*C. difficile* was relatively stable when it was incubated at neutral pH (73% activity remaining after 4 h).

When pepsin was present, bovine IgG degradation at acid pH was even more rapid (Fig. 2b). At pH 7 anti-*C. difficile* bovine IgG levels remained stable (84% activity remaining after 4 h) even in the presence of pepsin. Similar data were obtained when BIC-*C. difficile* was incubated in human gastric secretions (Fig. 2c). Again at pH 1 and 2 there was a rapid, near-complete loss of bovine IgG binding activity, whereas at pH 7, 83% activity remained after 4 h of incubation. Changes in total bovine IgG levels after exposure of BIC-*C. difficile* to hydrochloric acid, acid and pepsin or to human gastric aspirates were similar to those presented above for specific anti-*C. difficile* bovine IgG levels (data not shown).

The toxin-neutralizing activity of BIC-*C. difficile* was also reduced following acid exposure. BIC-*C. difficile* was incubated at 37°C for 1 h at pH 2. This acid treatment was expected to result in approximately 68% loss of anti-*C. difficile* bovine IgG activity, as measured by ELISA (Fig. 2a). The BIC solution was then titrated to pH 7, and its ability to neutralize the cytotoxicity of *C. difficile* culture filtrate was tested in the fibroblast tissue culture assay. There was a fourfold reduction in the cytotoxin inhibition potency of the acid-exposed *C. difficile* BIC compared to that of the control *C. difficile* BIC. This 75% loss of neutralizing activity correlates well with the 68% loss of reactivity seen by ELISA.

Study subjects and sample collection. Six volunteers participated in each stage of the study. Many of the volunteers participated in two or in all three study stages. Overall, 10 volunteers participated (5 males and 5 female; mean age, 26.5 years; age range, 21 to 40 years).

One of the 10 subjects was discontinued from the study during the first study period because of an inability to provide a complete stool collection in accordance with the study protocol. Another subject who had previously completed five study periods had an incomplete stool collection for the capsule study period of study stage 3. This was the result of an intercurrent gastrointestinal illness which was self-limited and was not believed to be related to BIC administration. Other than the two instances described above, all stool collections were complete.

Total bovine IgG levels in stool samples. The mean bovine IgG content of 3-day stool collections from subjects receiving 45 g of BIC-*C. difficile* containing 14.22 g of bovine IgG are presented in Fig. 3. Fasting subjects had a mean 3-day fecal

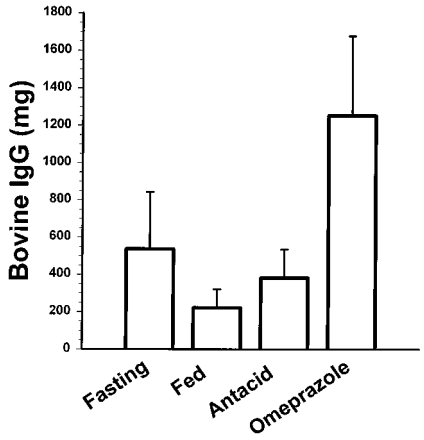

FIG. 3. Total bovine IgG levels in stool collections from subjects receiving anti-culture filtrate BIC-*C. difficile*. A single 45-g oral dose of BIC-*C. difficile* containing 14.22 g of bovine IgG was administered. BIC was administered in the fasting and fed states, with antacid, or during therapy with the proton pump inhibitor omeprazole. Data are presented as means ± standard errors ($n = 6$).

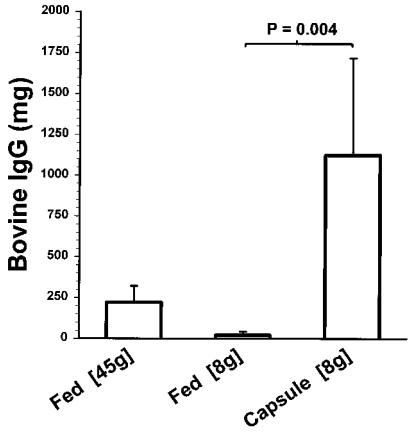

FIG. 4. Total bovine IgG levels in stool collections from subjects receiving anti-toxin A BIC-*C. difficile*. A single 8-g oral dose of BIC-*C. difficile* containing 3.44 g of bovine IgG was administered. BIC was administered in the fed state as a suspension or within enteric-coated capsules. Results for the fed (45 g) study group are also shown for comparison. Data are presented as means ± standard errors ($n = 6$ except for the capsule group, where $n = 5$). Statistical analysis indicates a significant difference between the fed (8 g) and capsule (8 g) groups ($P = 0.004$ by Mann-Whitney rank sum test).

