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Bovine Lactogenic Immunity against Cholera Toxin-Related Enterotoxins and *Vibrio cholerae* Outer Membranes

MARY BOESMAN-FINKELSTEIN,* NANCY E. WALTON,† AND RICHARD A. FINKELSTEIN

Department of Microbiology, School of Medicine, University of Missouri-Columbia, Columbia, Missouri 65212

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The newly parturient cow secretes large quantities of immunoglobulin G1, a relatively protease- and heat-resistant immunoglobulin, in its colostrum and milk. This study establishes the feasibility of producing protective colostral immunoglobulins by immunizing pregnant cows with cholera toxin (CT), a CT-related enterotoxin from *Escherichia coli*, and *Vibrio cholerae* outer membranes (OMs). The OMs were prepared from bacteria grown under iron-replete or iron-deficient (to simulate the in vivo environment) conditions. Immunoglobulins were purified from the colostrum of newly parturient control and immunized cows. The bovine anti-CT and anti-H-LT (CT-related heat-labile enterotoxin produced by diarrheagenic *E. coli* strains of human origin) antibodies were quantitated by enzyme-linked immunosorbent assays and by neutralization of toxin activity in both Y-1 adrenal cell and infant rabbit assays. The bovine anti-OM antibodies from both high-iron-grown and low-iron-grown vibrios were assessed by bacterial agglutination and by Western blot (immunoblot) analysis of polyacrylamide gel electrophoresis of high-iron-grown and low-iron-grown OMs. To test their protective effect, immunoglobulin preparations were administered orally in infant feeding formula to 6-day-old rabbits. Anti-CT and anti-OM immunoglobulins elicited statistically significant protection against diarrhea in infant rabbits challenged intraintestinally with virulent cholera vibrios.

Although marred by confounding variables, the vast majority of studies which have been conducted (3, 9, 21, 23, 25, 37) have found that breast-feeding reduced the risk of diarrheal disease morbidity and mortality in infants under 1 year of age. In light of these findings, the World Health Organization is engaged in a global effort to promote breast-feeding. Nevertheless, a substantial number of infants are, for a variety of reasons, not breast-fed. Additionally, it should be recognized that the protection passively imparted by breast-feeding may be limited by the immunological experience of individual mothers. Thus, it might be beneficial to consider that even more potent, broad-spectrum, and targeted immunity can be provided in infant feeding formulae supplemented with specific antibodies. Orally administered antibodies might also be protective in older age groups.

Although the figures vary at different times and in different places (4, 22, 29), overall a significant proportion of the diarrheal diseases in infants and young children are caused by bacteria that elaborate enterotoxins which are immunologically, structurally, and functionally related to cholera enterotoxin (CT), such as H-LT, a heat-labile enterotoxin produced by diarrheagenic *Escherichia coli* strains of human origin (17).

The newly parturient cow secretes large quantities of immunoglobulin G1 (IgG1), a relatively protease-resistant immunoglobulin, in its colostrum and milk (20, 24, 28). We have immunized pregnant cows with CT, with H-LT, and outer membranes (OMs) prepared from *Vibrio cholerae* grown under iron-replete or iron-deficient (to simulate the in vivo environment) conditions (15, 31–33). The immune response before and after parturition was followed by serum antibody measurements. Purified bovine colostral immunoglobulins containing antitoxic or antibacterial antibodies were found to protect infant rabbits from diarrhea resulting from direct intraintestinal live vibrio challenge. The results will be pertinent to future studies to define antibodies which, when administered orally, will contribute to protection against diarrheal disease in adults and, more importantly, in children.

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MATERIALS AND METHODS

Cow immunization regimen. Starting approximately 4 to 5 weeks preparturition, pregnant Holstein cows were immunized (at the University of Missouri-Columbia Foremost Dairy Farm), with either 2- or 10-mg doses of purified H-LT-1 (11, 17), which had been heated to reduce toxicity and to cause aggregation resulting in the formation of "procoligenoid" (16) of potentially increased immunogenicity. Additional cows were immunized with 10-mg doses of an analogous preparation of procholeragenoid (12) prepared from purified CT-1 (from *V. cholerae* 569B biotype *cholerae* serotype Inaba). The immunogens were emulsified 1:1 (vol/vol) in Freund incomplete adjuvant (FIA) and administered intramuscularly in 2.5-ml doses in each of the four quadrants of the cows. Toxin-immunized animals were boosted 2 weeks after the initial immunization with 2-mg doses of the antigens in the same volume of FIA. Controls were inoculated with only the same volume of FIA.

After 1 year, four of the original eight cows were available for study and pregnant again. Three weeks prior to parturition, these cows were bled to determine their antibody titers and boosted with 2 mg of toxin as described above. Colos-

* Corresponding author.
† Present Address: Department of Pediatrics, Memorial Hospital, University of North Carolina, Chapel Hill, NC 27514.

trum was collected, and a second lot of immunoglobulin was purified from each animal. Blood was collected at delivery and every 2 weeks until the immune response diminished.

