

Resistance of Bovine Colostral Anti-Cholera Toxin Antibody to In Vitro and In Vivo Proteolysis

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Pregnant cows immunized with cholera enterotoxin produce an immunoglobulin G class 1 antibody that enters the colostrum in high titer. After exposure to intestinal enzymes, this antibody remains immunologically reactive and inhibits intestinal fluid secretion in infant and adult rabbits exposed to cholera enterotoxin. Specific bovine colostrum antibodies may be a source of passive immune protection for human infants and adults at risk for cholera and other enteric diseases.

A recent report by Glass et al. (8) confirms the protective effect of disease-specific human milk antibodies against gastrointestinal infections in breast-fed infants. Unfortunately, bottle-fed and weaned infants, who do not receive these antibodies and the other host resistance factors in human milk (9), may have increased susceptibility to life-threatening gastrointestinal infections (2, 18, 22, 25). These infants could, at least theoretically, be protected by an infant formula fortified with antibodies. To be effective, these antibodies must resist digestion, thereby remaining functionally active in the gastrointestinal tract. Furthermore, the antibodies must be directed against a variety of potential gastrointestinal pathogens (12).

Petersen and Campbell (21) first suggested that orally administered bovine colostrum could provide passive immune protection for humans. Subsequently, Fernandez et al. (5) successfully used lyophilized bovine colostrum to treat infants with intractable diarrhea. Mietens et al. (20) had similar results in infants with *Escherichia coli* gastroenteritis treated with bovine colostrum whey (lactoserum) containing specific anti-*E. coli* immunoglobulin G class 1 (IgG1) antibodies. These anti-*E. coli* antibodies retained their immunological activity after passage through the gastrointestinal tracts of infants (13). The bovine colostrum whey used by these investigators (5, 12, 13, 20) may, however, have contained other host resistance factors that could have affected the infant diarrhea (24). Moreover, bovine colostrum contains a trypsin inhibitor that may have protected the bovine immunoglobulins from proteolysis (23).

In this report, we present evidence that isolated bovine colostrum anti-cholera enterotoxin antibodies resist in vitro inactivation by pepsin and trypsin and remain immunologically and functionally active against cholera enterotoxin in the gastrointestinal tracts of infant and adult rabbits.

MATERIALS AND METHODS

Immunization protocol. We immunized pregnant cows 5 to 6 weeks prepartum with 200 µg of cholera enterotoxin (CT) (Schwarz/Mann, Spring Valley, N.Y.) emulsified in 5 ml of sterile saline and 5 ml of Freund incomplete adjuvant by intramuscular injection. Two weeks prepartum, each cow received an intramammary booster injection consisting of 50

µg of CT in 10 ml of saline. Pooled colostrum was collected at calving and was stored at -20°C until ready for processing. We obtained control colostrum from cows that had not been immunized.

Immunoglobulin preparation. We removed the fat from anti-CT and control colostrum by centrifugation (14,000 rpm at 4°C, JA-21 rotor, Beckman Instruments, Inc., Irving, Calif.). Casein was precipitated from whey proteins by the addition of rennin (2 mg/liter; Sigma Chemical Co., St. Louis, Mo.) and calcium carbonate (4 g/liter), followed by incubation at 30°C until the curd formed. The bovine colostrum immunoglobulin fraction (BCI) was separated from the other whey proteins by three repetitive precipitations of the immunoglobulins with saturated ammonium sulfate. For each precipitation, saturated ammonium sulfate was added to the BCI in a ratio of 35 ml of saturated ammonium sulfate to 65 ml of BCI. The final immunoglobulin pellet was suspended in and dialyzed against borate-buffered saline, pH 8.4 (BBS) (6) at 4°C until it was free of detectable sulfate ions (no detectable precipitate formed after addition of a few drops of 10 g of BaCl₂ per deciliter).

Immunochemical and biological analyses. Bovine immunoglobulins (i.e., IgG1, IgG2, IgA, and IgM) were quantified with radial immunodiffusion kits (Miles Laboratories, Inc., Elkhart, Ind.). We characterized the anti-CT BCI by ion-exchange and affinity chromatography, anti-CT radioimmunoassay, rabbit ileal loop assay (15, 26), and quantitative precipitation (11).

