

Milk Immunoglobulin with Specific Activity against Purified Colonization Factor Antigens Can Protect against Oral Challenge with Enterotoxigenic *Escherichia coli*

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Enterotoxigenic *Escherichia coli* (ETEC) is the most commonly isolated pathogen responsible for travelers' diarrhea and the cause of up to 650 million cases of pediatric diarrhea per year in the developing world. As a safe alternative to the prophylactic use of antibiotics, a hyperimmune bovine milk antibody product with specific activity against purified colonization factor antigens (CFAs) was developed and evaluated in a human challenge study. Twenty-five healthy adult volunteers were challenged orally with 10^9 cfu of a virulent CFA/I-bearing ETEC. In the randomized double-blind trial, 7 of 10 volunteers receiving a lactose-free placebo developed clinical diarrhea after challenge, compared with only 1 of 15 cases in volunteers receiving active product (Fisher's exact test, $P < .0017$). It is concluded that antibodies against CFAs alone are sufficient for protection and that prophylaxis with milk-derived immunoglobulin is a feasible alternative to existing drug interventions.

Diarrhea is the most commonly reported illness in travelers from industrialized nations to the developing world. In patients in whom a known pathogen has been identified, enterotoxigenic *Escherichia coli* (ETEC) is commonly responsible for over half of all cases [1]. It is estimated that of the 16 million people traveling to these regions annually, 40%–70% contract ETEC-related diarrhea [1–3]. In addition, diarrheal disease in the developing world can account for as much as one-third of all deaths in children ≤ 2 years old [4].

Existing medical options for protecting travelers and children in developing countries from ETEC infection are limited to bismuth subsalicylate or prophylactic administration of antibiotics, such as trimethoprim-sulfamethoxazole or ciprofloxacin [5]. Neither of these interventions, however, is an ideal long-term solution. Preventative antimicrobial use in the context of world travel could increase greatly the outgrowth of antimicrobial-resistant pathogens into areas where these drugs are greatly needed therapeutically [3]. In fact, data from the Persian Gulf War reveal a striking number of resistant strains already in existence. Of 125 *E. coli* strains isolated, 39% were resistant

to trimethoprim-sulfamethoxazole, 63% to tetracycline, and 48% to ampicillin [6]. In the case of bismuth subsalicylate, long-term intake carries the risk of salicylate intoxication, especially among patients taking salicylates for other reasons [5].

Over the past 2 decades, a growing body of data has emerged supporting an important role for milk-derived antibodies in protection against gastrointestinal infectious disease in human and animal health. Principally, antibodies from bovine colostrum have been used in clinical studies of protection against infection with *Cryptosporidium parvum* [7, 8], rotavirus [9, 10], *Shigella flexneri* [11], *Clostridium difficile* [12, 13], and *Vibrio cholerae* [14]. Such products have been shown to be extremely safe and well tolerated. Oral administration of immunoglobulin with specific activity against whole ETEC cells has also been tested and shown to protect against oral challenge with a homologous ETEC strain [15].

Unfortunately, the lack of defined protective antigens and methods for producing large quantities needed for bovine immunization have stalled efforts to develop an effective, broad-spectrum product. In addition, all preparations tested to date have been made from colostrum, the IgG-rich first postparturition milkings. Purifying antibody from milk could be more economic in large-scale production than using colostrum. To overcome these obstacles, we have developed methods for large-scale production and purification of specific colonization factor antigens (CFAs) and used them to produce TravelGAM bovine anti-*E. coli* immunoglobulin (ImmuCell, Portland, ME), a milk-derived immunoglobulin concentrate with high specific activity against purified CFAs. In this report, we describe the composition and anti-CFA activity of this product and present results from a human challenge-protection study. Healthy adult volunteers who ingested either active product or a blinded placebo were challenged with 10^9 cfu of a virulent CFA/I-bearing ETEC. Incidences of clinical diarrhea were compared between groups as the primary measure of prophylactic efficacy.

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Informed consent was obtained from all patients prior to enrollment in the study in accordance with the human experimentation guidelines of the Department of Health and Human Services and those set out by the Institutional Review Board of the University of Maryland School of Medicine. Procedures using animals were done with US Department of Agriculture approval.

