CONCISE COMMUNICATIONS

Lack of Prophylactic Efficacy of an Enteric-Coated Bovine Hyperimmune Milk Product against Enterotoxigenic *Escherichia coli* Challenge Administered during a Standard Meal

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Orally administered bovine immunoglobulins with specific activity against colonization factors of enterotoxigenic *Escherichia coli* (ETEC) could provide passive protection against ETEC challenge in volunteers. Twenty healthy adult volunteers ingested either a placebo or a partially enteric-coated preparation of bovine immunoglobulins with activity against the colonization factor antigens CFA/I, CS3, and CS6 and then were challenged with ETEC strain E24377A ($CS1^+$, $CS3^+$) administered with a standard meal. There was no difference in the incidence or severity of diarrhea among the 10 volunteers who received the bovine immunoglobulins and the 10 who received placebo. Either the specificity or titer of anti–colonization factor antibodies or the formulation of antibodies in this product was not adequate to provide passive protection against ETEC challenge.

Diarrhea is the most common health problem among travelers to developing countries. Enterotoxigenic *Escherichia coli* (ETEC), associated with 30%–70% of the cases of traveler's diarrhea, is the single most prevalent pathogen [1–4]. ETEC is also an important cause of diarrhea in young children in developing countries [5–7].

The pathogenesis of diarrhea due to ETEC depends on 2 known virulence factors: (1) fimbrial adhesins, which allow the organism to stick to intestinal epithelium and resist the clearing action of peristalsis, and (2) elaboration of either or both of 2 toxins, a heat-stable toxin (ST) and a heat-labile toxin (LT). ETEC enterotoxins activate cyclic nucleotide synthesis in the gut epithelium that causes secretion by crypt cells of fluid and electrolytes into the small intestinal lumen, resulting in watery diarrhea.

Advances in understanding the pathogenesis of ETEC diarrhea have led to the development of vaccine candidates against ETEC [8–10]. A vaccine against a broad spectrum of ETEC fimbrial and toxin types, however, will require several additional years of basic research and clinical trials. Therefore, alternative methods of prophylaxis against ETEC diarrhea have been sought.

One such alternative is passively acquired local immunity afforded by immunoglobulins in hyperimmune cows' milk or colostrum [11–13]. Bovine anti–*E. coli* colonization factor antigen (CFA) milk immunoglobulin concentrate, produced by ImmuCell, is a potentially effective and safe preparation for prophylaxis of ETEC diarrhea. This antibody preparation, formulated as a lyophilized powder in foil packets, was previously studied in volunteers [12]. In a placebo-controlled, challenge-protection study, 25 healthy adult volunteers were challenged with an oral dose of 10^9 cfu of ETEC strain H10407 (O78 : H11 CFA/I^+) during a 5-day course of anti–*E. coli* milk immunoglobulin prepared as a lyophilate. In this previous study, 7 of 10 volunteers who received placebo but only 1 of 15 volunteers who received the bovine immunoglobulins with sodium bicarbonate developed diarrhea after challenge (Fisher's exact test, $P < .0017$; Neyman-Pearson, $P < .0012$) [12].

A new preparation of bovine anti–*E. coli* CFA milk immunoglobulin, called TravelGAM, has been produced by ImmuCell. In the previous study, volunteers ingested 2 g of sodium bicarbonate along with each dose of lyophilized bovine immunoglobulin to neutralize gastric acidity. That formulation was not well suited for the international traveler, since regular ingestion of antacids with meals would increase risk of infection by foodborne pathogens. However, bovine immunoglobulins are irreversibly denatured by stomach acid. Therefore, to administer these immunoglobulins without a buffer, a new formulation was developed that would permit high immunoglobulin loading, resist stomach acid, and release activity rapidly

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Informed consent was obtained from all volunteers, and human experimentation guidelines of the US Department of Health and Human Services and of the Institutional Review Board of the University of Maryland, Baltimore, were followed in the conduct of this clinical research.

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upon the shift in pH during transit from the stomach. This product was prepared by the formation of small (~1 mm), uniform, spherical granules through a proprietary extrusion-spheronization process.

Extrusion-spheronization is a common manufacturing technique in the pharmaceutical industry, but this technique is not widely used for protein formulation due to the possibility of denaturing labile proteins during the process. We adapted the process for immunoglobulin formulation by controlling the extrusion temperature, eliminating solvents during extrusion, and employing a proprietary excipient mixing process that resulted in preservation of immunoglobulin activity. Standard pharmaceutical excipients such as microcrystalline cellulose, hydroxypropyl methyl cellulose, and polyethylene glycol were used in the formulation. A pH-dependent polymer coating was used that resulted in dissolution of the granules at pH 6.0. In vitro studies showed that >90% of the CFA binding activity was released from the granules within 5 min of the pH shift.