(i) **Ion-exchange chromatography.** Anti-CT BCI (160 to 170 mg of total protein) (17) was applied to a DEAE-cellulose column (31 by 1.6 cm; DE-52, Whatman Ltd., Maidstone, Kent, United Kingdom). The immunoglobulins were eluted with a 0.017 to 0.2 M sodium phosphate buffer linear gradient (pH 8.0) at 4°C. We operated the column at 18 ml/cm² per h, collected 2-ml fractions, and monitored protein concentration spectrophotometrically (280 nm). We lyophilized all protein peaks and redissolved them in BBS. Two-dimensional gel diffusion with rabbit anti-bovine IgG1, IgG2, IgA, and IgM antisera (Miles Laboratories) identified bovine immunoglobulins.

(ii) **Affinity chromatography.** We coupled 5 mg of CT to 3 g of cyanogen bromide-activated Sepharose 4-B beads as instructed by the bead supplier (Sigma). To obtain anti-CT affinity-purified bovine colostrum antibodies, we incubated 9 ml of the anti-CT immunoglobulin preparation with all of the CT-coupled Sepharose on a rotary rack at 4°C overnight. This mixture was then placed in a sintered glass funnel.

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Unadsorbed immunoglobulins were washed from the affinity beads with 150 ml of BBS. Specific anti-CT antibodies were eluted from the beads with 20 ml of glycine-hydrochloride buffer (0.2 M; pH 2.0). We then dialyzed the anti-CT affinity eluate overnight at 4°C against 12 liters of BBS.

(iii) **Anti-CT radioimmunoassay (RIA).** We isolated the A subunit of CT (16) and labeled it with ^{125}I by the chloramine T method (10). For each assay, approximately 5,000 cpm of labeled A subunit in 100 μl of RIA buffer (1% ovalbumin in BBS) was added to duplicate tubes containing dilutions of anti-CT antibody (36 to 0.36 ng) in 300 μl of RIA buffer. These mixtures were incubated at 37°C for 3 h. To each tube, we then added 100 μl of second antibody (rabbit anti-bovine colostrum immunoglobulins, diluted 1:8 in RIA buffer) and incubated this mixture overnight at 4°C. After incubation, we decanted the supernatant from the centrifuged pellet and counted the pellet and supernatant in a gamma counter (Gamma 4000, Beckman). The percent antigen binding [(pellet cpm/total cpm) \times 100] was calculated. The concentration of anti-CT in unknown samples was determined from a standard curve.

(iv) **Rabbit ileal loop assay.** To evaluate the biological activity of anti-CT antibodies, we used the rabbit ileal loop assay (15, 26). For each assay, we fasted three New Zealand White male rabbits (1.5 to 2.0 kg) overnight. Ileal loops (16 to 22 per animal) were prepared. Into one loop in each of three animals, we injected 1 ml of the sample to be analyzed. Each sample was prepared by incubating 200 ng of CT per ml of saline with an equal volume of an anti-CT solution to be analyzed (37°C for 30 min). Three negative control loops (saline) and three positive control loops (100 ng of CT in 1 ml of saline) were included in each animal. After 18 h of *in vivo* incubation, we killed the animals, removed the intestines, and measured the length (L) and volume (V) of each loop. The V/L ratio of each sample loop was compared with the average V/L ratio of positive control loops. A simple percent neutralization of the 100-ng CT control loop was determined for each anti-CT sample. The mean percent neutralization by a given anti-CT sample represents the average response in three rabbits.