OMs were prepared from *V. cholerae* 3083 biotype El Tor serotype Ogawa grown either under low-iron or iron-replete conditions (15, 31–33). The immunogens were emulsified in FIA and administered intramuscularly in 2.5-ml doses, totaling 8 mg of low-iron-grown OMs or 15 mg of high-iron-grown OMs, in each of the four quadrants of additional cows. OM-immunized cows were boosted twice with 3 mg of OMs at 2-week intervals after the initial immunization, until 1 month prior to parturition. Controls were inoculated with the same volume of FIA only.

Purification of bovine colostral immunoglobulin. Colostrum/milk was collected for 1 to 4 days postdelivery. The whole colostrum was heated to 39 to 40°C to liquefy the fat. Cream was then removed by using a DeLaval model 104 centrifugal separator. Rennin (2 mg/liter; Sigma Chemical Co., St. Louis, Mo.) was added to the resultant skim milk, and the pH was lowered to about pH 6 with 1 N HCl. After 1 to 2 h at 30°C to set the casein clot, the clear yellowish whey was obtained either by filtration through plastic cheesecloth (Vitulis and Co., Frankfort, Ind.) or by centrifugation at 9,000 × *g* for 15 min. The immunoglobulin was precipitated from whey at 35% saturation (19.7 g/100 ml) of ammonium sulfate at 4°C overnight. The precipitated whey was then centrifuged at 9,000 × *g* for 15 min, and the pellets were dissolved in water, with stirring, overnight. The small amount of casein which remained in the whey did not solubilize and was removed by centrifuging the whey again at 9,000 × *g* for 15 min. The soluble immunoglobulin preparation was extensively dialyzed against normal saline by using a Pellicon system (Millipore Corp., Bedford, Mass.) with a 10,000-molecular-weight cassette until sulfate was no longer detectable (i.e., <3 mg/100 ml) by precipitation with 2% $BaCl_2$ and was finally dialyzed against phosphate-buffered saline, pH 7.0. The purified antitoxic immunoglobulin preparations were standardized to 50 mg of protein per ml (26) and stored at −20°C in 500-ml aliquots. The purified anti-OM immunoglobulin preparations were dialyzed into 0.05 M NaCl prior to storage. The preparations were >90% pure by cellulose acetate electrophoresis.

Antibody assays. Antitoxin antibody levels in serum, whey, and purified immunoglobulin were determined by enzyme-linked immunosorbent assays (ELISAs) as described previously (11), with the exception that the secondary antibody used was peroxidase-labeled sheep anti-bovine immunoglobulin (ICN Biomedicals, Inc., Cambridge, Mass.). The optical density was determined at 410/490 nm by using an MR600 microplate reader (Dynatech Laboratories, Inc., Alexandria, Va.). For comparison purposes, an ELISA unit was defined as the smallest amount of immunoglobulin giving an optical density of 0.4, a point early in the linear portion of the dose-response curve. ELISAs were sensitive (e.g., optical density of ≥0.2) to about 0.4 to 0.8 μg of purified immunoglobulin per well. Purified CT-2 (*V. cholerae* 3083 biotype *eltor* serotype Ogawa), which, although related, differs immunologically from CT-1 (11, 27, 36), was included in some assays.

The ability of serum, whey, and purified immunoglobulin to neutralize toxin was determined in Y-1 adrenal cells and in infant rabbits. In the former assay (27), equal volumes of serial dilutions of antibody were mixed with 100 pg (≈10 tissue culture doses) of toxin, incubated for 1 h at 37°C, and then distributed at 200 μl/well in microtiter plates containing confluent monolayers of cultured Y-1 cells. The endpoint was the smallest amount (highest dilution) of antibody required to reduce Y-1 cell rounding to a score of 1+. In the infant rabbit toxin neutralization assay, decrements of antibody were mixed with toxin (2 μg), incubated for 1 h at 37°C, and fed in 5 ml of 0.1 M Tris (pH 8.0) to suckling rabbits (6 to 8 days old). The choleragenic score (13) was then recorded at 18 h. The endpoint was the smallest amount of antibody which reduced the control choleragenic score by at least 50%.

The antibody activity in the bovine anti-OM sera and immunoglobulin preparations was assessed by bacterial agglutination (2) using high-iron-grown and low-iron-grown *V. cholerae* 3083 and by Western blot (immunoblot) analysis (31–33, 35) of 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) of the *V. cholerae* 3083 OM preparations.

The component of the total anti-OM contributed by anti-lipopolysaccharide (anti-LPS) antibodies was estimated by the vibriocidal antibody inhibition test, as previously described (10). *V. cholerae* LPS was prepared from strain 3083 by the method of Westphal and Jann (38).

