Evidence Supporting the Mechanisms of Enteric Protection Provided by Colostral Whey Fed Supplements

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Abstract

With the increasing public concern over the use of antibiotics in the production of meat and dairy animals, we decided to examine bovine colostral whey as a source of safe, effective biological agents to control enteric pathogens and promote the growth of neonatal and weaning animals. Colostral whey, its antibody rich and/or antibody poor fractions were found to have bactericidal activity against 0157 *E. coli* and bacteriostatic activity against a wide range of enteropathogenic bacteria. These same colostral whey preparations did not inhibit the growth of non-pathogenic strains tested. The colostral whey preparations inhibited cytotoxic activity of Shiga-like toxin, the infection of cultured cells with enteric viruses and exhibited some anabolic activity on cultured cells. In an *in vivo* experiment with gnotobiotic animals, the colostral whey reduced the severity of an experimental infection and partly blocked the induction of systemic immunosuppression. We feel these effects were mediated by inhibition of the growth of the challenge *E. coli* and inhibition of the action of the Shiga-like toxin, which appears to be involved in the development of the immunosuppression. These studies suggest that colostral components may be good candidates for biological replacements of antibiotics as supplements to young animals.

Introduction

The public has become increasingly concerned about agricultural use of antibiotics in meat and milk production. Recent years have seen debate over banning the use of antibiotics in agriculture in several state legislatures. The export market for U.S. meat, especially to the European Common Market, is greatly restricted by regulations concerning the use of antibiotics as feed additives and growth promotants. The public fears the development of "Superbugs" in meat animals which will cause untreatable infections in their elderly parents or young children; and the transmission of antibiotics through their food which will force the bacteria they have normal contact with to mutate into wild, untreatable agents of infection. Wide spread use of antibiotics does increase pressure on organisms to develop resistant strains, but it is likely that the public's perception of this situation will be a greater problem to livestock producers than the biological effects of the antibiotics in their animals. There is a growing demand for natural, biological alternatives to antibiotics as feed additives to control pathogens and for use as growth promotants. However, little hard scientific data about these biological "agents" has been available. It has been clear to producers and veterinarians that animals which receive colostrum at birth are better prepared to resist disease and are generally more vigorous. This complex mixture, delivered by the mother in a single dose provides an excellent model for the type of biological protection which would overcome the need for antibiotics in routine management of young animals. A role for the large quantity and broad diversity of antibody found in colostrum is evident, but the mechanism by which colostrum provides growth stimulation and continuing protection in the enteric tract remains to be described. This laboratory has undertaken a multifaceted program to examine the bacteriocidal, bacteriostatic, toxin inhibiting, viral infection inhibiting, and cellular growth promoting activities of colostral whey, the antibody from colostral whey and its aggregate non-antibody components *in vitro*. We have also examined the effects of whole whey on the course and consequences of a severe toxigenic *E. coli* infection model in gnotobiotic animals to determine if the *in vitro* effects were translated into *in vivo* protection. Our results suggest that both antibody and non-antibody components of colostrum have potential usefulness in management of disease and growth of young animals.

Materials and Methods

Colostral Whey and its Fractions

The colostral whey was produced either from the first two milkings of dairy cows from the South Dakota State University standing dairy production herd by acid curding, centrifugal removal of curd and serial filtra-

tion through glass fiber, 8.0, 2.0, 1.2, 0.8, 0.45 and 0.2 micron cellulose acetate filters, or as a sterile filtered, preservative free whey (from rennet curded first and second milkings) from Sterling Technology, Inc. of Toronto, SD. All whey was checked for sterility on tryptic soy agar and blood agar plates for five days before use. The whey was fractionated into IgG rich or IgG poor preparations by two methods. The first method was dialysis removal of the low molecular weight fraction across a 50kda molecular weight cutoff (mwco) membrane for 24 hours against phosphate buffered saline (PBS). The retentate used as IgG rich and the dialysate used as IgG poor. The second method for preparation was to separate the IgG from the remaining components with 45% ammonium sulfate. The precipitate dissolved in PBS representing the IgG rich and the soluble fraction as IgG poor. Each of these fractions was desalted in an 8kda mwco dialysis membrane against three changes of 10 volumes of PBS. The antibody concentration was measured for each colostral whey preparation and fraction using a commercial radial immunodiffusion kit (VMRD, Pulman, WA). The total protein concentration was determined using the Biorad protein assay. In each case, the IgG poor fraction contained essentially no IgG as measured by RID, while more than 80% of the IgG was recovered in the IgG rich fraction. Total protein recovered in the fractions was greater than 80% in all cases.