In vitro proteolysis. To determine the resistance of bovine colostrum IgG1 to *in vitro* proteolysis, we evaluated the biological activity of 33 μg of anti-CT immunoglobulins in the rabbit ileal loop assay after *in vitro* exposure to trypsin at an enzyme/substrate ratio of either 1:100 or 1:20 by weight (50 mM Tris-hydrochloride, 10 mM CaCl_2 [pH 8.0] for 8 h). Trypsin was inactivated by the addition of phenylmethylsulfonyl fluoride in isopropanol (1 mg/ml; Sigma). The biological activity of anti-CT was also evaluated after exposure to pepsin (enzyme/substrate ratio, 1:20 by weight) at a pH of either 4.0 (0.07 M sodium acetate) or 2.0 (0.05 M KCl-0.001 M HCl buffer) for 4 h at 37°C. Pepsin was inactivated by the addition of Na_2CO_3 to a pH of 8.0. The *in vitro* digestions were otherwise performed as described by de Rham and Isliker (3). Enzymes were obtained from Sigma. Trypsin and pepsin activities were confirmed by the methods of Hummel (14) and Anson (1), respectively.

In vivo proteolysis. To determine the resistance of bovine colostrum immunoglobulins to *in vivo* digestion in adult rabbits, we fed three groups of New Zealand White rabbits (seven rabbits per group) anti-CT colostrum whey, control whey, or saline in three equal feedings over 24 h (total, 90 ml per animal). Six hours after the last feeding, the animals were killed and their cecal contents were removed. To the cecal contents of each rabbit (\approx 45 ml) we added 10 ml of BBS; we then homogenized each sample and centrifuged

them at 20,000 rpm for 60 min (Beckman JA-21 rotor). The supernatants were lyophilized, reconstituted in a minimum volume of BBS, and stored at -20°C until analyzed. The rabbit cecal anti-CT antibody content and activity were evaluated in the anti-CT RIA and rabbit ileal loop assay, respectively.

In vivo protection. To evaluate the protective effect of orally administered anti-CT bovine colostrum immunoglobulins, we fed 80 to 120-g, 3- to 5-day-old infant rabbits (New Zealand White and Checker Giant strains) per orogastric tube anti-CT bovine colostrum immunoglobulins (immune BCI), control immunoglobulins (nonimmune BCI), or 5% glucose water (D5W). Immune and nonimmune BCIs were prepared from the respective immunoglobulin fractions by dialysis against 5% glucose in water to remove BBS. Each animal received three feedings over 24 h (total, 9 ml per animal). Five to six hours after the last feeding, we anesthetized the animals with ketamine, ligated the intestine at the appendix-cecum, and injected 100 ng of CT in 1 ml of saline into the intestine, just proximal to the ligature. Animals from each feeding group were randomly selected and similarly injected with 1 ml of saline. These animals served as sham-operated controls. In an effort to reduce postoperative dehydration, each animal received 2 ml of D5W (\approx 20 ml/kg) subcutaneously. The mortality and intestinal fluid response (Fluid Accumulation Index, FAI [4]) of each group was assessed at 18 h. For statistical analysis of mortality and FI data, we used the chi-square test with Yates correction, and unpaired *t* tests with Bonferroni adjustment for multiple comparisons (7).

RESULTS

Immunochemical analyses. The immunoglobulin contents of the anti-CT bovine colostrum whey, precipitated immunoglobulin fraction (BCI), and affinity eluate are shown in Table 1. The major immunoglobulin class present in all preparations was IgG1. The anti-CT antibody content of the immunoglobulin fraction, determined by quantitative precipitation, was 2.65 mg/ml or 3.1 mg per 100 mg of IgG1.

Ion-exchange chromatography separated the anti-CT BCI into three peaks (Fig. 1). The first peak is predominantly IgG2, whereas the second peak contains IgG1 with some IgA and IgM. The third peak is mainly IgM with some IgG1. We evaluated each peak for precipitating antibody to CT and found anti-CT antibody in the early portion of the second peak, suggesting that our anti-CT was an IgG1 or an IgA antibody or both. In the anti-CT eluate from the affinity column, only IgG1 was quantifiable (0.7 mg/ml). Precipitin lines to IgA and IgG2 were just detectable but could not be quantified by radial immunodiffusion.