Because of the possibility that the bovine anti–*E. coli* immunoglobulins used in the previous study may have exerted their protective effect by inactivation of the *E. coli* inoculum in the stomach, half of the immunoglobulins used in this study were formulated into granules but not coated with the pH-dependent polymer; this material released activity rapidly, in a non–pH-dependent manner. The granules were contained within 00-sized gelatin capsules. The new bovine anti–*E. coli* CFA milk immunoglobulin formulation has the same anti-ETEC antibody activity as the product studied in the successful challenge trial described above, and the new formulation is convenient, portable, and shelf-stable.

The new bovine immunoglobulin preparation contains immunoglobulin with specific activity against fimbrial adhesins representing the 3 major classes of CFA: CFA/I, CS3 (a component of CFA/II), and CS6 (a component of CFA/IV). The current study was designed to assess the effectiveness of the bovine immunoglobulin preparation in the prevention of CS1/CS3-expressing ETEC enteritis as measured by the incidence and severity of diarrhea in a challenge-protection trial in healthy adult volunteers.

Methods

Study design. Twenty healthy adult volunteers were admitted to the Research Isolation Ward located in Kernan Hospital (Baltimore), which is part of the University of Maryland Medical System. After being screened, volunteers were randomly assigned in a double-blind fashion to receive 2 capsules (690 mg total) of a new preparation of bovine anti–*E. coli* CFA milk immunoglobulin (TravelGAM, ImmuCell; 50 : 50 coated : uncoated; hereafter referred to as bovine immunoglobulins) or 2 capsules (690 mg) of placebo. The placebo consisted of an identical preparation from nonimmunized cows. The study medication was administered to the volunteers 3 times daily, 10 min after each meal, for 5 days. During consumption of a standard lunch on the second day of the course of bovine immunoglobulins or placebo, volunteers were given 10^8 cfu of ETEC strain E24377A (O139 : H28 LT^+ ST^+ $CS1^+$ $CS3^+$). In previous dose-response studies evaluating the meal-challenge model, an inoculum of strain E24377A as low as 10^7 cfu produced diarrhea in 6 of 6 volunteers, with a mean diarrheal stool volume of 0.4 L. After an inoculum of 10^9 cfu of this strain was given with a standard meal, 4 (57%) of 7 volunteers developed diarrhea, with a mean diarrheal stool volume of 0.7 L (present authors' unpublished data). The apparently lower attack rate in volunteers given a higher inoculum may be related to the small number of subjects studied.

All stools were collected, and their consistency was graded on a scale of 1–5. Diarrheal stools were weighed. Study participants who developed diarrhea received an oral solution of glucose and electrolytes to prevent dehydration, and they received a 5-day course of ciprofloxacin (500 mg, twice daily) beginning 24 h after the definition of diarrhea was met. All other volunteers received ciprofloxacin before discharge from the study. Participants provided blood samples on days 14, 21, and 28 for the determination of serum anti-CS3 activity and anti-LT activity by ELISA [12].

Study medication. The study medication consists of bovine immunoglobulin purified and concentrated from the milk of dairy cows immunized with CFAs CFA/I, CS3, and CS6 purified from ETEC [12]. The bovine immunoglobulin was assayed for anti–colonization factor activity by ELISA as previously described [12]. Because titers reported as a reciprocal dilution vary from assay to assay, a reference standard preparation of defined potency was developed. Each ELISA assay yields units of activity of the test preparation that have been interpolated from the standard curve on the basis of the reference preparation. This process generates potency data that can be compared from preparation to preparation. In the case of anti-CFA/I activity, for example, the reference preparation was lot 43218, the material associated with protection in a previous challenge-protection study [12]. This material had an ELISA potency value of 72,000 ELISA units (EUs) per gram of IgG powder (13.9 μg of material yielding an average of 1.2 optical density units in several assays). The test preparation in the current study had an anti-CFA/I activity of 72,800 EU/g of powder (similar to that used in the previous study). After formulation, each dose contained 53,520 EU/dose of anti-CFA/I, 88,400 EU/dose of anti-CS3, and 35,646 EU/dose of anti-CS6.