Bacteria

A non-pathogenic strain of *E. coli* was obtained from the American Type Culture Collection (ATCC 25922). The remaining pathogenic strains of *E. coli* were obtained from Dr. R.A. Wilson of the Penn State *E. coli* Reference Center (State College, PA). These strains represented *E. coli* serotypes 0157:H7, 0111:NM, 055, 026 and 0126. Two salmonella strains, *S. typhimurium* and *S. javiana*, from clinical outbreaks of food borne disease associated with milk products, were provided by Dr. D. R. Henning of South Dakota State University. The strain of *E. coli* used to produce shiga-like toxin (SLT), C600-933J, and its non toxic parent were obtained from Dr. D.H. Francis of South Dakota State University. Stock cultures were grown in Mueller Hinton broth and frozen in 15% glycerol containing Mueller Hinton broth and stored at -80°C until needed. SLT containing culture fluid was produced by incubating the C600-933J *E.coli* aerobically for 48 hours in iron depleted syncase broth. The bacterial cells were removed and the culture supernatant was filter sterilized through a 0.2 micron nylon filter. Identically treated cultures of the C600 parent strain were processed as a metabolic control.

Cell Cultures

The human tumor cell lines, HeLa and HEp-2, were obtained from the ATCC. MA-104 cells and porcine ST cells were obtained from the South Dakota State Veterinary Diagnostic Laboratory. The cells were grown and maintained in Dublecco's minimal essential medium (DMEM) with 10% heat inactivated fetal bovine serum and 50μg/ml of gentamicin sulfate (all obtained from Sigma, St. Louis, MO). The cells were grown for stock in T-75 flasks and put into 96 well flat bottom plates for assays.

Assays of Microbial Growth

Frozen cultures were thawed and 0.1 ml was inoculated into 10 ml of Mueller Hinton broth. The cultures were incubated overnight at 37°C. The cultures were inoculated into our own minimal medium (2% nonessential MEM amino acids, 1% MEM vitamins, 10mM glucose in PBS) $1x10^6$ or $1x10^2$ bacterial per ml. The bacterial were placed in minimal medium alone or minimal medium supplemented with 1:10, 1:20. 1:40 or 1:80 of one of our colostral whey preparations.

Two types of assays were performed. First, bacteriocidal activity was determined by seeding $1x10^6$ bacteria/ml into minimal medium and monitoring the number of surviving organisms after 0, 15, 30 and 60 minutes of incubation by performing plate counts on tryptic soy agar (TSA). Second, the bacteriostatic activity of the preparations was accessed by seeding the medium with $1x10^2$ bacteria/ml and incubating the cultures for 18 hours and monitoring growth by performing plate counts on TSA.

Assay of Bacterial Binding

Bacterial binding was measured using radiolabelled bacteria. The bacterial cells were metabolically labelled using 100μCi of ^3H thymidine (ICN, 6.7Ci/mM) in 2.5 ml of DMEM with 10% fetal bovine serum without antibiotics. The bacteria were incubated for 18 hours at 37°C, washed three times with PBS and suspended at about $1x10^8$ bacteria/ml in DMEM without supplementation. The bacteria were added to five replicate wells of HEp-2 cells for each treatment tested. Two types of assays were performed. In one type the HEp-2 cells were incubated with 100μl of serially diluted preparations of the colostral whey or its fractions for two hours, washed and 100 μl of bacterial suspension plus 100μl of DMEM were added to each well. In the second type of assay, 100μl of radiolabelled bacteria and 100μl of serially diluted colostral whey or its fractions were added at the same time. Once the labelled bacteria were added in either assay, the cells were incubated for two hours at 3°7C, washed three times with PBS, the HEp-2 cells lysed to release bound bacteria and the lysate counted in a scintillation counter. The results were expressed as percent of control binding.

Assays of SLT toxicity

The effects of colostral whey and its fractions in SLT cytotoxicity assays were measured. DMEM (100 µl) containing one LD_{50} unit of SLT was added to 96 well plates of confluent HeLa cells. To triplicate wells 100µl serially diluted samples of colostral whey or its fractions were also added. Wells with only DMEM or DMEM and toxin were used as negative and positive controls. The plates were incubated for 24 hours and read for cytotoxicity.