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Methods

Bacterial strains for vaccine production. All ETEC strains used in the manufacture of this product were obtained from the culture collection of the Center for Vaccine Development (University of Maryland, Baltimore). *E. coli* M424C1 (CS1, CS3) and E9034A (CS3) were used for production of CS1 and CS3; *E. coli* H10407 was used for production of CFA/I.

Production of bovine vaccines. Pure cultures were made from frozen vaccine master seed stocks of bacteria maintained at -80°C . Crude, CFA-containing, cell-free extracts were obtained by shearing CFAs from the surface of broth-grown cells, followed by centrifugation. The predominant CFA-containing fraction was partitioned from the extract by differential ammonium sulfate precipitation. The precipitating salt was removed by dialysis against 50 mM phosphate buffer, pH 7.2 (Spectra/Por 2, 12–14 kDa cutoff membrane tubing; Spectrum, Laguna Hills, CA). At this stage, extracts were monitored for approximate purity and yield by SDS-PAGE using a 5%–20% polyacrylamide gradient slab gel and standard buffer conditions [16]. Depending on the antigen, CFAs were further purified by either size-exclusion or ion-exchange chromatography. SDS-PAGE analysis was repeated and purity determined by scanning densitometry (model GS300; Hoefer Scientific Instruments, San Francisco). Preparations in which $>70\%$ of all Coomassie brilliant blue staining protein was CFA were considered acceptable for use as bovine vaccines. The CFAs were concentrated, diafiltered, and sterilized by passage through a $0.45\text{-}\mu\text{m}$ filter. All vaccine preparations were tested for bacterial and fungal sterility and the presence of $<100,000$ EU/mL endotoxin, as determined by limulus amoebocyte lysate assay (QCL 1000/LAL; BioWhittaker, Walkersville, MD). The final vaccine was prepared by mixing the appropriate dose of antigen 1:1 (vol/vol) with a synthetic, metabolizable, oil-based adjuvant.

Bovine vaccinations. All vaccinations were performed under the direction of a licensed veterinarian. All animals used were healthy Holstein dairy cows. Health records were maintained, and only healthy, mastitis-free animals were included in the milk pool. A series of intramuscular vaccinations was administered deep into the rear thigh muscle. A total volume of 2 mL was administered at a single site and the animals were monitored for adverse reactions. None were observed. Milk was collected regularly beginning shortly after administration of the final vaccination. Although the anti-CFA titers for each batch were known, no attempt was made to use only the highest titer milk for production. To simulate continuous manufacture, milk from seven different batches, collected over a 4-week period, were pooled to make the clinical test material described herein.

Preparation of anti-*E. coli* bovine milk immunoglobulin. Hyperimmune milk was processed into cheese by standard dairy practices. The whey fraction (containing the immunoglobulin) was pasteurized and fat was removed by centrifugation. Defatted whey was enriched for immunoglobulin by ion-exchange chromatography using a continuous contacting, pilot scale, automated chromatography system (ISEP, model L0604; Advanced Separation Technologies, Lakeland, FL) and concentrated by hollow-fiber filtration (model UFP-30-C-75; A/G Technology, Needham, MA) using a series of four polysulfone filtration cartridges (30,000 molecular weight cutoff). Phospholipids and residual non-immunoglobulin proteins were precipitated chemically and removed by continuous-flow centrifugation (model AS-16; Alfa-Laval/Sharples, Warmin-

ster, PA). The centrifugation supernatant was collected and concentrated to $\sim 10\%$ solids using the hollow-fiber filtration system. During this concentrating, residual lactose, milk peptides, and other salts were removed by step-wise diafiltration against 3 vol of 15 mM potassium citrate, pH 6.5. The buffered immunoglobulin fraction was frozen and subsequently contract-lyophilized (Oregon Freeze Dry, Albany, OR) to produce a stable, dry powder.