Protection studies. The protective effect in the infant rabbit model of feeding both antitoxic and anti-OM immunoglobulin preparations (in infant feeding formula) against intraintestinal challenge with virulent cholera vibrios was tested as follows. Infant rabbits (6 days old) were fed 4 ml of infant feeding formula (Enfamil; Mead Johnson Nutritional Division, Evansville, Ind.) containing the immunoglobulin preparation to be tested, on the evening before challenge, the morning of challenge (about 4 h before challenge), and 2 h after challenge. Approximately 10^5 vibrios (strain 569B or 3083) were injected directly into the small intestine by laparotomy. The following day, a diarrheagenic score (+1 to +4 by degree of wetness up the abdomen; 5, moribund; 6, dead) was taken hourly. Those scores at 11 a.m. (≈20 h after challenge) were used in the studies reported here, although observations were continued until all the animals had succumbed or 48 h postchallenge (whichever was earlier). Mean, standard deviation, standard error of the mean, and *P* values comparing groups were determined (18).

RESULTS

The amounts of purified colostral immunoglobulin recovered per cow and their antibody titers are summarized in Table 1. Immunoglobulin recovery ranged from 143 to 794 g per cow. As the highest concentration of immunoglobulin was obtained on day 1, the inclusion of later milkings did not substantially increase the yield of immunoglobulin in proportion to the intensity of labor involved in processing the larger volumes. For example, cow 271 secreted 14.7, 22.6, and 52 liters on days 1, 2, and 3 and 4 (combined) postpartum, respectively, and the ELISA titers of the colostral whey were 1,600, 500, and 100 for the respective samples. Colostrum was collected for 4 days in the earlier part of the study for cows 384, 269, and 271; for 3 days for cow 238; for 2 days for cows 323, 417, 376, and 388; and for 1 day for cows 351, 382, 373, 380, 352, and 312. Cows which recalved the second year (cows 384, 238, 417, and 388) were harvested on day 1 only, except cow 388 which was harvested for 2 days (no colostrum on day 1).

The time course of the immune response was followed by determining serum antibody titers during immunization and before and after parturition. Results from two cows (anti-H-LT cow 238 [Fig. 1A] and anti-CT cow 388 [Fig. 1B]) which calved again the second year are shown in Fig. 1. In

TABLE 1. Recovery of purified colostral immunoglobulin

Cow no.	Immunogen (dose)[a]	Purified immuno-globulin (g)	Antibody titer[b]
384	Adjuvant only	335 (254)[c]	260 (68)[c]
269	Adjuvant only	370	95
271	H-LT (2 × 2)	794	1,700
238	H-LT (2 × 2)	359 (508)	1,575 (2,170)
323	H-LT (10 + 2)	385	9,600
417	H-LT (10 + 2)	538 (403)	5,300 (8,200)
376	CT-1 (10 + 2)	353	1,700
388	CT-1 (10 + 2)	170 (226)	2,000 (920)
351	Adjuvant only	392	320
382	Adjuvant only	176	40
373	3083 OM[d] at low Fe (8 + 2×3)	170	80
380	3083 OM at low Fe (8 + 2×3)	394	320
352	3083 OM at high Fe (15 + 2×3)	273	1,280
312	3083 OM at high Fe (15 + 2×3)	143	640

[a] See the text for explanation of dose.

[b] Immunoglobulin fractions were adjusted to 50 mg of protein per ml in phosphate-buffered saline and stored in 500-ml aliquots at −20°C. Titers are from ELISAs for H-LT and CT-1 and agglutination tests for OMs.

[c] Values in parentheses show yield/titer from colostrum at the second delivery 1 year after the initial immunization.

[d] 3083 OM, *V. cholerae* 3083 OMs, grown under low-iron or high-iron conditions.