Assays of Viral Infection of Cultured Cells

Stock of poliovirus type 1 (vaccine strain), the OSU and Godfried strains of rotavirus and TGE virus were obtained from Dr. D.A. Benfield, South Dakota State University. Working stocks were produced in culture and the $TCID_{50}$ were determined for each virus. Two types of assays were performed with the viruses. First, cells were incubated for two hours with serial dilutions colostral whey or its fractions, then after the cells were washed three times with PBS, five replicate wells were inoculated with 0.5, 1, 2, 5 and 10 $TCID_{50}$ of the stock. The cells were incubated at 37°C and scored for cytopathic effect at 24 and 48 hours. In the second type of assay, the virus was added to the wells at the same time as the dilutions of colostral whey or its fractions into five replicate wells. The cells were incubated at 37°C and again scored for cytopathic effect at 24 and 48 hours.

Assays of Cellular Growth

Cultures of HEp-2 cells and HeLa cells were seeded into triplicate wells of 96 well flat bottom plates at $1x10^3$ per well. The medium in the wells contained DMEM, 2% fetal bovine serum and 50µg/ml of gentamicin plus either serial dilutions of colostral whey, its fractions or additional fetal bovine serum (as a positive control). Each treatment was plated into triplicate wells. The cells were pulsed with 0.2uCi of ^3H thymidine at 40 hours after plating and the DNA was harvested at 48 hours. The amount of thymidine incorporated into new DNA was compared between the samples.

Assessment of Feeding Colostral Whey on E. coli Infection

Gnotobiotic piglets were maintained in a specific pathogen free (SPF) environment. They were fed either SPF-lac or SPF-lac supplemented with 10% sterile colostral whey for 14 days, followed by only SPF-lac. The pigs were challenged on day two with an 0111:NM *E. coli* (strain 10049), which has been shown to cause reproducible chronic infections with serious immunosuppression in gnotobiotic piglets. The animals were maintained under gnotobiotic conditions for four weeks and each week blood was collected from the animals. The animals were also injected with sheep red blood cells on days five and eighteen to induce antibody responses. The blood samples were analyzed for hemoglobin, hematocrit, complete and differential white cell counts, and for response to B and T cell mitogens according to the methods of Christopher-Hennings *et al.* (1993). At the end of the experiment the production of antibodies to sheep red blood cells was measured using a complement fixation assay.

Results

Effects of Bacterial Growth

Colostral whey and the antibody rich fractions of colostral whey had substantial bacteriocidal activity against *E. coli* 0157:H7 (Table 1), but did not have any reproducible bacteriocidal effect against *E. coli* 0111, 055 or either of the salmonella strains tested.

Table 1. Bacteriocidal Effects of Colostral Whey Preparations on *E. coli* 0157:H7

Whey preparation	Length of Exposure			
	0 min	15 min	30 min	60 min
Whole Whey	100*	75	55	42
IgG rich (dialysis)	100	83	71	70
IgG rich (salt)	100	73	51	53
IgG poor (dialysis)	100	98	102	110
IgG poor (salt)	100	85	105	100

* the results are average percent of control of two trials.

Colostral whey inhibited the growth of all the pathogenic strains of *E. coli* and salmonella against which we tested it. The inhibition was dose dependent and the inhibition was generally much greater with the IgG rich preparations than with the IgG poor (Table 2.) None of the colostral whey preparations inhibited the growth of the non-pathogenic strains tested, for example the ATCC strain. We found that in general the more virulent the pathogen the greater the inhibition of its growth.

In preliminary studies, we also observed that the colostral whey had growth promoting effects on lactic acid producing bacteria. Confirmation of these results and testing of the growth inhibiting activity of the colostral preparations under anaerobic conditions are currently in progress.

Table 2. Inhibition of Bacterial Growth by Colostral Whey Preparations

	Whey Preparation				
	Whole Whey	IgG rich (Dialysis)	IgG rich (salt)	IgG Poor (Dialysis)	IgG Poor (salt)
***E. coli* sero type**					
0157	98	99.99	99.8	65	60
0111	55	60	15	15	95
055	20	33	48	12	90
026	60	78	85	50	92
0126	15	5	0	10	88
ATCC	8	4	12	0	6
Salmonella strain					
javiana	68	95	82	0	60
typh	35	61	58	69	82

The values represent the mean percent inhibition from two trial run in minimal medium. The values represent the inhibition seen at a 1:10 dilution of the colostral preparation. The inhibition was generally dose dependent, however some preparations caused nearly complete inhibition at all doses tested.