Biological activity of anti-CT colostrum preparations. The maximum dilutions of anti-CT bovine colostrum whey, anti-CT BCI, and anti-CT affinity eluate that completely neutral-

TABLE 1. Immunoglobulin contents of bovine colostrum anti-CT preparations

Prepn	Concn (mg/deciliter) of the following immunoglobulin ^a :			
	IgG1	IgG2	IgM	IgA
Anti-CT whey	18.2 (4.9)	1.1 (0.08)	2.45 (0.09)	3.15 (0.78)
Anti-CT BCI	85.6 (10.4)	3.36 (0.96)	10.0 (0.16)	17.9 (3.2)
Anti-CT affinity eluate	0.7 (0.03)			

^a Each value is the mean of three lots. Numbers in parentheses are standard deviations.

ized 100 ng of CT in the rabbit ileal loop assay were 1:500, 1:2,000 and 1:800, respectively. Control colostral globulins and no neutralizing activity in the ileal loop assay.

In vitro and in vivo proteolysis of anti-CT. After exposure to trypsin at an enzyme/substrate ratio of 1:100 and to pepsin (1:20) at pH 2.0 or 4.0, anti-CT BCI retained activity against CT in the rabbit ileal loop assay. Fifty microliters of both pepsin digests and the 1:100 trypsin digest completely neutralized 100 ng of CT. Fifty microliters of the 1:20 trypsin digest of anti-CT immunoglobulins neutralized only $48 \pm 25\%$ of the fluid secretion induced by 100 ng of CT. The inactivated enzymes in buffer had no neutralizing activity in the ileal loop assay.

Six of seven adult rabbits fed anti-CT whey had sufficient anti-CT activity in 50 μ l of reconstituted cecal fluid to neutralize completely 100 ng of CT in the rabbit ileal loop assay. Only two of seven control whey-fed animals and one of seven saline-fed animals had 100% neutralizing activity in their cecal extracts ($P = 0.05$ by Fisher exact test). Cecal extracts from anti-CT whey-fed animals exhibited a mean (\pm

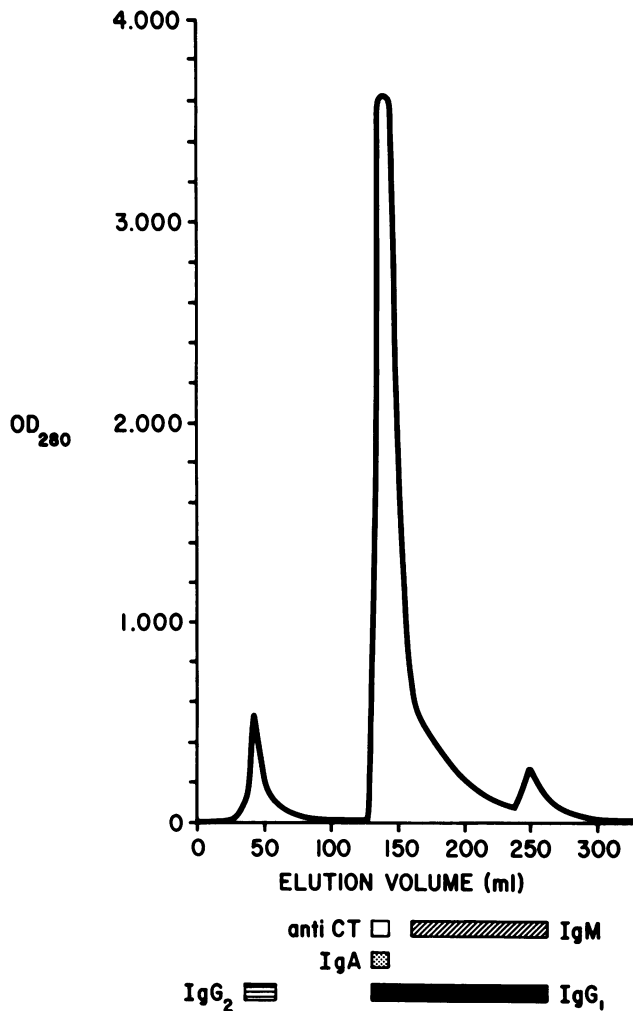


FIG. 1. Chromatographic profile of anti-CT BCIs over DEAE-cellulose on a column (31 by 1.6 cm), eluted with a 0.017 to 0.2 M sodium phosphate buffer gradient (pH 8.0). Bars represent fractions containing class-specific bovine immunoglobulins as determined by two-dimensional gel diffusion.