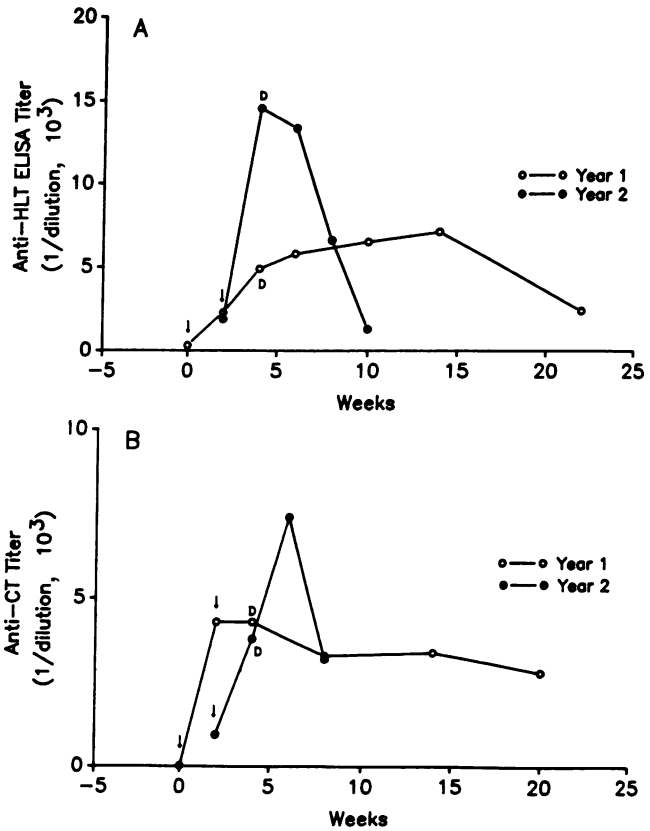

FIG. 1. Time course of bovine immune response to H-LT and CT immunization, as determined by serum ELISA antitoxin antibody titers. These are results of two calving periods for each cow. (A) Cow 238, immunized initially in year 1 with 2 mg of H-LT, followed by one 2-mg boost 2 weeks before parturition. In year 2, the cow was given a second 2-mg boost 2 weeks before calving. (B) Cow 388, immunized as described above with CT. Arrows represent time of immunization; D denotes time of delivery.

general, serum antibody levels in year 1 continued to rise postparturition, with individual cows peaking 2 to 10 weeks postdelivery and declining 6 to 18 weeks postdelivery. In year 2, on the other hand, titers rose to higher levels earlier and were already declining 4 weeks postpartum. The higher serum antibody levels at the second delivery did not result in proportionally higher colostral immunoglobulin titers (compare Fig. 1 and Table 1). Cows immunized with higher doses of H-LT antigen (cows 323 and 417) exhibited higher titers in both serum (results not shown) and colostrum (Table 1) than cows immunized with lower doses (cows 271 and 238).

The purified colostral antitoxic immunoglobulin preparations were evaluated for their ability to neutralize and cross-neutralize homologous and heterologous toxins in Y-1 adrenal cells, and the results were compared with their reactivity in ELISAs (Table 2). With occasional exceptions, results in the two assays paralleled each other. However, colostral immunoglobulins from cows immunized with CT, although of lower titer, were relatively more cross-reactive in both ELISAs and neutralization assays than the anti-H-LT immunoglobulin preparations. For example, immunoglobulins from the H-LT-immunized cows required more protein per ELISA unit or in neutralization against the CTs than against H-LT, whereas the anti-CT activities against the CTs and H-LT were more similar. Relatively more immunoglobulin was required for neutralization (versus 100 pg of toxin) than for detection in ELISAs (versus 100 ng of toxin). Even larger amounts of immunoglobulin (2 to 10 mg) were required for neutralization of toxicity as evaluated in vivo in infant rabbits (Table 3).

To test the effect of heat on the stability of bovine IgG1 antibody activity, one of the anti-H-LT immunoglobulin preparations (cow 271) was diluted to 2 mg/ml in infant feeding formula and heated for 1 h at 56 or at 63°C (pasteur-

TABLE 2. Comparison of antibody titrations by ELISA and Y-1 neutralization[a]

Immunogen and amt (mg)	Cow no.	H-LT		CT-1		CT-2	
		EU[b]	Y-1 U[c]	EU	Y-1 U	EU	Y-1 U
H-LT (2)	271	2.4	5.0	14.3	>40	43.5	>40
	238	1.7	1.25	25.0	20.0	25.0	20.0
H-LT (10)	323	0.8	0.3	12.5	20.0	50.0	5.0
	417	1.0	0.3	16.0	20.0	8.6	10.0
CT-1 (10)	376	19.2	20.0	14.3	1.25	17.8	10.0
	388	6.3	2.5	12.5	0.63	19.2	1.25

[a] H-LT data were derived from four independent duplicate titrations. CT data were derived from three independent duplicate titrations.

[b] EU, ELISA units, i.e., number of micrograms of purified colostral immunoglobulin per 100 μl required for an optical density of 0.4 with 100 ng of toxin.

[c] Y-1 U, Y-1 units, i.e., number of micrograms of purified colostral immunoglobulin per 100 μl required to reduce Y-1 cell rounding to a score of 1+ with 100 pg of toxin.

TABLE 3. Toxin neutralization by bovine colostral immunoglobulin in infant rabbits

Cow no.	Immunogen	Toxin challenge	Choleragenic score (no. of rabbits) with the indicated amt (mg) of immunoglobulin: 0	0.08	0.4	2	10	50
238	H-LT	H-LT	7.7 (11)	5.7 (3)	5.3 (6)	0.8[a] (6)	0 (5)	0 (6)
		CT-1	10.0 (3)	10.0 (2)	10.0 (3)	9.0 (3)	0[a] (3)	0 (3)
376	CT-1	H-LT	8.0 (3)	5.7 (3)	4.3 (3)	4.0 (3)	0[a] (3)	0 (3)
		CT-1	9.1 (3)	8.7 (3)	3.2[a] (6)	1.6 (5)	0 (5)	0 (6)

[a] >50% neutralization of biological activity of 2.0 μg of toxin.

ization temperature). The anti-H-LT ELISA antibody activity was stable to heating for 1 h at 56 or 63°C under these conditions (Fig. 2).

