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Interaction between Bovine Casein and *V. Cholerae* Enterotoxin in the Rabbit Ileal Loop

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Summary

Secretory IgA from human breast milk neutralizes cholera enterotoxin in the rabbit ileal loop system. No similar protection by purified bovine milk proteins could be demonstrated; however, one bovine milk protein, casein, had a deleterious effect on intestine exposed to very small quantities of enterotoxin. Highly purified cholera toxin (10 or 100 ng) was incubated with bovine protein solutions for 60 min at 37°C. One-milliliter aliquots were then injected into prepared rabbit intestine loops. The animals were sacrificed at 18 h and the intestinal loop contents were aspirated, and a volume to length of loop ratio (V/L) was determined. The activity of 100 ng of toxin was not enhanced by the majority of bovine milk proteins, but bovine casein caused a 14–40% increase in the fluid production (V/L of casein + toxin *versus* toxin, 1.05 *versus* 0.92 and 1.82 *versus* 1.30). All of the bovine proteins but casein inhibited the action of low dose enterotoxin. Bovine casein caused a 78–90% increase in fluid production by loops exposed to a suboptimal toxin dose (10 ng) (V/L of casein + toxin *versus* toxin, 1.12 *versus* 0.63 and 0.95 *versus* 0.50). Virtually all of this enhancement of enterotoxin fluid response resided in the purified alpha-casein fraction.

Abbreviation

V/L, volume to length of loop ratio

Numerous gram-negative bacteria cause diarrhea by producing an enterotoxin that induces fluid loss into the intestine. *Vibrio cholerae* is the prototype for enterotoxin-induced disease, but milder forms can be induced by a wide variety of coliforms, including *E. coli* and *Klebsiella sp.* (9, 11, 12). Although toxigenic *E. coli* diarrhea is seldom fatal in adults, its effect on the neonate can be devastating. (2, 14, 20). Clinically, the effects of these bacterial intoxications can be either ameliorated or exacerbated by multiple factors, one of which is secretory IgA directed against enterotoxin (19). These protective factors in breast milk may confer resistance to diarrhea in infants receiving mother's milk in underdeveloped countries (8).

While studying this protective effect of breast milk, we examined other milk proteins for their effect on enterotoxin-induced diarrhea. A number of bovine proteins were examined, and no additional factors were found that were specifically protective. We found that there is one milk protein, alpha-casein, present in large amounts in cow's milk that has a deleterious effect on intestine exposed to very small quantities of enterotoxin.

MATERIALS AND METHODS

Proteins. Highly purified *Vibrio cholerae* enterotoxin (Schwarz Mann, Orangeburg, NY, Lot BZ 2487) was initially suspended at a concentration of 10 μg/ml in phosphate buffered saline and stored at 4°C. Before use, it was diluted in phosphate buffered saline to a concentration of either 10 ng or 100 ng/ml. Bovine casein hydrolysate (Sigma, St. Louis, MO, Lot 59B21000), bovine casein (Pentex, Kankakee, IL, Lot 96-005-1), bovine Beta lactoglobulin A (Pentex, Lot 96-003), bovine Beta lactoglobulin B (Pentex, Lot 96-004), bovine lactalbumin (Nutritional Biochemicals, Chagrin Falls, OH), and bovine gamma globulin (Schwarz Mann, Lot Y3145) were dissolved in phosphate buffered saline at a concentration of 10 mg/ml just before use.

Isolation of human casein. One liter of pooled human breast milk was collected via breast pump from 15 normal nursing mothers at University Hospitals, Cleveland, OH. The milk was

defatted by centrifuging at 9000 rpm for 60 min and concentrated by vacuum dialysis against normal saline to 300 ml. Casein was prepared from this skim, concentrated milk by slow acidification to pH 3.0 with glacial acetic acid. The pH was readjusted to pH 4.0 with 1 N NaOH. The casein precipitate was removed by centrifugation at 9000 rpm for 60 min. The semi-solid slurry was suspended in distilled water and centrifuged at 5000 rpm for 30 min. The pellet was resuspended in distilled water and thoroughly dialyzed against two changes of phosphate buffered saline. The casein solution was then lyophilized and dissolved in phosphate buffered saline (10 mg/ml) just before use.

