Bovine immunoglobulin concentrate—Clostridium difficile retains C difficile toxin neutralising activity after passage through the human stomach and small intestine

M Warny, A Fatimi, E F Bostwick, D C Laine, F Lebel, J T LaMont, C Pothoulakis, C P Kelly

Abstract
Background—Bovine immunoglobulin concentrate (BIC)—Clostridium difficile is prepared from the colostrum of cows immunised against C difficile toxins and contains high concentrations of neutralising IgG antitoxin.

Aims—To determine the proportion of BIC-C difficile which survives passage through the human stomach and small intestine.

Methods—Six volunteers with an end ileostomy took 5 g of BIC-C difficile containing 2.1 g of bovine IgG on four occasions: alone, with an antacid, during treatment with omeprazole, and within enteric coated capsules.

Results—When BIC-C difficile was taken alone, a mean (SEM) of 1033 (232) mg of bovine IgG was recovered in the ileal fluid representing 49% of the total ingested dose. Bovine IgG recovery was not significantly increased by antacid (636 (129) mg) or omeprazole (1052 (268) mg). The enteric capsules frequently remained intact or only partially opened in the ileal effluent and free bovine IgG levels were low in this treatment group (89 (101) mg). Bovine IgG recovery was higher in volunteers with shorter (less than two hours) mouth to ileum transit times (68% versus 36%, p<0.05). Specific bovine IgG against C difficile toxin A was detected in ileal fluid following oral BIC. Toxin neutralising activity was also present and correlated closely with bovine IgG levels (r=0.95, p<0.001).

Conclusion—BIC-C difficile resists digestion in the human upper gastrointestinal tract and specific anti-C difficile toxin A binding and neutralising activity was retained. Passive oral immunotherapy with anti-C difficile BIC may be a useful non-antibiotic approach to the prevention and treatment of C difficile antibiotic associated diarrhoea and colitis.

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Clostridium difficile infection is a common cause of diarrhoea in hospital and nursing home patients.1 2 Antibiotic therapy alters the normal colonic microflora and allows opportunistic infection by C difficile.1 3 Infection is more likely to occur in hospital than in outpatients receiving antibiotics because hospital patients and their environment frequently harbour C difficile and its spores. C difficile diarrhoea and colitis are caused by two protein exotoxins produced by pathogenic strains of the organism.4 Toxin A is a 308 kDa protein with cytotoxic, enterotoxic, and proinflammatory effects. Toxin B, a 280 kDa protein, is a more potent cytotoxin than toxin A, but is not enterotoxic for rodent intestine. However, toxin B does cause injury to human colonic mucosa in vitro.4 Toxin A and toxin B show considerable sequence homology and both share the same intracellular mechanism of cytotoxicity. They catalyse the monoglucosylation of small GTP binding rho proteins leading to disruption of the cytoskeleton, cell rounding, and cell death.5 6 C difficile colitis is currently treated by antimicrobial therapy using metronidazole or vancomycin.1 7 These agents are effective in the short term. However, when treatment is discontinued up to 20% of patients suffer a recurrence of diarrhoea and, in some instances, multiple recurrences occur requiring repeated courses of therapy. Treatment with metronidazole or vancomycin may predispose to recurrent C difficile diarrhoea by causing further disruption of the colonic microflora, a primary event in the pathogenesis of C difficile colitis.7 The use of antimicrobial agents to treat antibiotic associated diarrhoea has also led to concern regarding nosocomial bacterial antibiotic resistance; in particular vancomycin resistance in enterococcal species.1 8 These issues have led to a search for an effective, non-antibiotic agent, for prevention or treatment of nosocomial C difficile diarrhoea.