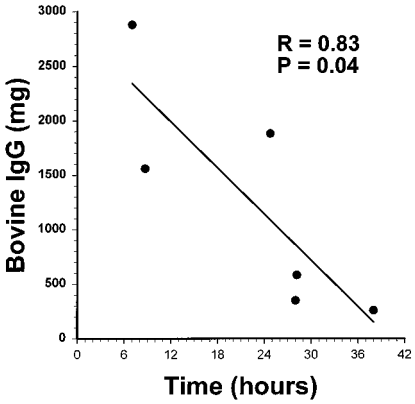

FIG. 5. Gastrointestinal transit time and stool bovine IgG content. Gastrointestinal transit times and bovine IgG levels in stool collections are shown for subjects receiving BIC-*C. difficile* during therapy with omeprazole. Transit time was measured by the time of first appearance of carmine red in the stool. Linear regression analysis indicates an inverse relationship between transit time and stool bovine IgG content.

bovine IgG content of 536 mg, which represents 3.8% of the total ingested dose (Fig. 3). The mean fecal bovine IgG content for the fed group was 221 mg (1.6% of the total dose), and for the antacid group it was 381 mg (2.7% of the total dose). The omeprazole group had the highest fecal bovine IgG content at 1,253 mg (8.8% of the total dose). However, statistical analysis did not reveal any significant difference between the four treatment groups ($P = 0.07$).

Two study groups received 8 g of BIC-*C. difficile* containing 3.44 g of bovine IgG orally either alone or within enteric-coated capsules. When administered alone, without encapsulation, 22 mg (0.6% of the total dose) was recovered in the stool (Fig. 4). However, when the same dose of BIC-*C. difficile* was administered in enteric-coated capsules, a mean of 1,124 mg of bovine IgG was present in the subsequent 3-day stool collections. This represents 32.7% of the total ingested dose of bovine IgG. Statistical analysis indicated a highly significant difference between these two treatment groups ($P = 0.004$ by the Mann-Whitney rank sum test).

Gastrointestinal transit time and stool bovine IgG content. Gastrointestinal transit time was measured by the time of first appearance of carmine red in the stool (range, 5 to 46 h). There appeared to be an inverse relationship between transit time and stool bovine IgG content for the 45-g BIC-*C. difficile* groups. This was most clearly evident in the omeprazole group ($R = 0.83$; $P = 0.04$) (Fig. 5). For the fasting, fed, and antacid groups, linear regression analysis did not reveal a significant correlation between transit time and survival of bovine IgG ($R = 0.44$; $P = 0.07$). However, when a single outlying value was excluded from the analysis, a highly significant inverse correlation was evident ($R = 0.85$; $P < 0.0001$).

In some instances bovine IgG levels were measured in individual stool samples as well as in the combined 3-day stool collections. Two representative examples of the results of these studies are presented in Fig. 6. Figure 6a illustrates the bovine IgG content of stool samples from a subject who took 45 g of *C. difficile* filtrate BIC after an overnight fast. The first stool sample with carmine red marker was passed 24 h and 17 min after BIC ingestion and contained substantial amounts of bovine IgG. A second stool passed after 48 h and 30 min contained little bovine IgG. Subsequent stools contained no detectable bovine IgG. A similar pattern was seen with the second example from a subject who took 8 g of *C. difficile* toxin A BIC in enteric-coated capsules (Fig. 6b).

Anti-*C. difficile* toxin activity in stool samples. As illustrated in Fig. 6 the specific anti-*C. difficile* bovine IgG activity of stool samples correlated with their total bovine IgG content. Anti-*C. difficile* IgG activity, as measured by ELISA, was highest in conjunction with the early peak of fecal total bovine IgG content and was not evident in subsequent stool samples, which lacked detectable bovine IgG.