V. cholerae agglutination titers of sera and immunoglobulin preparations from cows immunized with OMs, compared with those of nonimmune controls, are summarized in Table 4. Interestingly, many preimmune and nonimmune sera and immunoglobulin preparations had agglutinating activity against *V. cholerae*, which may reflect cross-reactive antibodies produced in response to contact with antigens (dietary or normal flora) with antigenic similarities to surface components of *V. cholerae* or as a result of the herd immunization, given 3 weeks preparturition, which contained (among other antigens) a K99-enriched *E. coli* bacterin preparation. Each of the immunized cows responded with fourfold or greater rises in agglutinating antibody levels. Results of vibriocidal inhibition by *V. cholerae* LPS (from strain 3083) showed that the vibriocidal activity of the preimmune serum from cow 380 was markedly inhibited, whereas that of immune serum and immunoglobulin was not affected by treatment with LPS (results not shown). The vibriocidal activity of sera and immunoglobulin from the other cows tested was not affected by LPS. Thus, the predominant anti-*V. cholerae* antibodies of the immunized preparations were not directed against LPS. The origin of the cross-reactive antibodies is not clear, but earlier studies (10, 14) have noted the presence of complement-dependent vibriocidal antibody in the serum of Americans with no known exposure to *V. cholerae* antigens.

TABLE 4. Bovine immune response to immunization with *Vibrio cholerae* OMs

Antigen and cow no.	Antigen culture condition	Agglutination titer[a] Preimmune	Delivery	Immunoglobulin (50 mg/ml)
High-iron-grown OM				
352	High Fe	<20	320	1,280
	Low Fe	40	320	320
312	High Fe	80	1,280	640
	Low Fe	<20	1,280	1,280
Low-iron-grown OM				
373	High Fe	20	640	80
	Low Fe	20	2,560	80
380	High Fe	40	320	320
	Low Fe	<20	320	320
No antigen (FIA only)				
351	High Fe		<20	320
	Low Fe		160	320
382	High Fe		<20	40
	Low Fe		160	160

[a] Agglutination titer with live *V. cholerae* 3083.

Figure 3 shows Western blot analyses of the spectrum of antibodies present in OM-immunized and nonimmune cows against OM components of both high-iron-grown and low-iron-grown *V. cholerae* 3083. Arrows (Fig. 3, lanes 2A, 3A, and 4A) indicate areas of the blot in which one or several bands were present only in the low-iron-grown OMs, probably proteins whose synthesis is induced by low-iron stress (shown to simulate in vivo conditions [15, 31–33]). It should also be noted that control immunoglobulin (lane 2) reacted with a number of bands in both OM preparations, including some low-iron-grown membrane antigens. Although immunization increased both the number and, especially, the intensity of the immunostained bands, marked differences between immunoglobulins raised against high- (lane 3) and low- (lane 4) iron-grown OMs were not observed. However,

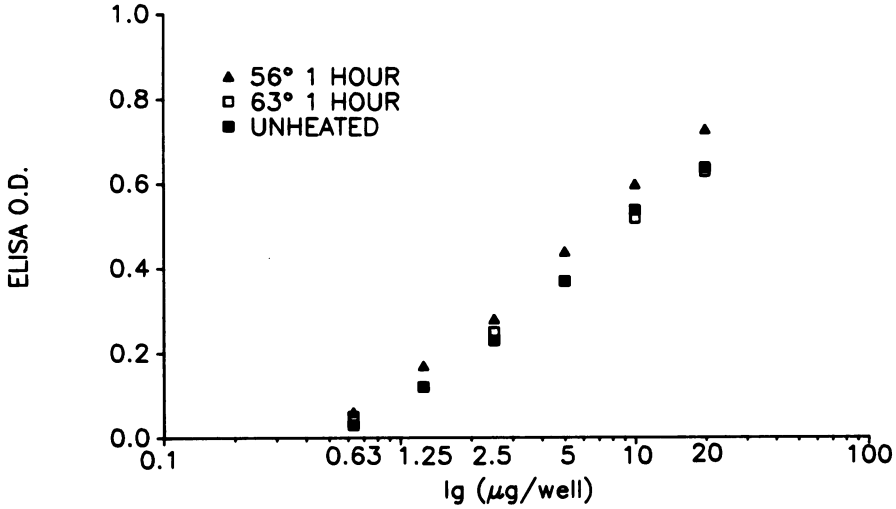

FIG. 2. Effect of heating on bovine colostral anti-H-LT activity (cow 238). Purified immunoglobulin at a concentration of 2 mg/ml in formula was heated for 1 h at 56 and 63°C. Antibody was serially diluted and tested in the H-LT ELISA. O.D., optical density; Ig, immunoglobulin.