We previously reported the production of a hyperimmune bovine colostral antibody preparation against C difficile.9 10 Holstein cows are immunised against C difficile and its toxins during the gestation period and later an immunoglobulin concentrate is prepared from their colostrum. The bovine immunoglobulin concentrate–Clostridium difficile (BIC-C difficile) contains high levels of IgG against C difficile toxins, neutralises the biological effects of C difficile toxins in vitro and, in animal studies,
protects against *C difficile* toxin induced enterocolitis. BIC-*C difficile* is designed for use as an oral passive immunotherapy against *C difficile* and its toxins. To be therapeutically active, oral BIC-*C difficile* must exert toxin neutralising activity in the human colon. In an earlier study of healthy volunteers, we found detectable levels of bovine IgG as well as *C difficile* toxin neutralising activity in the stool after oral BIC-*C difficile* administration. In that study protection from acidic gastric secretions and short gastrointestinal transit times both appeared to increase faecal bovine IgG concentrations.

The survival of intact bovine IgG in the faeces is relevant to the therapeutic use of BIC-*C difficile*. However, of equal or even greater importance, is the delivery of intact bovine IgG to the human colon. In this study, volunteers with an end ileostomy but otherwise apparently normal gastrointestinal function took BIC-*C difficile* orally and their ileal fluid effluent was collected for analysis. The main study aims were to measure accurately the amount of bovine IgG surviving passage to the distal small intestine in humans and to determine whether specific *C difficile* toxin binding and neutralising activity was preserved. Additional aims were to determine whether reduced exposure to acidic gastric secretions or individual variations in upper gastrointestinal transit times would significantly alter bovine IgG survival.

**Methods**

**PREPARATION OF BOVINE IMMUNOGLOBULIN CONCENTRATE—*CLOSTRIDIUM DIFFICILE***

BIC-*C difficile* was prepared from the colostral milk of Holstein cows, as previously described. For this study, cows were immunised with a toxoid of purified *Clostridium difficile* toxin A. The resulting BIC-*C difficile* contained 42 mg of bovine IgG per 100 mg of powdered concentrate. This preparation of BIC-*C difficile* showed high concentrations of anti-*C difficile* toxin A activity as demonstrated by: (1) ELISA; (2) inhibition of toxin A binding to its brush border membrane receptor; (3) neutralisation of the cytotoxic effects of toxin A in the tissue culture cytotoxicity assay; and (4) inhibition of the enterotoxic effects of toxin A in the rodent ileal loop assay.

**HUMAN STUDY PROTOCOL**

A single site, open, phase I study was performed in volunteers with an end ileostomy to examine the safety and bioavailability of a single oral dose of BIC-*C difficile*. The study protocol was approved by the Beth Israel Deaconess Medical Centre Committee on Clinical Investigations. An outline of the study protocol is illustrated in fig 1.

**Study subjects**

Six volunteers (four men, two women; mean age 49 years, range 35–69) with a surgically created end ileostomy were enrolled into the study. In all cases, end ileostomy was performed following colectomy. The indications for colectomy were ulcerative colitis in five patients and indeterminate colitis in one. At study enrolment none of the patients had a history or any clinical evidence of upper gastrointestinal tract disease apart from ileostomy. All volunteers reported stable ileostomy function during the six month period prior to study entry.

Exclusion criteria included: a history of other gastrointestinal diseases or surgery which might be expected to affect normal gastrointestinal function, a history of clinically significant cow’s milk protein allergy or other allergy to milk products, lactose intolerance, evidence of ongoing systemic or infectious disease, or use of medication that might be expected to affect normal gastrointestinal function. All volunteers received monetary compensation for study participation.

**Administration of BIC-*C difficile***

BIC-*C difficile* 5 g, containing 2.1 g of bovine IgG, was administered orally. Volunteers adhered to a clear liquid diet from eight hours until four hours prior to BIC-*C difficile* administration at which time they consumed a standard meal (fig 1). They then fasted for four hours after which the BIC-*C difficile* was consumed, reconstituted in 250 ml of an isosmotic polyethylene glycol solution containing 421 mg of sodium bicarbonate. The polyethylene glycol was used as a non-absorbable transit marker. After a further two hours they were again allowed to take clear liquids until the end of the six hour ileostomy fluid collection period. Each volunteer was studied on four separate occasions with an intervening washout period of at least 72 hours. The BIC-*C difficile* was taken alone, with an antacid (30 ml of BICTRA), during therapy with the proton pump inhibitor omeprazole (Prilosec 20 mg, twice daily for four days prior to and on the day of BIC ingestion), or within enteric coated capsules. Treatments were administered in random order except omeprazole which was given last to avoid any carryover effect. The enteric coated capsules were designed to increase colonic delivery of BIC-*C difficile* and were prepared by the Pharmaceutical Service Division, College of Pharmacy, University of Iowa, as described previously.