Quantitation of anti-CFA activity by ELISA. Anti-CFA titers were determined by measurement of binding of milk antibodies to purified antigen-coated plates by ELISA. Ninety-six-well microtiter plates (Immulon II; Dynatech Laboratories, Chantilly, VA) were coated with a solution of $>90\%$ pure CFA antigen at $0.5\ \mu\text{g/mL}$ overnight at 4°C . Coating antigen was removed, and the wells were blocked with 0.1% porcine gelatin in PBS for 1 h at 37°C . A 10% (wt/vol) aqueous solution of the lyophilized product was prepared in sterile PBS. Serial 2-fold dilutions were made by mixing $50\ \mu\text{L}$ of sample with an equal volume of PBS in microtiter wells. Plated samples were incubated for 1 h at room temperature (RT) on a rotating shaker. After a wash with PBS plus 0.1% Tween 80, a horseradish peroxidase-conjugated goat anti-bovine IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was added at a dilution of 1/100,000 and incubated for 1 h at RT with shaking. After another wash, H_2O_2 substrate and chromagen (3,3',5,5'-tetramethylbenzidine-2 HCl) were added. Color development was stopped by addition of 40% H_2SO_4 , and absorbance was measured at 450 nm using a kinetic microplate reader. Because the absolute ELISA titer or optical density is variable and dependent primarily on the antigen preparation used to coat the wells, the most accurate and meaningful comparison of multiple samples was made by establishing a reference standard from which all unknown sample titers were interpolated. Dilutions of our anti-CFA milk standard were run on each plate containing unknown samples, and a standard curve was constructed. Titers for unknown samples were then interpolated from the standard curve. This normalized all ELISA data and permitted meaningful comparisons between samples run on different assays to be made.

Comparison of anti-CFA, anti-lipopolysaccharide (LPS), anti-heat-labile toxin (LT), and anti-type I pilus-specific activity by ELISA. Microtiter plates were divided into four quadrants so that comparative analyses could be run side-by-side. This minimizes error due to plate-to-plate variation. To each quadrant was applied a different coating antigen: CFA/I, *E. coli* LT, *E. coli* LPS (O78, O127), or type I somatic pilus. CFA/I and LPS O78 were purified from challenge strain *E. coli* H10407, type I somatic pilus was purified from *E. coli* strain H10407P, and LPS O127 was obtained commercially (Sigma, St. Louis). Lyophilized product was used to prepare a fresh 10% (wt/vol) solution in microfiltered, deionized water, serially diluted (1/2000–1/640,000) and subjected to ELISA analysis by methods described above.

IgG purity. Total protein was determined by the Dumas Classical Digestion Assay (Association of Official Analytical Chemists [AOAC] method). The percentage of total protein that was bovine IgG was determined by resolving all proteins by SDS-PAGE and quantitating IgG by scanning densitometry. Briefly, lyophilized product was reconstituted in PBS to an approximate protein concentration of 1 mg/mL and mixed 1:1 with standard running buffer. Samples ($10\ \mu\text{L}$ each) were applied to 5%–20% polyacrylamide slab gels and electrophoresed using the system of Laemmli [16]. After Coomassie brilliant blue staining, the percentage of total

protein that was bovine IgG was determined using an automated scanning densitometer.

Volunteer study. Twenty-five healthy adult volunteers, housed as in-patients in the isolation ward at the Center for Vaccine Development (University of Maryland School of Medicine), were randomly assigned to 3 groups—placebo ($n = 10$), high-dose treatment ($n = 11$), and low-dose treatment ($n = 4$)—by assigning subject identification numbers to identically packaged foil pouches containing measured doses of each test article. The randomization list was generated and secured by ImmuCell's Quality Assurance Supervisor. All investigators and volunteers were blinded to these treatment group assignments throughout the study and during assessment of outcome. Each placebo pouch contained a single dose of a lactose-free infant formula (Lactofree; Mead Johnson Nutritional, Evansville, IN).

All 25 individuals received three doses/day of either the placebo or one of the two doses of active product 15 min after meals for 2 days. Each dose consisted of the assigned powder dissolved in 5 ounces (150 mL) of water containing 2 g of sodium bicarbonate. On day 3, 2 h after consuming the morning dose, all volunteers drank 4 ounces (120 mL) of water containing 2 g of sodium bicarbonate. One minute later, each received an oral challenge inoculum containing 10^9 cfu of H10407 (O78:H11), a CFA/I-bearing ETEC strain suspended in 1 ounce (30 mL) of water containing sodium bicarbonate. Fifteen minutes later, a second dose of either product or placebo was given. The usual evening dose was administered after dinner. On days 4–7, three doses/day were administered, as previously done, after each meal.

Volunteers collected every bowel movement produced during the study. Stools were graded for consistency, weighed, and logged to record number and volume produced per day. Daily stool samples were taken for bacteriologic examination. Daily medical rounds were conducted to monitor symptoms. Before discharge from the hospital, all volunteers were given a 3-day course of ciprofloxacin (500 mg twice per day) to eradicate the challenge organism.