Purification of alpha-casein. The proteins in bovine casein were fractioned into the alpha and non-alpha-portions by virtue of their differing charges. Alpha-casein was separated from the remainder of the caseins by DEAE cellulose for exchange chromatography (21) using an ascending salt gradient and descending pH gradient (Fig. 1A). The high positive charge of bovine alpha-casein causes it to bind preferentially to the negatively charged diethylaminoethyl groups and it can be eluted in pure form (a single band on polyacrylamide gel electrophoresis, Fig. 1B).

Pure alpha-casein and the mixture of non-alpha-caseins were tested at a concentration of 10 mg in the rabbit intestinal loop with a low dose of enterotoxin (10 ng, less than 1 ED50).

Rabbit ileal loop assays. Intestinal loops were prepared by Sack's modification of the method of Kasai and Burrows (10, 15). New Zealand white, weanling, male rabbits, weighing 1.5–2.4 kg were fasted overnight before use. They were anesthetized with sodium pentobarbital (25 mg/kg IV) and ether. The small intestine was ligated into 3–5-cm loops. *V. cholerae* toxin (10 or 100 ng) was incubated with 1 ml of 10 mg/ml of the previously mentioned protein solutions for 60 min at 37°C and injected with a 26 gauge needle into the loops.

Eighteen hours later, the animals were sacrificed, and the intestines removed for study. The small intestine was photographed and examined for the presence of gross hemorrhage. The loops were drained into a syringe, the volume of fluid noted, and the length of the loop measured. The activity of the toxin or toxin-protein combinations was determined by the ratio of volume within the intestinal loop to the length of the loop. A V/L ratio of >0.5 is characteristic of enterotoxin activity.

Statistical analysis. Each set of experiments was analyzed by analysis of variance where the treatments were fixed, and the