Twelve hard gelatin capsules were consumed, each containing 420 mg of BIC and coated with two layers of cellulose acetate phthalate.

**Collection of ileostomy fluid**

Fluid was completely drained from the volunteers’ ileostomy appliance immediately prior to
BIC-\textit{C difficile} ingestion and, at 30 minute intervals, for six hours thereafter. The fluid volume was recorded and an aliquot taken which was filter sterilised and used for cytotoxicity neutralisation experiments. Protease inhibitors were added to the remaining fluid which was immediately frozen and stored at \(-20^\circ\text{C}\). The final concentrations of protease inhibitors were as follows: aprotinin 10 \(\mu\text{g/ml}\), leupeptin 10 \(\mu\text{g/ml}\), phenylmethylsulphonyl fluoride 10 \(\mu\text{g/ml}\), \(N\text{-p-tosyl-L-lysine}\) chloromethyl ketone 10 \(\mu\text{g/ml}\), and \(\text{L-1-tosylamido-2-phenylethyl chloromethyl ketone}\) 10 \(\mu\text{g/ml}\).

\textbf{SPECIAL LABORATORY STUDIES}

Total bovine IgG levels in the ileostomy fluid were measured by single radial immunodiffusion.\textsuperscript{10, 11} Specific anti-\textit{C difficile} toxin A bovine IgG levels were measured by enzyme linked immunosorbent assay (ELISA) as previously described.\textsuperscript{10, 11} Cytotoxicity was determined by rounding of Chinese hamster ovary (CHO) cells (American Type Culture Collection, Rockville, Maryland, USA) in monolayer culture after exposure to purified \textit{C difficile} toxin A. The minimum 50\% cytotoxic dose of toxin A was defined as the minimum dose resulting in 50\% cell rounding at 24 hours (40 ng/ml in these experiments). Inhibition of cytotoxicity was quantified by adding serial twofold dilutions of the ileostomy fluid to purified \textit{C difficile} toxin A to achieve an end concentration of twice the minimum 50\% cytotoxic dose of toxin A. After 30 minutes the mixture was added to the cell monolayers and cell rounding was assessed after 24 hours as previously described.\textsuperscript{10, 11}

\textbf{STATISTICAL ANALYSES}

Statistical analyses were performed using SigmaStat for Windows version 1.00 (Jandel Scientific Software, San Rafael, California, USA). Unless stated otherwise, analysis of variance was used for intergroup comparisons and the Spearman rank order test was used to evaluate correlations.

\textbf{Results}

\textbf{STUDY SUBJECTS AND SAMPLE COLLECTION}

Six volunteers participated in the study and all completed the study protocol. However, during the study one volunteer developed symptoms of ileostomy dysfunction and, two weeks after study termination, required hospital admission because of small bowel obstruction. This resolved without surgical intervention. Ileostomy samples from this volunteer were excluded from the data analysis. No other significant adverse experiences were encountered by the study volunteers.

\textbf{ILEAL RECOVERY OF BOVINE IgG AFTER ORAL BIC-\textit{C difficile}}

Figure 2 shows the total free bovine IgG content of the six hour ileostomy fluid collections. When the BIC-\textit{C difficile} was taken alone the mean (SEM) recovery of bovine IgG from the terminal ileum was 1033 (232) mg. This represented 49\% of the total ingested dose of 2100 mg of bovine IgG. The total ileal recovery of bovine IgG was 636 (129) mg (30\% of the oral dose) when additional acid buffering capacity was provided in the form of an oral antacid. The omeprazole group had the highest ileal bovine IgG content at 1052 (268) mg (50\% of the oral dose). However, analysis of variance did not reveal any significant difference between these three treatment groups (\(p=0.13\)).