The primary effectiveness variable was the clinical diagnosis of diarrhea, defined as one liquid stool of ≥ 300 mL or two liquid stools totaling 200 mL during any 48-h period within 120 h after challenge.

Bacteriology and serology. Daily stool samples were tested for the presence and enumeration of the challenge organism. Identification of the inoculum strain was confirmed by screening 10 individual colonies picked from eosin methylene blue (EMB) plates for agglutination with anti-O78 and anti-CFA/I serum.

Blood samples were drawn from volunteers before the study began (baseline) and on days 10 and 31 for serology, blood cell counts, and determination of anti-CFA, anti-LT, and anti-LPS responses.

Anti-CFA/I. Serum levels of IgG against CFA/I were determined by ELISA essentially as described for the active product, with the following modifications. Antigen-coated plates were blocked with PBS containing 0.1% fetal bovine serum instead of the 0.1% porcine gelatin used for the product assays, as this was found superior for reducing background. In addition, the reference standard comprised a pool of human serum from patients in this study rather than the hyperimmune milk reference standard used to assay the product.

Anti-LT. Serum levels of IgG against *E. coli* LT were also determined by ELISA. Plates were coated with 1.0 $\mu\text{g}/\text{mL}$ purified enterotoxin. The assay was carried out as described above.

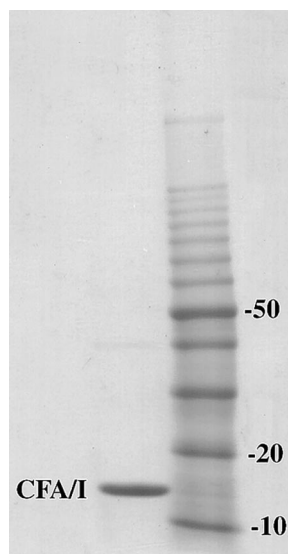


Figure 1. SDS-PAGE of purified CFA/I: Coomassie brilliant blue–stained, 5%–20% polyacrylamide gradient gel showing CFA/I that was purified from broth cultures of enterotoxigenic *E. coli* H10407 (O78:H11). Solution (500 $\mu\text{g}/\text{mL}$) of antigen in PBS was mixed 1:1 with sample buffer containing 20 mM dithiothreitol and run under reducing conditions. Prominent band at 15 kDa is CFA/I. This preparation is representative of antigens used in vaccines to inoculate dairy cattle for production of anti-*E. coli* bovine milk immunoglobulin. Protein ladder (10-kDa multimers) was run alongside pilus antigen preparation as size marker. Nos. at right correspond to M_r ($\times 10^3$) for subset (10, 20, 50 kDa) of these markers shown for reference.

Anti-O78 LPS. Serum levels of IgG against O78 LPS were also determined by ELISA. Plates were coated with 5 $\mu\text{g}/\text{mL}$ O78 LPS purified from H10407. The assay was carried out as described above.

Results

Composition of the bovine vaccine. By proprietary methodologies, CFAs were expressed and purified from broth-grown ETEC cells. Purified concentrated antigen preparations were analyzed by SDS-PAGE and scanning densitometry (figure 1). Antigen preparations that were $>70\%$ CFA were incorporated into the bovine vaccines.

Composition of the hyperimmune bovine milk immunoglobulin. Proximate analysis of the active product (lot 43218) revealed that the lyophilized powder was 2.7% moisture, 78% protein, 5.5% fat, 1.1% carbohydrate, 10.5% ash (due to added potassium citrate buffer), and 2.2% residual ash. IgG constituted 76% of the total protein as revealed by scanning densitometry and SDS-PAGE (figure 2). Since the powder is 78% protein, of which 76% is IgG, it is 59% IgG by weight. Additional milk proteins present include β -lactoglobulin, α -lactalbumin, serum albumin, and trace amounts of casein.