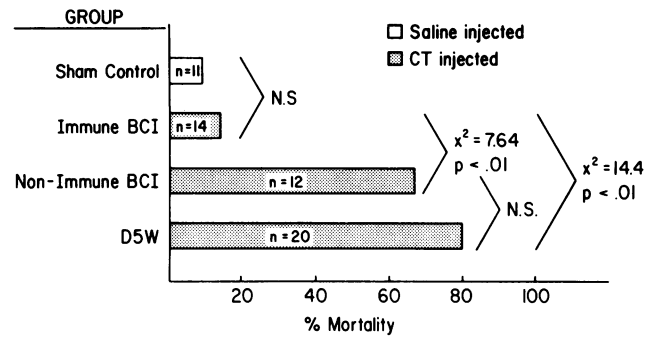


FIG. 2. Mortality of infant rabbit feeding groups 18 h after direct intestinal injection of 100 ng of CT. Before CT injection, each group received three feedings over 24 h (total, 9 ml per animal) of anti-CT bovine colostrum immunoglobulins (immune BCIs), nonimmune BCIs, or D5W. Animals selected randomly from each feeding group were injected with saline in lieu of CT and served as sham controls. The mortality of each group was: immune BCI, 14.2%; nonimmune BCI, 66.7%; D5W, 80%; and sham controls, 9.1%.

standard deviation) anti-CT antibody content of $5.7 \pm 2.0 \mu$ g/ml as determined by anti-CT RIA. Neither the non-immune-fed nor the D5W-fed animals had CT binding activity in their cecal extracts as determined by RIA. The bovine immunoglobulin content of the cecal extracts from rabbits fed anti-CT or control colostrum contained 0 to 0.56 mg of IgG1 and 0 to 4.2 mg of IgA per ml.

In vivo protection. After dialysis against D5W, the immune (anti-CT) BCI preparation had 62.4 mg of IgG1 and 2.7 mg of IgA per ml, whereas the control immunoglobulins (nonimmune BCIs) contained 38.4 and 8.0 mg/ml, respectively. When we examined the gross mortality and FAI of each feeding group, animals fed immune BCI were protected from the adverse effects of CT (Fig. 2 and 3). The mortality and FAI data of the immune BCI group were statistically the same as the data in the sham-operated, saline-injected control group. The mortality of immune BCI-fed animals (14.3%) was significantly less than that of the nonimmune BCI- or D5W-fed animals (66.7 and 80%, respectively [$P < 0.01$]; Fig. 2). Too few toxin-injected control animals (non-immune-fed and D5W-fed) survived to allow meaningful comparison of group FAIs; however, when survivors of these control groups were combined, they exhibited an FAI significantly greater than that of either the immune BCI-fed or sham control animals ($P < 0.01$; Fig. 3).

DISCUSSION

In this report, we demonstrate that pregnant cows immunized with cholera enterotoxin produce primarily an IgG1 antibody that is secreted in large quantities into the colostrum. This antibody represents approximately 3% of the total colostral IgG1 content. After in vitro exposure to trypsin at an enzyme/substrate ratio of 1:100, these anti-CT IgG1 antibodies still retained their ability to neutralize completely 100 ng of CT in the ileal loop assay. In vitro exposure of anti-CT BCI to trypsin at a higher ratio of enzyme to immunoglobulin resulted in a 50% decrease in neutralizing activity against CT. Exposure of anti-CT antibody to pepsin at pH 4.0 and 2.0 had no appreciable effect on anti-CT neutralizing activity in the ileal loop assay. Furthermore, a protective concentration of anti-CT antibodies could be found in the cecal contents of adult rabbits fed anti-CT BCI. When anti-CT antibodies were fed to infant rabbits, the