Effects on Bacterial Binding

As the adherence of bacteria to cells of the enteric tract appears to have an important role in the pathogenesis of enteric infections, we expected that colostral whey would inhibit binding of bacteria to HEp-2 cells. To our surprise this was not the case. Two things about these experiments surprised us. First, the non-pathogenic strain of *E. coli* exhibited the highest level of binding to HEp-2 cells, around 20% of the bacteria compared with 1-5% for the pathogenic *E. coli* and salmonella strains. Second, the addition of colostral whey or the IgG rich fraction of colostral whey enhanced the binding of the bacteria to the HEp-2 cells. The strains most enhanced were *E. coli* 0157 and *S. javiana*. The binding of the other strains tested was not markedly changed by the presence of colostral whey.

Effects on SLT Cytotoxicity

Toxins are important virulence factors of many enteric bacteria. We tested the ability of colostral whey, the IgG rich fraction prepared by dialysis and the IgG poor fraction prepared by dialysis to inhibit SLT induced cytotoxicity on HeLa cells. We demonstrated a dose dependent inhibition of the cytotoxic effect (Table 3).

Table 3. Inhibition of SLT Cytotoxicity by Colostral Whey Preparations

	Whey Preparation		
	Whole Whey	IgG rich	IgG poor
Dilution			
1:10	100±5	100±3	100±5
1:100	100±5	100±5	75±8
1:1000	76±6	65±2	60±6
1:10000	50±6	50±2	50±4

The values represent the mean± standard deviation of the percent inhibition of toxic activity from three trials.

Preliminary studies suggest that colostral whey and both the fractions we tested also inhibit the activity of other bacterial toxins including, the staphylococcal enterotoxins, Pseudomonas toxin A, cholera toxin and *E. coli* stable toxin a. These trials are in progress in our laboratory at present.

Effects on Viral Infection of Cultured Cells

Our studies demonstrated that colostral whey and both its high and low molecular weight fractions inhibited infection of cultured cells by enteric viruses. We found that both treatment of the cells with the colostral whey preparations before addition of the viruses and addition of colostral whey preparations to the cultures with the virus resulted in dose dependent inhibition of viral infection. The addition of the colostral whey preparations at the same time as the virus generally gave greater inhibition of infection than treatment of the cells prior to exposure to the virus, suggesting that components of the whey interact with both the virus and the cell surface in interfering with viral infection. The results of the virus inhibition were more completely described in Hurley and Talib (1992) and Bury and Hurley (1993), a summary of the inhibitory activity is shown in Table 4.

Table 4. Effects of Colostral Whey Preparations on Enteric Viral Infection of Cultured Cells.

	Virus		
Conditions tested	Poliovirus	Rotavirus	TGE virus
Whole whey (@ about 80-100 μg/ml)			
cells treated first	50	50	35
added with virus	75	75	50
IgG rich (@ about 70-100 μg/ml)			
cells treated first	30	70	15
added with virus	30	90	20
IgG poor (@ about 15-25 μg/ml)			
cells treated first	65	50	30
added with virus	70	55	40

Values represent typical maximal percent inhibition of viral infection under the conditions described.

Effects on Cell Growth

While doing the toxin inhibition assays and the viral infection inhibition assays, we observed that the colostrum only control cultures often grew faster than the control cultures. Therefore, we tested the effects of the colostral whey preparations on the growth of HeLa and HEp-2 cells. When the colostral whey preparations were added to DMEM in place of most of the fetal bovine serum, we found that the IgG rich fraction had little or no growth promoting activity and even inhibited cell proliferation in some assays. The whole whey enhanced cell division by about 15% at its optimal dose, about 20 μg/ml. However, the IgG poor preparations tested often enhanced cell proliferation to about 30% of the level of optimal fetal bovine serum. Further testing will be necessary to determine the nature of the growth promoting activities and if they will offer clinical application.

Effects on Infection In Vivo

In a trial using an infection model characterized at South Dakota State University, we demonstrated that supplementation of the diet of gnotobiotic piglets with 10% sterile colostral whey improved their response to an *E. coli* which causes severe inflammation, chronic infection and systemic immunosuppression. The uninfected animals during the course of the trial aver-