We next examined whether the ability of BIC-*C. difficile* to neutralize the biological effects of *C. difficile* toxins survived passage through the human intestinal tract. Toxin neutralizing activity was studied by the tissue culture cytotoxin assay (17). As shown in Table 1, stool samples collected shortly after *C. difficile* filtrate BIC administration (Table 1, samples 1a and 2a) neutralized the cytotoxicities of both toxin A and toxin B. Subsequent stool samples from the same individuals which contained no detectable bovine IgG lacked toxin-neutralizing activity (Table 1, samples 1b and 2b). Similarly, *C. difficile* toxin A BIC ingestion resulted in toxin A-neutralizing activity in the

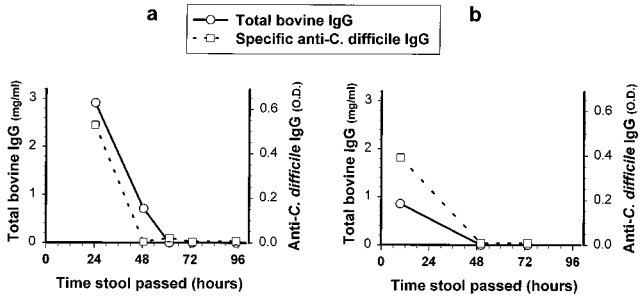

FIG. 6. Time course of detection of bovine IgG in stool after a single oral dose of BIC-*C. difficile*. The total bovine IgG concentration (○) and specific anti-*C. difficile* bovine IgG activity (□) of individual stool samples are shown. BIC-*C. difficile* was administered at time zero, and the carmine red marker appeared with the first stool sample indicated. (a) Data for a subject who received 45 g of anti-culture filtrate BIC-*C. difficile* after an overnight fast. (b) Data for a subject who took 8 g of anti-toxin A BIC-*C. difficile* in enteric-coated capsules. O.D., optical density.

TABLE 1. Cytotoxin neutralizing activity in stool samples taken from four subjects after *C. difficile* BIC ingestion

Stool sample	BIC administered	Time stool passed[a]	Bovine IgG concn (mg/ml)[b]	% Cytotoxicity[c]	
				Toxin A	Toxin B
None			0	>60	>60
1a	Anti-*C. difficile* filtrate BIC[d]	24:15	2.90	0	20
1b	Anti-*C. difficile* filtrate BIC[d]	95:00	<0.10	>60	>60
2a	Anti-*C. difficile* filtrate BIC[d]	7:00	2.41	0	20
2b	Anti-*C. difficile* filtrate BIC[d]	64:00	<0.10	>60	>60
3a	Anti-*C. difficile* toxin A BIC[d]	25:00	6.97	0	30
3b	Anti-*C. difficile* toxin A BIC[d]	48:45	2.41	0	>60
4a	Anti-*C. difficile* toxin A BIC[d]	9:30	0.82	0	>60
4b	Anti-*C. difficile* toxin A BIC[d]	73:15	<0.10	>60	>60

[a] Time in hours:minutes after BIC ingestion.
[b] Bovine IgG concentration of filter-sterilized fecal slurry supernatant.
[c] Percentage of cells rounded in the tissue culture cytotoxicity assay: >60% denotes the absence of any detectable neutralizing activity, and 0% denotes complete neutralization of cytotoxicity.
[d] 45 g, fasting.

stool (Table 1, samples 3a, 3b, and 4a). In this instance, however, toxin B-neutralizing activity was absent (Table 1, samples 3b and 4a) except at very high bovine IgG concentrations (Table 1, sample 3a). The latter observation is consistent with our previous finding that the *C. difficile* toxin A BIC preparation used in this study shows some activity against toxin B at high concentrations (17). Thus, toxin-neutralizing activity is retained, parallels the bovine IgG content of the stool samples, and correlates with the specific antitoxin activity of the ingested BIC-*C. difficile* preparation.

DISCUSSION

This study demonstrates that a portion of orally administered BIC-*C. difficile* survives passage through the human intestinal tract while retaining its specific toxin-neutralizing activity. Previous studies have found that hyperimmune bovine colostral immunoglobulins, taken orally, may provide protection against challenge with various intestinal pathogens including *Shigella flexneri*, enterotoxigenic *Escherichia coli*, rotavirus, and *Cryptosporidium parvum* (7, 12, 25, 31, 32). These reports suggest that bovine colostral immunoglobulins are somewhat resistant to digestion in the human intestinal tract. In a previous study bovine antibodies against *S. flexneri* lipopolysaccharide were detected by immunoassay in the stools of volunteers who had taken anti-*S. flexneri* lipopolysaccharide BIC (32). In this study we also used an immunoassay (ELISA) to detect anti-*C. difficile* bovine IgG in the stool. We further demonstrate that this immunoreactivity is associated with toxin-neutralizing activity. Stool samples which contained anti-*C. difficile* bovine IgG were capable of neutralizing the biological effects of *C. difficile* toxins in the tissue culture cytotoxicity assay. Moreover, the fecal toxin-neutralizing activity correlated with the antitoxin activity of the ingested BIC preparation.