Immunologic evaluation of milk antibody product by ELISA. To establish a specific role for anti-CFA antibodies in protection against ETEC challenge, we wished to determine the potency of anti-CFA/I activity in the product relative to activity against O78 LPS, LT, and type I (somatic) pili. A comparative ELISA was performed, and the relative titers of anti-CFA/I, anti-LPS, anti-LT, and anti-type I pilus activity were determined (table 1). As a negative control, side-by-side samples of a commercially available, hyperimmune anti-K99⁺ bovine *E. coli* immunoglobulin preparation (First Defense; ImmuCell, Portland, ME) were run. These results reveal that, although there is measurable non-CFA activity in the product, the vast

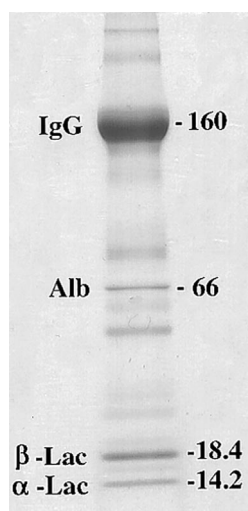


Figure 2. SDS-PAGE of anti-*E. coli* immunoglobulin: Coomassie brilliant blue-stained, 5%–20% polyacrylamide gradient gel showing bovine milk anti-*E. coli* immunoglobulins. Solution (10% wt/vol) of final product was resuspended in PBS, mixed 1:1 with sample buffer, and run under nonreducing conditions. Bovine IgG1 (prominent band at 160 kDa) represents >75% of all stainable proteins as determined by scanning densitometry. Major nonimmunoglobulin milk proteins present in bovine immunoglobulin concentrate include albumin (Alb), α-lactalbumin (α-Lac), and β-lactoglobulin (β-Lac); numbers at right correspond to M_r ($\times 10^3$). This preparation is representative of clinical material that was administered in study described here. Individual doses of lyophilized powder were dissolved in 150 mL of water containing sodium bicarbonate and ingested orally.

majority of anti-*E. coli* activity is specific for CFA (32-fold higher).

Clinical. Seven of the 10 volunteers in the placebo group had diarrhea after challenge, compared with only 1 of 15 volunteers in the 2 groups receiving the active product (table 2, Fisher's exact test, $P < .0017$). In comparing the attack rate in the placebo group (70%) with the attack rate in the treated groups (6.7%), prophylactic administration of the hyperimmune milk immunoglobulins brought about a protection rate of ~90%. The mean stool volume in volunteers with diarrhea was 1327 mL (range, 263–4421), and the mean number of stools was 7.4 (range, 2–21). The mean incubation time was 58.8 h (range, 19.4–100.3).

Daily medical rounds were conducted to record the incidence of several other symptoms besides diarrhea (table 3). Anorexia

Table 1. Comparison of anti-*E. coli*-specific activities in the clinical material.

| Antigen tested | Titer | |
|---|-------------------|-------------------|
| | Clinical material | Negative control* |
| CFA/I | 640,000 | <2000 |
| LT | 20,000 | <2000 |
| O78 LPS (purified from H10407) | 20,000 | <2000 |
| O127 LPS (heterologous <i>E. coli</i> serotype) | 2000 | <2000 |
| Type I (somatic) pilus | 20,000 | NA |

NOTE. Titers reported are inverse of highest dilution that gave OD >0.2 (450–650 nm). Titers were normalized for coating antigen concentration and represent comparisons of samples run side by side on quadrants of same microtiter plates to minimize plate-to-plate variation. LT, heat-labile toxin; LPS, lipopolysaccharide; NA, not applicable.

* Dilutions of commercially available, hyperimmune antibody preparation against K99⁺ bovine enterotoxigenic *E. coli* were prepared and analyzed side by side with samples of hyperimmune anti-CFA product tested here.

Table 2. Incidence of clinical diarrhea in volunteers receiving either placebo or active product followed by oral enterotoxigenic *E. coli* challenge (ETEC).

| Group, subgroup | n | Volunteers with diarrhea* (%) | Protection rate† | P | |
|------------------|----|-------------------------------|------------------|--------|----------------|
| | | | | CNPA‡ | Fisher's exact |
| Treatment | | | | | |
| High dose | 11 | 1 (9.09) | — | 0.0061 | 0.0075 |
| Low dose | 4 | 0 | — | 0.0264 | 0.0650 |
| Treatment totals | 15 | 1 (6.67) | 90.5 | 0.0017 | 0.0017 |
| Placebo totals | 10 | 7 (70.0) | — | — | — |

NOTE. Study of 25 healthy adults challenged with 10^9 cfu of H10407 ETEC and randomly assigned to 3 groups: placebo or high- or low-dose treatment with either milk-derived bovine anti-*E. coli* immunoglobulin or lactose-free infant formula (see Methods).