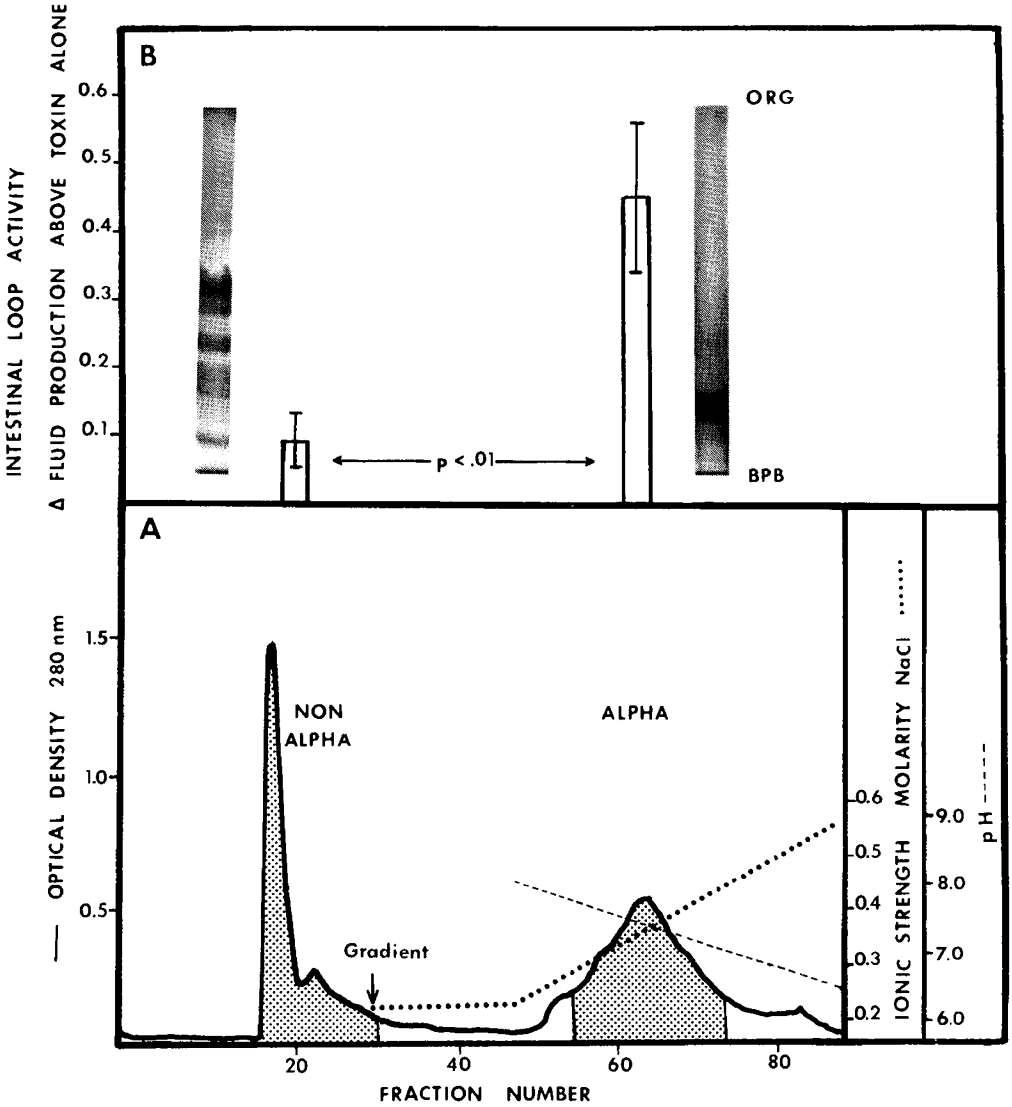

Fig. 1. Fluid production by intestine exposed to bovine casein fractions and enterotoxin. *(A)* DEAE chromatography of bovine casein. Optical densities of casein fractions obtained by DEAE exchange chromatography separation of whole bovine casein. The positively charged casein bound to the DEAE was eluted in pure form by an ascending salt gradient and a descending pH gradient. *(B)* Intestine exposed to 10 ng *V. cholerae* enterotoxin. The mean ± S.D. of the change in fluid production above toxin alone was determined for both alpha- and non-alpha bovine casein. Superimposed upon this graph are photographs of the polyacrylamide gels of the electrophoresis of the casein fractions. ORG (origin), BPB (Bromphenol Blue–rapidly moving marker dye.)

rabbits were considered a random variable (18). Scheffe's criterion was applied for multiple contrasts (16). If, for example, a factor in analysis of variance is represented by k + 1 levels with k degrees of freedom and is tested by an appropriate "error" term with n degrees of freedom, then Scheffe's criterion is $S = \sqrt{k\ F(k,n)}$ at the α level of type 1 error. The F(k,n) is the tabular F value for this α level.

If the F(k,n) computed in the analysis of variance does not exceed the tabular F(k,n) for a given level of confidence, then the factor is considered not significant at this level of confidence. Scheffe has shown that under these conditions a t test of any comparison of treatments will never yield a value in excess of S (16).

If F(k,n) is greater than tabular F(k,n) so that the factor is considered significant at the α level, then one is free to do all the t-tests he devises using the S as a criterion. Those that pass (*i.e.*, $t > S$) are significant at the α level and those that fail are not significant at that level.

Ordinarily $t = \sqrt{F}$, but it is apparent that Scheffe's criterion is a modified "t" that is larger than the ordinary t because k is generally 1. Although F(k,n) decreases with increasing k, the magnitude of the decrease of F(k,n) is much smaller than the increasing k. There is some loss of power in using Scheffe's criterion but it gives a conservative estimate of what is and what is not significant when testing for relationships.

RESULTS

General description of intestinal loops. When the rabbit peritoneum was opened 18 h after surgery, the distended loops of bowel readily came into view. At this time the vascularity of the intestine was examined, and one rabbit with compromised blood flow to the intestine was discarded. Loops exposed to enterotoxin alone were tense and friable, but when the lumen was opened and the mucosa washed, no evidence of hemorrhage in the mucosa was observed. Occasionally, all the loops exposed to enterotoxin and casein were bloody. In these loops, although the vasculature was intact, blood was observed within the intestinal wall. Although the extent of bowel wall hemorrhage could not be easily quantitated, approximately 14% (4/29) of the animals in one series of experiments had enterotoxin-casein loops that were hemorrhagic. In these experiments, hemorrhage in loops containing purified enterotoxin alone was extremely rare. Histologic examination of these affected loops revealed hemorrhage within the tips of the villi and at the junction of the mucosa and submucosa. There was little evidence of an acute inflammatory infiltrate anywhere. The only histologic abnormalities in loops containing enterotoxin or enterotoxin and non-alpha-casein-rich proteins were thinning of the mucosa (associated with distension) and discharge of mucous from the goblet cells.