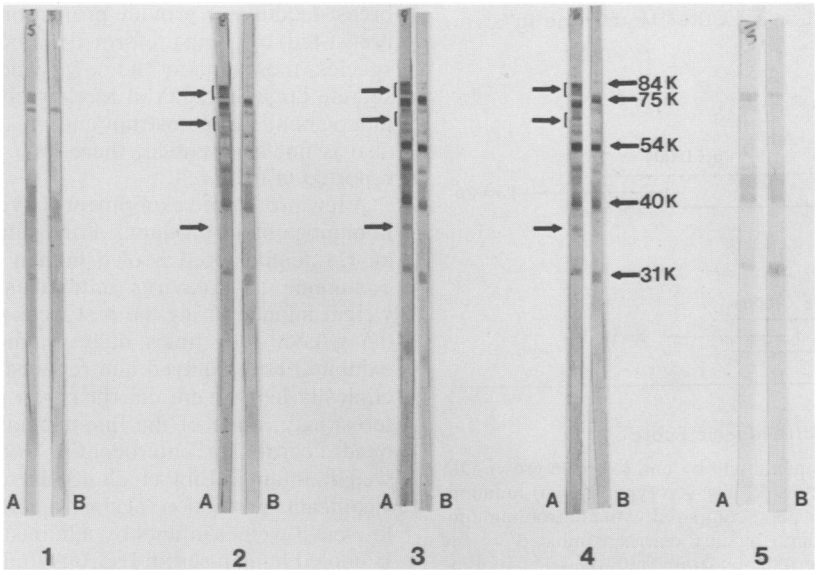

FIG. 3. Western blot analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) showing the spectrum of antibodies present in normal and immune bovine immunoglobulin preparations. The OMs were grown with low (A) or high (B) levels of iron. Lanes: 1, no primary antibody; 2, normal bovine colostral immunoglobulin (cow 351); 3, anti-high-iron-grown-OM immunoglobulin (cow 352); 4, anti-low-iron-grown-OM immunoglobulin (cow 380); 5, Ponceau S-stained nitrocellulose prior to bovine serum albumin blocking. OM preparations were boiled and reduced prior to electrophoresis. Molecular weights (in thousands) of some major membrane antigens are indicated by arrows (lane 4B).

it is clear that more stained bands are evident in the low-iron-grown OMs (A lanes versus B lanes).

Protection against *V. cholerae* 3083-induced diarrhea (live vibrio challenge) in infant rabbits by feeding bovine anti-OM immunoglobulin from strain 3083, anti-CT-1 immunoglobulin, and control immunoglobulin is shown in Fig. 4 through 6. The oral administration of three 50-mg doses of bovine anti-OM (both high-iron-grown and low-iron-grown) immunoglobulin (Fig. 4, bars 3 and 5) resulted in statistically significant protection ($P < 0.001$) against *V. cholerae* 3083-induced diarrhea 20 h following challenge and 18 h after the last feed. A lower-dose regimen of anti-low-iron-grown-OM immune immunoglobulin (three 10-mg doses) was also protective (bar 4). Control immunoglobulin (cow 351) (bar 2) resulted in protection ($P < 0.1$) compared with placebo-fed rabbits (bar 1); this effect was markedly less than that seen in animals fed immune immunoglobulin. Protection was also achieved by feeding three 50-mg doses of bovine anti-OM immunoglobulin (cow 380, upper bars) against challenge with 10^5 live virulent vibrios of the heterologous strain *V. cholerae* 569B (Fig. 5). Lower bars represent three 50-mg feeds of control immunoglobulin (cow 351).

Figure 6 shows that orally administered bovine anti-CT-1 immunoglobulin (pool of immunoglobulin from cows 376 and 388) was significantly more protective than control immunoglobulin (cow 351) against live vibrio challenge with both homologous (strain 569B) and heterologous (strain 3083) *V. cholerae* ($P < 0.005$ and $P < 0.001$, respectively).

It should be mentioned that the protective effects observed were primarily manifested as a delay in the onset of diarrheal disease. Although some passively immunized animals remained symptom-free during the entire 48-h observation period, many did eventually succumb or express symptoms (later than the controls). We attribute these breakthroughs to the fact that feeding of antibody was not continuous after challenge.

DISCUSSION

The concept of preventing infectious disease by passive administration of antibody is not new; in fact, one form of passive immunization, breast-feeding, has been practiced since long before the advent of *Homo sapiens*. It is widely