Challenge. On the second day of the 5-day course of bovine immunoglobulin or placebo, each volunteer ate breakfast, and 10 min later, each received a dose of study medication. For lunch, a standard meal consisting of chicken, green beans, rice, and applesauce was served to all volunteers. Approximately midway through the meal, the applesauce, containing an inoculum of 10^8 cfu of ETEC strain E24377A (O139 : H28 $CS1^+$ $CS3^+$), was served. The ETEC challenge was followed 10 min later by the scheduled dose of study medication after lunch.

Outcome measurements. The primary effectiveness variable was the incidence of diarrhea, defined as 1 diarrheal stool of $\geq$300 mL or $\geq$2 diarrheal stools totaling $\geq$200 mL passed during a 48-h period within 96 h after challenge. The secondary effectiveness variables included diarrheal stool volumes, the number of diarrheal stools after challenge, and serum IgG responses to LT and CS3.

Results

There was no difference in the incidence of diarrhea, the total diarrheal stool volume, or number of diarrheal stools among the 10 volunteers who received bovine immunoglobulins and the 10 who received placebo (table 1). In addition, all volunteers shed the challenge organisms in the stools to an equivalent extent. Of the 20 volunteers, 19 developed active serum IgG responses to LT, and 2 of 10 in each group developed serum IgG to CS3 after challenge.

Table 1. Clinical, bacteriologic, and immune responses to challenge with enterotoxigenic *Escherichia coli* strain E24377A (O139 : H28 $CS1^+$ $CS3^+$) administered during a standard meal after ingesting a preparation of bovine anti–*E. coli* colonization factor antigen immunoglobulins or placebo.

Response	Bovine Igs (n = 10)	Placebo (n = 10)
Attack rate for diarrhea	5/10	3/10
Mean diarrheal stool volume, L	0.6	0.8
Mean no. of diarrheal stools	3.8	5.3
No. of volunteers who shed challenge strain in stool	10/10	10/10
Geometric mean peak stool excretion $\times$ 10^7 cfu/g	7.2	9.2
Rate of seroconversion with anti-LT IgG antibody	9/10	10/10
Rate of seroconversion with anti-CS3 IgG antibody	2/10	2/10

NOTE. All P values for bovine Igs vs. placebo were nonsignificant. Ig, immunoglobulin; LT, heat-labile toxin.

Discussion

Several factors could explain the failure of the bovine immunoglobulin capsules to protect against challenge with a CS1/CS3-expressing ETEC. This same preparation, formulated as a lyophilized powder of bovine immunoglobulins with activity against CFA/I, had previously provided significant protection against diarrhea that resulted from challenge with ETEC expressing CFA/I [12]. The newly formulated antibodies in the current study had the same activity as those used in the previous successful trial against a CFA/I-bearing strain; however, in the study described here, unlike the previous study, volunteers were challenged with a strain bearing CS1 and CS3 and were given the challenge organisms with food. Perhaps the titer of bovine anti-CS3 in the new preparation was not adequate for protection. Alternatively, passively administered anti-CS3 antibodies alone may not provide sufficient interference with the colonization of a $CS1^+$ $CS3^+$ challenge strain to prevent disease. However, in previous volunteer studies, challenge of volunteers with E1392-75/2A, an O6 : H16 $CS1^+$ $CS3^+$ strain, protected volunteers against subsequent challenge with an O139 : H28 $CS1^+$ $CS3^+$ strain, suggesting that anti-CS1 and -CS3 immune responses, at least in combination, may contribute to protection against diarrhea [10]. Another explanation is that the bovine immunoglobulins were not released from their enteric coating at the appropriate time or location to interfere with the colonization of the challenge organism.

The ETEC challenge model in which organisms are given with a standard meal was recently developed to better simulate conditions under which enteric pathogens might be ingested by a traveler. The ETEC challenge model previously consisted of live ETEC given along with 2 g of sodium bicarbonate in 150 mL of water to volunteers fasting for 90 min [12]. In the meal-challenge model, organisms are given in applesauce halfway through the standard meal, without the protection of sodium bicarbonate buffer. The meal challenge may be a useful model for testing the efficacy of vaccines and other prophylactic agents against the mild diarrhea associated with foodborne ETEC in US adults, although the attack rate for diarrhea may not be as consistently reproducible as a fasting challenge with sodium bicarbonate buffer.

Future work into the development of passive bovine antibody products for protection against traveler's diarrhea should focus both on broadening the spectrum and potency of anti-CFA and on a practical formulation to deliver these antibodies. A convenient, inexpensive product providing solid prophylaxis against traveler's diarrhea, without the use of antimicrobial agents used therapeutically, would probably be well received by travelers at high risk of diarrhea.

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