Fluid production by intestine exposed to milk proteins and enterotoxin. Because there are a large number of proteins in milk, the major classes of milk proteins were injected into rabbit loops to study their effect upon enterotoxin-induced fluid production. The activity of 100 ng of toxin (>1 ED50) was not increased by bovine serum albumin (Fig. 2A), or bovine gamma globulin (Table 2). It should be remembered that human natural antibody to enterotoxin can easily inhibit this dose of enterotoxin (20). Bovine caseins in one experiment caused a 40% increase (Fig. 2A) in the fluid production induced by 100 ng of enterotoxin (toxin V/L, 1.30 ± 0.13 S.D. *versus* casein + toxin, 1.82 ± 0.11 S.D., $P<0.01$). In a second experiment bovine casein resulted in a 14% increase (Table 1) in fluid production induced by 100 ng

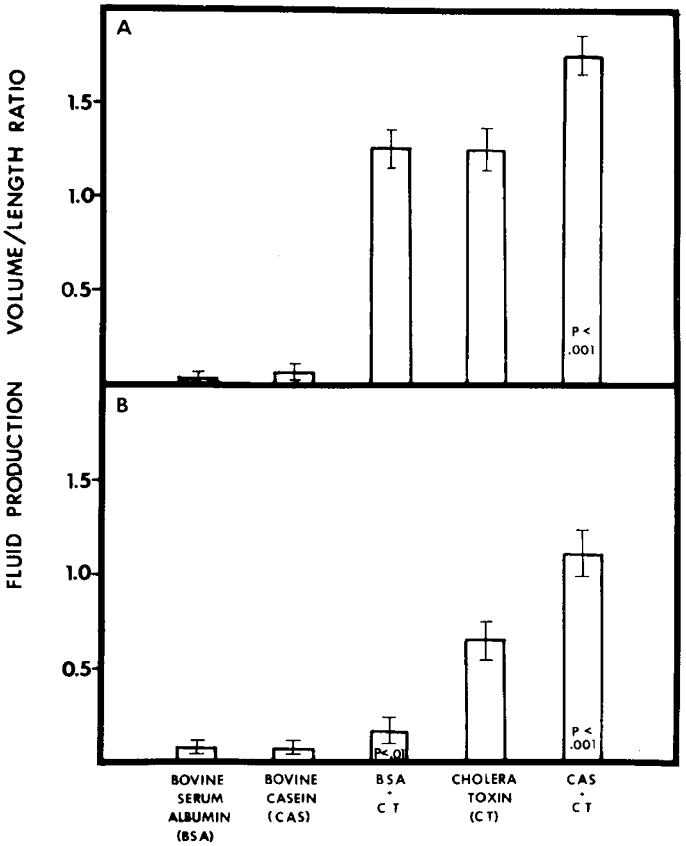

Fig. 2. Fluid production by intestine exposed to milk proteins and enterotoxin. *(A.) Intestine exposed to 100 ng of cholera enterotoxin.* Fluid response/length of intestine for enterotoxin alone, protein alone (BSA or bovine casein) and the protein enterotoxin (100 ng) combinations was determined in each of 12 rabbits. (mean ± S.D.). *(B.) Intestine exposed to 10 ng of cholera enterotoxin.* Fluid response/length of intestine with the above proteins was determined at a lower toxin dose (10 ng) in eight animals. (mean ± S.D.).

Table 1. *Effect of various proteins upon fluid production induced by 100 ng of* V. cholerae *toxin*

Protein[1]	Number of loops	Volume/length ratio mean	% change *vs* toxin alone	Significance *vs* toxin by Scheffe's criteria
Cholera toxin alone	11	0.92		
Bovine gamma globulin + toxin	11	0.75	18% decrease	<0.01
Bovine serum albumin + toxin	11	0.92	no change	n.s.
Bovine casein + toxin	11	1.05	14% increase	<0.05
Human casein + toxin	11	0.66	28% decrease	<0.01
S.E. = 0.025 based on analysis of variance; $F_{4,40} = 36.53$ $P \equiv 0$				

[1] Protein concentration, 10 mg/ml.

of enterotoxin ($P < 0.05$ by Scheffe's criterion). In no experiment did any other protein in the presence of enterotoxin induce fluid production above that of the saline controls. At the 100 ng enterotoxin dose, there was no inhibitory effect of bovine serum albumin, but bovine gamma globulin and human casein each were able to reduce the expected fluid production (Table 1).