The mean recovery of free bovine IgG from the ileal fluid after ingestion of BIC-\textit{C difficile} in enteric coated capsules was 89 (45) mg (4\% of the oral dose). This was significantly less than for the BIC-\textit{C difficile} alone and omeprazole groups (\(p<0.05\) for both). However, many of the capsules were recovered intact or only partially opened. Furthermore, when free bovine IgG was detected it was only found at the latter end of the six hour collection period (table 1). For all other treatment groups peak bovine IgG levels were detected much earlier (table 1) and negligible amounts of bovine IgG were found at six hours (fig 3). Since the release of bovine IgG was delayed and a substantial amount remained encapsulated the measurement of free bovine IgG in the ileal fluid is an incomplete measure of BIC-\textit{C difficile} survival in this treatment group.

\textbf{TIME COURSE OF BOVINE IgG TRANSIT THROUGH THE UPPER GASTROINTESTINAL TRACT}

Figure 3 depicts the time course of bovine IgG recovery from ileal fluid. Bovine IgG first reached the distal ileum 30 to 60 minutes after oral administration of reconstituted BIC-\textit{C diffic-
Figure 3 Time course of bovine IgG recovery from the ileum after oral BIC-C difficile. The mean amounts of bovine IgG recovered at each collection time are shown for each treatment group (n=5).

Figure 4 Comparison of specific anti-C difficile toxin A activity with total bovine IgG in ileal fluid after oral BIC-C difficile. (A) Levels for subject 2, receiving BIC-C difficile alone. Total recovery was 1558 mg or 74% of the ingested dose of bovine IgG. (B) Levels for subject 3, receiving BIC-C difficile alone. Total recovery was 611 mg or 29% of the ingested dose of bovine IgG.

**Discussion**

The main finding of this study is that almost half of the bovine IgG in an oral dose of BIC-C difficile can be retrieved intact from the distal small intestine in humans. The surviving IgG...
oral BIC-C diarrhoea are notably increased when acid degradation. The findings of this study suggest that enteric capsules may protect against bovine IgG degradation by delaying the release of BIC rather than by acid protection alone. This interpretation is further supported by our finding that volunteers with slower mouth to ileum transit times had significantly lower bovine IgG recovery than those with more rapid transit. For example, when BIC-C difficile was taken alone 76% of the oral dose was recovered from the distal ileum in volunteers with transit times of less than two hours whereas only 31% was recovered from those with transit times of more than two hours. A prolonged gastrointestinal transit time was also found to reduce bovine IgG recovery from the stool. Thus, it appears that enteric encapsulation raises faecal bovine IgG levels by delaying the release of BIC until the distal small intestine or colon. Formulation of BIC-C difficile for targeted release in the colon may increase its efficacy in neutralising C difficile toxins within the colonic lumen and at the same time allow a reduction in the oral dose.

Animals immunised against C difficile toxins are protected against challenge with toxigenic C difficile whereas non-immunised animals develop fatal enterocolitis. Some, but not all, clinical microorganisms indicate that patients with severe or recurrent C difficile colitis have an inadequate antibody response to C difficile and its toxins. The first animal study describing passive immunotherapy for C difficile enterocolitis was reported in 1979; hamsters were treated with parenteral Clostridium sordellii antitoxin, which cross reacts with C difficile toxins A and B, and protected against clindamycin induced colitis. Protective oral immunisation of hamsters using breast milk was reported in 1987. There are also limited reports of passive immunotherapy against C difficile diarrhoea and colitis in humans. A number of patients with recurrent, or refractory, C difficile diarrhoea have been treated using pooled normal human IgG administered intravenously. This treatment increases serum anti-C difficile toxin antibody concentrations and was effective in controlling symptoms in the small number of cases reported. In the present study, we showed that oral administration of BIC-C difficile provides C difficile toxin binding and neutralising activity in the distal ileum in human volunteers. A randomised, controlled, clinical trial examining the efficacy of BIC-C difficile in treating mild to moderately severe C difficile diarrhoea is now underway.

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