Our in vitro studies indicate that exposure of BIC-*C. difficile* to acidic gastric secretions results in a substantial loss of antitoxin activity (5, 8). These findings led us to administer *C. difficile* BIC in combination with an antacid given at a sufficient dosage to temporarily neutralize gastric acid (28). This maneuver did not result in any evident increase in bovine IgG survival. However, when gastric acid secretion was blocked with a proton pump inhibitor, there was a two- to threefold increase in stool bovine IgG levels. An even more dramatic increase (45-fold) in survival was seen when BIC was sheltered from gastric acid by enteric encapsulation. These results bear relevance to the clinical use of BIC-*C. difficile* and other hyperimmune bovine colostral antibody preparations. They also suggest a role for targeted-release BIC preparations analogous to the delivery systems developed to release 5-aminosalicylates in the small intestine and/or colon for the treatment of inflammatory bowel disease. In the case of *C. difficile* colitis, the release of hyperimmune bovine IgG in the colon would be ideal. For other enteric pathogens such as *C. parvum*, release in the small intestine would be preferable (25).

We observed an inverse relationship between gastrointestinal transit time and bovine IgG survival in the stool. We believe that prolonged exposure of IgG to proteases and other enzymes released by colonic bacteria is likely to contribute to this effect. In this study we used fecal bovine IgG content as a convenient measure of BIC survival. However, this measurement underestimates delivery of intact bovine IgG to the colon since substantial degradation may occur during colonic transit and dwell time. This hypothesis is supported by a study which found that in fasting subjects, 19% of orally administered bovine colostral IgG could be detected intact in the ileum (30), compared to 3.8% recovery from the stool under similar conditions in our study. It is worth noting that gastrointestinal transit times are altered in patients with infectious diarrhea. This may alter BIC-*C. difficile* bioavailability under such conditions.

Active immunization by the parenteral or mucosal route is effective in preventing *C. difficile* enterocolitis in animals (11, 18, 21). Immunized hamsters are colonized with *C. difficile* but have no active toxin in the lumen of their intestine (19). Thus, the mechanism of protection is by toxin neutralization rather than prevention of *C. difficile* infection. Immunization of animals against toxin A alone may protect them from *C. difficile*-induced enterocolitis (6, 19). In humans, however, toxin B also causes damage to the colonic mucosa (29). Hence, effective human immunization is likely to require neutralization of both toxin A and toxin B.

Passive immunization also protects animals against *C. difficile* enterocolitis. Protection may be conferred by the parenteral administration of preformed antitoxin antibodies (1, 6). Oral passive immunization is also effective, as evidenced by protection of infant hamsters after ingestion of breast milk

from immunized mothers (19). Previous animal studies have also shown that BIC-*C. difficile* protects against *C. difficile* toxin-mediated intestinal injury and inflammation (17, 24).

This study, together with our previous in vitro and animal experiments, suggest that BIC-*C. difficile* may be effective in neutralizing the biological effects of *C. difficile* toxins within the colonic lumen. The efficacy of BIC-*C. difficile* may be enhanced by refinements to the animal immunization procedures, leading to the generation of even more potent antitoxin preparations (17). Efficacy may be further improved by enteric coating of BIC to increase the delivery of intact immunoglobulin to the colon. BIC-*C. difficile* is safe and has the theoretical advantage of allowing reconstitution of the colonic microflora. These microflora are arguably the most effective defense against *C. difficile* infection (4, 15). If BIC-*C. difficile* is effective in preventing toxin-mediated diarrhea while simultaneously allowing the normal colonic flora to return, it may be especially beneficial in preventing relapsing diarrhea, which is the most frequent complication of current antibiotic therapies for *C. difficile* colitis (3, 14, 15, 18).

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