When milk proteins were added to very low dose enterotoxin (10 ng, approximately 1/10 of an ED50) two types of effects were observed. All bovine non-casein proteins inhibited the action of cholera toxin by 77–90% (Table 2 and Fig. 2B). Rather than inhibiting enterotoxin, in two experiments whole bovine casein caused an increase in fluid production by loops exposed to a suboptimal dose of enterotoxin (10 ng).

Figure 2B demonstrates a 78% increase in fluid production/intestine length (10 ng toxin, 0.63 ± 0.10 S.D. *versus* casein + toxin, 1.12 ± 0.12 S.D.) in nine animals exposed to low dose toxin and casein. An increase of 90% was noted in the second experiment (Table 2). Hydrolyzed casein failed to increase enterotoxin induced fluid production (Table 2).

Effect of enterotoxin and various doses of bovine caseins upon the intestine. In order to determine whether this casein-enterotoxin effect occurs at physiologically relevant casein concentrations, a casein dose-response curve was run at a constant enterotoxin dose of 10 ng. As demonstrated in Figure 3, there was not a striking relationship between casein dose and response in 14 animals; 100 μg of casein caused a 72% increase in enterotoxin-induced fluid response whereas 10 mg of casein caused a 134% increase. All concentrations of casein tested with enterotoxin yielded significantly more fluid than toxin alone, and they were not statistically different from each other. Very small amounts of whole casein (100 μg) were able to increase the fluid response.

Effect of enterotoxin and purified casein upon the intestine. Human casein, which has no alpha-casein, did not have the same effect on enterotoxin-exposed intestine as alpha-casein-rich bo-

Table 2. *Effect of various bovine milk proteins upon fluid production induced by 10 ng (less than 1 ED_{50}) of* V. cholerae *enterotoxin*

Protein[1]	Number of loops	Volume/length ratio mean	% change	Significance *vs* cholera toxin alone by Scheffe's criterion at $\alpha = 0.001$
Cholera toxin alone	9	0.498		
Casein + toxin	9	0.945	90% increase	<0.001
Alpha lactalbumin + toxin	9	0.051	90% decrease	<0.001
Beta lactoglobulin A + toxin	9	0.053	90% decrease	<0.001
Beta lactoglobulin B + toxin	9	0.058	89% decrease	<0.001
Serum albumin + toxin	9	0.117	77% decrease	<0.001
Hydrolyzed casein + toxin	9	0.098	80% decrease	<0.001

S.E. = 0.0248 for analysis of variance; $F_{6,48} = 189.96$
$P \equiv 0$

[1] Bovine proteins were used at a concentration of 10 mg/ml.