* 1 liquid stool of ≥ 300 mL or 2 liquid stools totaling 200 mL during any 48-h period within 120 h after challenge.

† Calculated for combined treatment groups: diarrhea incidence in control group (70%) minus diarrhea incidence in combined treatment groups (6.67%) divided by diarrhea incidence in control group (70%) = $(70 - 6.67)/70 = 90.5$.

‡ Conditional Neyman-Pearson algorithm (CNPA) of Coleman (W. P. Coleman, personal communication) was used to provide an exact correction for use of Fisher's exact twice in simultaneous nonindependent tests to compare 2 doses of active product against placebo.

was reported by 6 of 10 controls compared with 1 of 15 treated. Malaise was found in 3 of 10 controls and 1 of 15 treated. Five of 10 controls experienced headaches compared with 4 of 15 treated subjects. Finally, while all 10 volunteers receiving the placebo experienced abdominal cramps, only 2 of 15 volunteers receiving the product did. No adverse side effects attributable to ingestion of the bovine antibody were observed in any volunteer.

Bacteriology. Daily stool samples were analyzed for the presence of the challenge organism to quantitate shedding over time. The average number of challenge organisms shed by volunteers receiving the placebo at the time of maximal shedding was 4.5×10^8 cfu/g of stool. The equivalent mean bacterial count for volunteers in the 2 active groups was 6.2×10^7 g. The average number of days that volunteers excreted the challenge organism was virtually the same between groups: 5.3 days for controls versus 5.4 days for volunteers receiving active product (range, 4–6 days).

Serology: anti-CFA/I, anti-LPS, and anti-LT. To assess the extent of infection and to quantify the humoral immune response of volunteers to the challenge organism, serum titers against CFA/I, O78 LPS, and LT were determined. Blood samples were drawn on day 0 (admission/preimmune), day 10, and day 31, and sera were analyzed by ELISA against purified antigens. The mean peak titers for each group were calculated for comparison. The results of these analyses are presented in table 4. Although none of the group differences observed are statistically significant, there are some suggestive trends. First, volunteers in each group were able to mount substantial immune responses to all three antigens. Second, mean peak serum

Table 3. Clinical symptoms reported by volunteers during daily medical rounds conducted after oral challenge with enterotoxigenic *E. coli*.

| Group, subgroup | n | Volunteers reporting symptoms, no. (%) | | | | |
|------------------|----|--|---------|--------|----------|----------|
| | | Anorexia | Malaise | Gas | Cramps | Headache |
| Treatment | | | | | | |
| High dose | 11 | 1 (9) | 1 (9) | 2 (18) | 2 (18) | 3 (27) |
| Low dose | 4 | 0 | 0 | 0 | 0 | 0 |
| Treatment totals | 15 | 1 (6.7) | 1 (6.7) | 2 (18) | 2 (18) | 3 (27) |
| Placebo totals | 10 | 6 (60) | 3 (30) | 5 (50) | 10 (100) | 5 (50) |

responses to all three *E. coli* surface antigens are lower in both active groups than in the placebo group. The mean peak responses to LT and LPS were 2.7 and 2.5 times lower, respectively, and the mean peak response to CFA/I was nearly 2-fold lower.

Discussion

Oral prophylaxis with hyperimmune bovine milk immunoglobulin provided 90% protection against clinical diarrhea caused by oral challenge with ETEC in a placebo-controlled, human challenge study (Fisher's exact test, $P < .0017$). Reduction in concomitant disease symptomatology and microbial stool counts were also observed. In addition, there were no adverse events due to ingestion of the milk antibodies.

Although prevention of ETEC infection with oral milk antibodies has been demonstrated before [15], identification of the molecular target of protective immunity has remained elusive. Drawing analogies to virulence mechanisms utilized by other enteric pathogens, colonization factors, LPS, LT, and type I (somatic) pilus have all been investigated as potential candidate molecules. The milk antibodies produced here provided a means of directly testing the relative importance of anti-CFA immunity. By using purified CFAs in place of killed whole cells to immunize donor dairy cattle, the milk produced was greatly enriched for anti-CFA immunity. Specifically, the anti-CFA/I titer was 32 times greater than the comparable titers

raised against the three other antigens (table 1). Given that anti-CFA immunity was the predominant anti-ETEC activity in the concentrate, these results strongly suggest a central role for CFA-specific antibodies in protection against oral ETEC challenge.