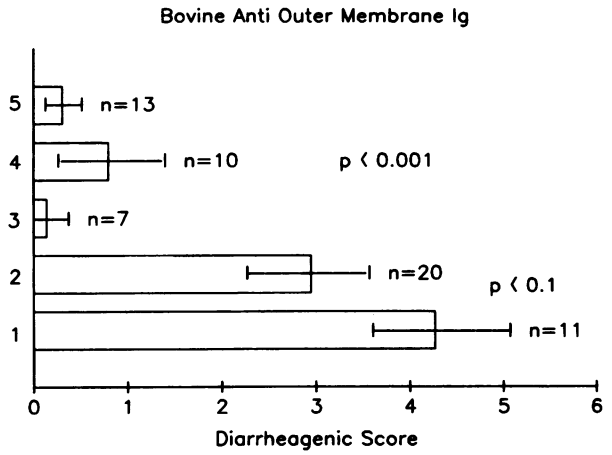

FIG. 4. Protection by bovine immunoglobulin in 6-day-old rabbits against diarrhea induced by intraintestinal vibrio challenge with 10^5 live virulent *V. cholerae* (strain 3083) per rabbit. Rabbits were fed (using a size 8 French feeding tube) 4 ml of infant feeding formula (unsupplemented, supplemented with normal immunoglobulin, or supplemented with immune immunoglobulin [Ig]) on the afternoon of arrival, the next morning before surgery, and again after surgery. Bars: 1, formula alone; 2, formula containing 50 mg of control immunoglobulin (cow 351); 3, formula containing 50 mg of anti-high-iron-grown-OM immunoglobulin (cow 352); 4, formula containing 10 mg of anti-low-iron-grown-OM immunoglobulin (cow 380); 5, formula containing 50 mg of anti-low-iron-grown-OM immunoglobulin (cow 380).

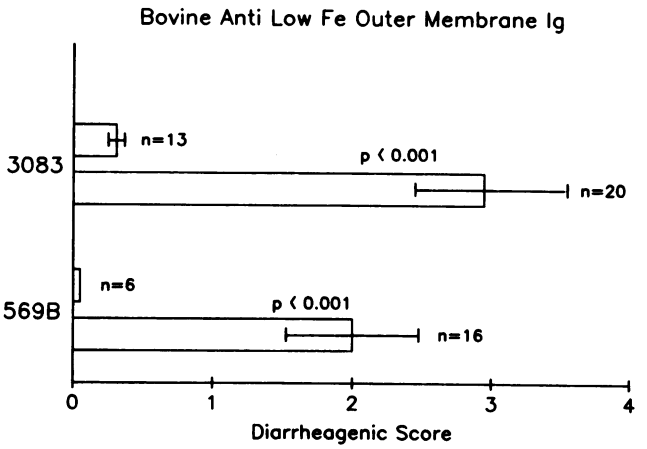

FIG. 5. Protection in infant rabbits by anti-low-iron-grown-OM (from *V. cholerae* 3083 biotype El Tor serotype Ogawa) immunoglobulin (Ig) (cow 380, upper bars) compared with control immunoglobulin (cow 351, lower bars) against diarrhea induced by the homologous strain or by heterologous strain 569B (classical biotype Inaba serotype). Intraintestinal challenge was 10^5 live virulent vibrios per rabbit.

accepted that breast-fed babies fare better than their formula-fed counterparts, although, as mentioned above, almost all of the supporting studies have inherent methodological problems. Nevertheless, the abundance of evidence is convincing in itself, even though the mechanisms are not clearly understood. The protective effects may be due in part to avoidance of environmental contamination (e.g., infant feeding formula may be prepared with polluted water in dirty containers), but to a more significant degree, they probably depend on protective elements in breast milk including, especially, preformed maternal secretory IgA. The protective activity of maternal antibody would, of course, be limited by the immunological background of the mother.

The thrust of the present work is that it should be possible to complement or supplement the protection afforded by

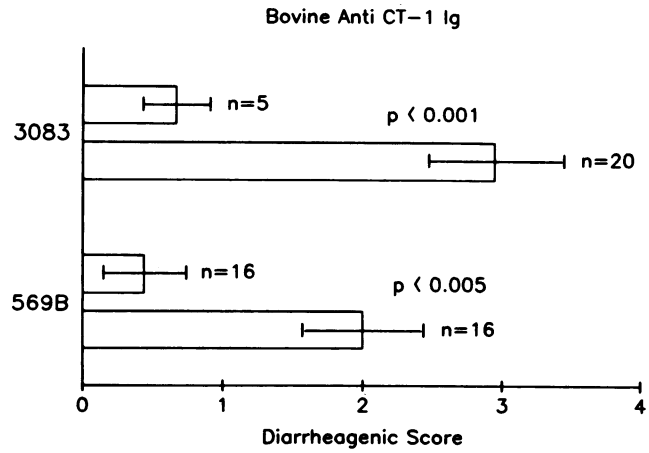

FIG. 6. Protection in infant rabbits by anti-CT-1 (three 50-mg feeds of a pool of immunoglobulin [Ig] from cows 376 and 388) against diarrhea induced by the homologous (569B) and heterologous (3083) *V. cholerae* strains, as described in the legend to Fig. 5. The upper bars represent bovine anti-CT-1 immunoglobulin, and the lower bars show three 50-mg feeds of control immunoglobulin (cow 351). Intraintestinal challenge was 10^5 live virulent vibrios per rabbit.

breast-feeding (or provide protection to infants who are not breast-fed) by using preformed antibody produced in another species, most notably the cow, which has been reported to secrete large amounts of a relatively protease-resistant immunoglobulin in colostrum and milk (24, 28). Although this also is not a new idea, there have been very few studies reported in this area.