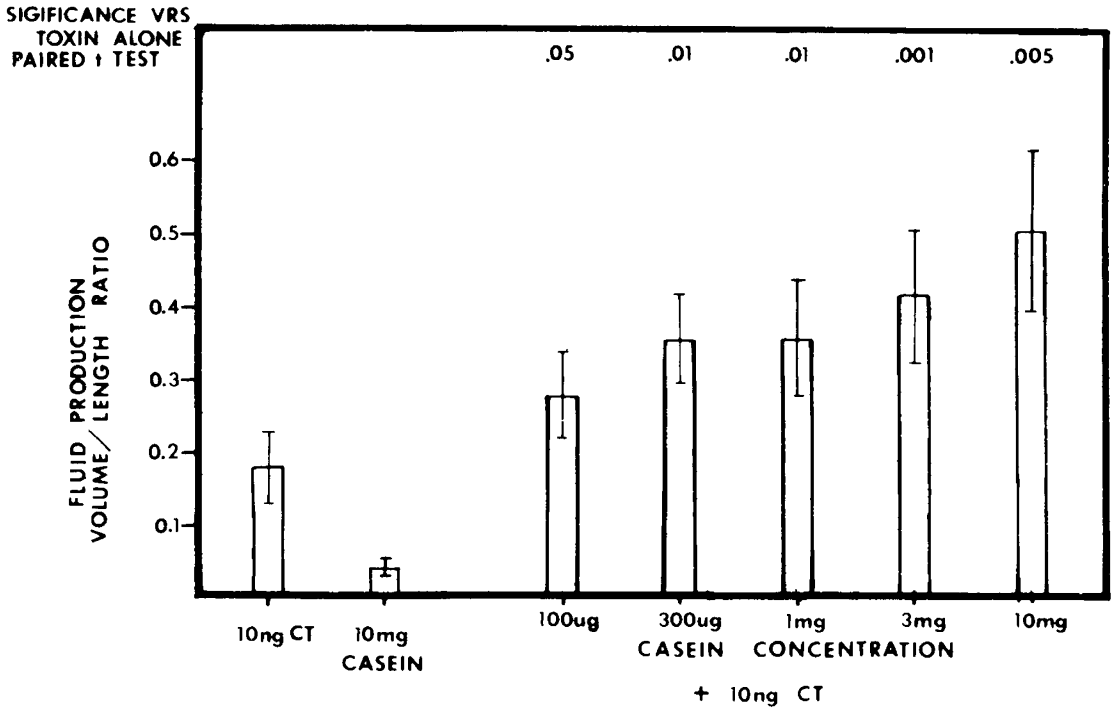

Fig. 3. Casein dose response curve. Fluid response/length of intestine was determined in each of 14 rabbits comparing casein-toxin loops to loops containing toxin alone (mean ± S.D.).

vine casein (Table 1). The bovine alpha-casein fraction exhibited increased fluid production in the enterotoxin exposed intestine whereas the bovine non-alpha-fraction did not.

Alpha-casein caused a strong, consistent increase in fluid production in six rabbits (Fig. 1B). In combination with enterotoxin, non-alpha-caseins caused a slight but not significant increase in fluid production. This non-alpha-casein-induced increase was significantly less than that achieved with pure alpha-casein ($P < 0.01$).

DISCUSSION

In vivo, purified enterotoxin and *bovine* casein interacted to increase fluid production in the small intestine. We hypothesize that if the casein-enterotoxin interaction is operant in humans, individuals in the following two groups would be most affected.

(*1*) Individuals in underdeveloped countries, afflicted with diarrhea caused by an enterotoxigenic organism (6, 7) could suffer exacerbation of the diarrhea if the intestinal transit time is rapid enough to allow inadequate digestion of cow's milk protein.

(*2*) Bottle-fed infants receive the majority of their protein intake as casein from cow's milk. The preterm infant has well defined deficiencies in digesting fats (13, 17) and disaccharides (1, 4) and may have difficulties in digesting protein. If a preterm infant is colonized with an organism that normally produces insufficient amounts of enterotoxin to cause fluid production, then the presence of undigested casein in the ileum might induce gastrointestinal symptoms.

The mechanism of interaction between cholera enterotoxin and bovine casein is not clear. The molecular weight, solubility, and other characteristics of proteins are very similar in the alpha-casein and non-alpha-casein fractions of bovine casein. The major difference is electrical charge. The casein molecule may alter the receptor sites for enterotoxin, thereby promoting attachment and increased fluid response.

The enterotoxins from *Vibrio cholerae* and *E. coli* are very similar in their structure and modes of action (5). A similar interaction may occur with enterotoxin from *E. coli* and casein (*E. coli* enterotoxin is not currently commercially available to test in this system). Cushing (3) recently reported an outbreak of necrotizing enterocolitis associated with *E. coli* enterotoxin (3). We speculate that very small amounts of enterotoxin may be potentiated by dietary bovine casein and may help to explain some of the outbreaks of necrotizing enterocolitis in preterm infants.

There are three ways that enterotoxin can react with milk proteins. First, the natural antibodies in human milk can bind and inactivate toxin preventing diarrhea. This interaction occurs over a wide range of milk and toxin doses. Second, at extremely low enterotoxin doses (10 ng), most bovine milk proteins and human casein seem to inactivate enterotoxin [although they have no effect on the doses of toxin (100 ng) usually used in rabbit ileal loop experiments]. Last, bovine casein exacerbates enterotoxin-induced gut-fluid distension. This increase in enterotoxin-induced fluid production occurred over a variety of casein and enterotoxin concentrations. Activity appeared to reside almost exclusively in the bovine alpha-casein, a protein absent from human breast milk. Associated with this increased fluid production was bowel wall hemorrhage in some animals; thus, in a small bowel exposed to bovine milk proteins we envision a picture of fluid production and hemorrhage induced by amounts of enterotoxin (10 ng) that are normally without effect in bowel containing human breast milk.

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