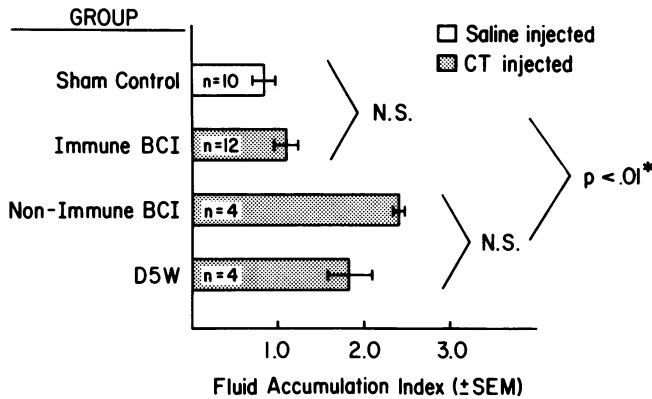


FIG. 3. Fluid accumulation index FAIs of infant rabbits surviving direct intestinal injection of 100 ng of CT after prophylactic feedings of anti-CT bovine colostrum immunoglobulins (immune BCIs), nonimmune BCIs, or D5W. FAIs for feeding groups were: immune BCI, 1.09 ± 0.15 ; nonimmune BCI, 2.41 ± 0.06 ; D5W, 1.81 ± 0.24 ; and sham controls, 0.85 ± 0.15 . FAI, Grams of intestinal content per gram of intestinal tissue (4). *, Result of unpaired *t* test with Bonferroni adjustment for multiple comparisons (7).

animals were protected from the toxic effects of a direct intestinal injection of 100 ng of cholera enterotoxin.

Although we found measurable anti-CT activity in the cecal fluid from adult rabbits fed anti-CT colostrum whey, less than 0.56 mg of IgG1 per ml of cecal fluid was found. Bovine colostrum IgG1 antibodies are partially digested to $F(ab)_2$ components by intestinal proteolytic enzymes (12, 13, 20). Since the $F(ab)_2$ fragment retains the antigen binding site, an $F(ab)_2$ component of the anti-CT antibody could be protective in the rabbit ileal loop assay and immunologically active in our anti-CT RIA. This immunoglobulin fragment may not, however, be accurately measured by the subclass-specific immunodiffusion plates we used to quantify cecal immunoglobulins. This technical problem would lead to falsely low values for cecal IgG1 content.

The anti-CT immunoglobulin fraction may have contained some secretory IgA antibody with anti-enterotoxin activity. These secretory IgA anti-CT antibodies would also resist proteolysis. They could account for the presence of anti-CT activity and the apparently low concentration of bovine IgG1 in the ceca of rabbits fed colostrum whey. Nevertheless, the amount of IgA found in the affinity-purified fraction of the anti-CT immunoglobulins was very low; therefore, we believe that specific anti-CT secretory IgA antibodies contributed very little to the rabbit cecal anti-CT activity.

We have not evaluated the anti-CT BCI for the presence of bovine colostrum trypsin inhibitor or other host resistance factors such as lactoperoxidase, lactoferrin, and lysozyme. Trace quantities of these proteins may be present, although it is unlikely that these proteins would be precipitated by 35% saturated ammonium sulfate. In addition, it seems quite unlikely that such proteins would have adsorbed to the CT-Sepharose beads or eluted from the DEAE-cellulose column in the same fractions as the anti-CT immunoglobulins. Moreover, the total protein content of BCI measured by the method of Lowry et al. (17) is very similar to the total immunoglobulin content measured by quantitative radial immunodiffusion (19).

Our findings are consistent with the results of earlier investigators (12, 13, 20, 27, 18) and add information regarding the activity of a specific bovine colostrum antibody

directed against the enterotoxin of *Vibrio cholerae*. Our results suggest that specific bovine colostrum antibodies, administered orally, may be a means of providing passive immune protection of infants at risk for enteric disease. We have not yet determined the resistance of these bovine colostrum anti-CT antibodies to passage through the human infant gastrointestinal tract, or the ability of these antibodies to neutralize toxin produced by viable organisms. Furthermore, we have not compared anti-CT BCIs with established antitoxin antibody standards.

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