A few prototypic experiments have been done with human immunoglobulin products. For example, in 1979 Barnes et al. (1) administered pooled human serum immunoglobulin containing antirotavirus antibodies per os to low-birth-weight infants during the first week of life. The dosage was 0.5 g/feed, four times daily. Immunoglobulin-fed infants exhibited both delayed and reduced excretion of virus and clinically less severe diarrhea than placebo-fed infants. Interestingly, none of the immunoglobulin-fed infants developed necrotizing enterocolitis, a high risk in low-birth-weight infants. Eibl et al. (8) have very recently shown significant protection against necrotizing enterocolitis in low-birth-weight infants by administration of Cohn fraction II pooled human serum IgG-IgA (0.6 g/day, as a supplement to their formula or pasteurized human milk, for a period of 28 days). None of the immunoglobulin-treated infants developed necrotizing enterocolitis, whereas nearly 7% of infants in the control group did.

In 1979, Mietens and Kleinhorst (30) treated infants with acute gastroenteritis with a bovine milk immunoglobulin concentrate (MIC) containing antibodies to enteropathogenic strains of *E. coli* and reported that the duration of positive stool cultures was significantly reduced in the treated patients, but no therapeutic effects were noted. The MIC preparation, containing 40% immunoglobulin, was given for 10 days in doses of 1 g/kg (body weight) per day (or, as an order of magnitude, about 10 g of MIC per day per child or 4 g of immunoglobulin per day per child). At this rate, and according to our study, a single cow would yield perhaps 100 treatment days (about 400 g of immunoglobulin).

More recently, Brüssow et al. (6) and Hilpert et al. (20) treated infants with acute gastroenteritis with another MIC preparation containing rotavirus antibody, at a dose of 2 g/kg (body weight) per day for 5 days. Three preparations, differing quantitatively in antibody level, were used; none was therapeutic, and only the highest-titer preparation reduced the period of excretion of rotavirus.

While the present work was in progress, and most significantly, adult American volunteers fed 9 g of MIC from cows immunized with a multivalent *E. coli* preparation were found to be protected against challenge with an enterotoxigenic *E. coli* (34). Minimal protective doses of MIC were not defined, nor were the essential antibody (or antibodies) (i.e., antisomatic, antimembrane, antiadhesin, antiflagellar, anti-H-LT, or other). At this level, and again according to our results, a single cow would also provide about 100 days of prophylaxis. Preparations of higher specific activity would go further.

In experimental animal studies, McLead and Gregory (28) found that bovine colostral anti-CT immunoglobulin neutralized CT and could be detected in the cecal contents of rabbits fed anti-CT whey. Live cholera vibrio challenges were not attempted. More recently, Yoshiyama and Brown (39) reported that milk from rabbits immunized with live *V. cholerae* protected rats against *V. cholerae* challenge in intestinal loops in situ. Adsorption studies indicated that antitoxic antibodies were the only protective components of the immunized milk. It should be noted that Glass et al. (19)

associated the protection against cholera in breast-fed children with antibodies in breast milk.

We are using experimental cholera, namely, CT-related enterotoxins and *V. cholerae* OMs, as a prototype testable model for the evaluation of the protective effect of orally administered bovine colostral antibodies, recognizing that other antibodies could well be incorporated in similar formulations to protect against additional diseases. The present study establishes the feasibility of immunizing pregnant cows with CT-related enterotoxins and *V. cholerae* OMs to obtain substantial (about 150 to 800 g/cow) quantities of purified colostral immunoglobulin with antitoxic or antibacterial activity. The bovine anti-CT and anti-*V. cholerae* OM immunoglobulin preparations have clearly been shown to protect against (or delay the onset of) diarrhea in infant rabbits challenged intraintestinally with live vibrios. Immunized cows were demonstrated to have a significant serum anamnestic response after 1 year, although this was not translated into proportionately higher colostral antibody titers.

It was of interest to note that normal colostral immunoglobulin from unimmunized cows had demonstrable antibodies which were reactive with CT and with *V. cholerae* OMs and exhibited some protective effect. This antibody is probably directed against antigenically cross-reactive conserved epitopes of dietary or normal flora origin (as toxinogenic *V. cholerae* is not present in Missouri). Similar observations were noted earlier with milk from human mothers in Missouri (5, 7).

Further studies in experimental animal models and in human beings will be needed to determine the feasibility and practicality of bovine lactogenic immunity. While our studies have been confined to essentially pure immunoglobulin, the antibodies have been demonstrated to be stable to pasteurization in infant feeding formula. If further studies demonstrate the effectiveness of antibody at lower concentrations than have been used previously (34), it may be possible to use whole milk as a source of protective antibody, and it is likewise conceivable that a cow or a herd of cows could be immunized with multiple antigens.

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