As travelers' diarrhea caused by ETEC is primarily a food-borne infection, the dosing schedule utilized in this study was designed to model ingestion of milk antibodies with or shortly after meals. The positive results of this trial confirm that prophylaxis using this type of dosing schedule can be highly effective. Since the initial dose was administered shortly after challenge, it is not known whether a less frequent dosing regimen would be equally efficacious.

Microbiologic analyses revealed that although all volunteers shed high numbers of challenge organisms in their stool, the mean peak number of challenge organisms shed by individuals in the placebo group (4.5×10^8 /g of stool) was ~ 1 log higher than that for the treatment groups (6.2×10^7 /g of stool). This difference is not significant, but these data do suggest that eradication of the organism is not the primary mechanism of protection. Since ETEC diarrhea occurs when sufficient toxin is expressed in close proximity to the intestinal epithelium to cause secretory disease, oral immunoglobulins may confer protection by effectively reducing the number of organisms that can attach and locally elaborate toxin below the threshold necessary to cause disease.

A central issue relating to practical use of oral passive antibodies is whether such treatments dampen the development of natural immunity. In this study, milk immunoglobulins did have a measurable effect on mucosal immune responses to CFA/I, LPS, and LT (table 4). For example, the mean peak serum titers to LT and LPS were almost 3 times lower and mean peak serum titers to CFA/I 2 times lower in volunteers receiving antibody than in control individuals. Although this is not statistically significant, it is consistent with a mode of action whereby passive antibodies effectively restrict the number of organisms capable of maintaining close proximity to the antigen-processing cells of the intestine. Thus, the milk antibodies provided passive protection while still permitting development of active immunity. It is not known whether the magnitude of these responses would be sufficient to protect an individual against a subsequent ETEC challenge, but it is

Table 4. Serum immune responses to CFA/I and two other important *E. coli* surface antigens in volunteers receiving either active product or placebo followed by oral enterotoxigenic *E. coli* challenge.

| Group, subgroup | n | Serum titers* | | |
|------------------|----|---------------|----------|---------|
| | | Anti-CFA/I | Anti-LPS | Anti-LT |
| Treatment | | | | |
| High dose | 11 | 23.4 | 44.2 | 21.7 |
| Low dose | 4 | 26.5 | 2.5 | 13.5 |
| Treatment totals | 15 | 24.2 | 35.3 | 19.5 |
| Placebo totals | 10 | 47.1 | 89.4 | 52.2 |

NOTE. LPS, lipopolysaccharide; LT, heat-labile toxin.

* Peak mean titers for each group.

conceivable that some longer-term protection might be possible. Confirmation of such long-term effects will require further study.

Effective prophylaxis against ETEC diarrhea with passive antibodies in the natural setting will require high specific activity against a broad range of CFAs and improvements in the dose formulation to allow inclusion of multiple specificities. Regarding CFA coverage, there is a growing body of data indicating the relative prevalence and clinical importance of three adhesin classes: CFA/I, CFA/II, and CFA/IV. Epidemiologic surveys of travelers to Argentina [17], Bangladesh [18], Saudi Arabia [19], Mexico [20], Burma [21], and Chile [22] who contracted ETEC-related diarrhea, for example, have revealed the presence of these antigens in a majority of clinical isolates. Work currently underway in our laboratory includes expanding this database and augmenting the anti-CFA profile of the milk antibody concentrate.

The current dose form (powder neutralized in solution with sodium bicarbonate) would be inappropriate for use in countries with endemic severe gastrointestinal pathogens. A more feasible formulation, in which antibodies are enteric-coated to preserve functional activity during passage through the stomach, would obviate coadministration of antacid.

The need for a safe and effective means of protecting infants and travelers to the developing world from diarrhea caused by ETEC has been well established [23–27]. Oral administration of hyperimmune bovine immunoglobulin would provide an attractive alternative to antibiotic prophylaxis. From the results reported here, we conclude that antibodies against CFAs alone are sufficient for protection and that prophylaxis with milk-derived immunoglobulin may become a feasible alternative to existing drug interventions.

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