aged about 1000 neutrophils per mm^3 (range of 200-2000). The animals which received only the *E. coli* had neutrophil counts averaging 7000 to 9000 per mm^3 during all three weeks after infection. However, the animals receiving the colostral whey supplement and *E. coli* infection had a neutrophil spike at about 7000 per mm^3 the week of the infection, a reduction to about 3000 per mm^3 the next week and a level equivalent to controls the remainder of the study. These results suggest that the infection caused a less severe systemic inflammatory response in the colostral whey supplemented animals. In addition, the lymphocytes from animals receiving the *E. coli* only never mounted a significant mitogenic response to concanavalin A, phytohemagglutinin or pokeweed mitogen after the infection, nor did they mount a significant response to sheep red blood cells. The animals supplemented with colostral whey had depressed mitogen responses for two weeks post-infection, but the lymphocytes from these animals mounted equivalent or higher responses to T and B cell mitogens than control animals during the remainder of the study. In addition, they mounted similar antibody responses to sheep red blood cells as the control animals. The strain 10049 used in this experiment is moderately invasive and produces a large quantity of SLT. The SLT has been postulated to play a role in the immunosuppressive effects of the infection. The data suggests that both control of the growth and toxic activities of this bacteria occurred *in vivo*. The less prolonged systemic increase in neutrophils and the transient, rather than prolonged immunosuppression in the colostral whey supplemented animals suggest that pathogen inhibiting mechanisms similar to those seen *in vitro* occur *in vivo*.

Discussion

The studies reported here suggest that colostral whey has activities which control the growth of bacteria both by bacteriocidal and bacteriostatic mechanisms. The binding of bacterial pathogens is altered, but was enhanced rather than reduced. The actions of the *E. coli* SLT was inhibited and other enterotoxins may be similarly inhibited. Infection of cultured cells by enteric viruses was inhibited and the interaction of colostral components with both the cells and the viruses were involved. There appeared to be some anabolic effect on cultured cells, especially in the antibody poor fraction. Finally, the activities measured *in vitro* appear to have similar effects *in vivo* in reducing the severity and consequences of enteric infection.

Our studies further confirm the indications that bovine colostrum is an excellent source of antibody for oral immunotherapy (Stephan *et al.*, 1990). They found neutralizing titers against a wide variety of pathogenic bacteria which were higher than typical human serum titers. However, they did not consider the action of other colostral components in managing infection. Silva and Giampaglia (1992) demonstrated that fresh human colostrum would almost completely block the binding of enteropathogenic *E. coli*. This is the opposite of our finding, but we were using only whey or further fractionated material. Some ionic or saccharide components of their whole colostrum may be important to the activity. McClead and Gregory (1984) demonstrated that colostrum from immunized cows will block the action of cholera-toxin. They proposed using such preparations as oral therapy. They found that most of the toxin inhibition was associated with the antibody in their study, in contrast to our findings which suggest that both antibody and non-antibody components appear to play a role. Previous *in vivo* studies have shown that enteric virus infection can be blocked by oral colostrum (Schaller *et al.*, 1992, Micheals, 1965, and Sabin and Fieldsteel, 1962). In fact, Sabin and Fieldsteel proposed the use of colosturm as a preventative for polio infection of human children before the development of the Sabin vaccine. There have been few previous *in vitro* studies of the effects of colostral components on viral infection. Several studies have suggested that bovine colostrum is a good source of growth factors and has anabolic activity (Ramirez *et al.*, 1990, Francis *et al.*, 1986, Francis *et al.*, 1988). Each of these studies represented an analysis of a single component or activity of colostrum. The total effect appears to lie in the synergistic effect of several of these components, and only a multifaceted examination will reveal the interelationship among these activities.

Our studies and the studies discussed above suggest that a better understanding of the activities contained in colostrum would provide a basis for the development of biological alternatives for antibiotics in the management of enteric infections of neonatal and weaning animals, and possibly the basis for biological growth promotants as well. A more complete fractionation of the IgG rich and IgG poor fractions of colostral whey may uncover biological molecules which are readily available in milk or could be engineered in yeast which would provide safe, effective biological protection of calves and overcome the public's rising fear of meat and milk as a source of antibiotic resistant disease causing organisms.

Field studies have suggested that bovine colostrum may be useful in preventing rotavirus infection in calves and piglets (Schaller *et al.*, 1992). These studies found a negative correlation between the supplementation with colostral protein and the development of rotavirus scouring. Another application of colostral protein has been to treat the enteric sequelae of AIDS and its murine models (Watzl *et al.*, 1993, Ebina *et al.*, 1983). Feeding colostral protein to AIDS patients or mice with MAIDS

resolves the symptoms of the enteric infections (Watzl *et al.*, 1993). However, if the supplements are removed the symptoms return. Application to mastitis control has also been tested (Kerhli *et al.*, 1989, Owens *et al.*, 1992) The studies indicate that colostrum contains components which enhance neutrophil function and reduce inflammation in the mammary gland. The application in each case was intramammary, but similar effects in the intestine are possible.

The producers you serve will soon be placed in a difficult situation. The public will demand complete withdrawal of antibiotics as feed additives, and unless biological alternatives are developed, tested, and approved as replacements, they will suffer a great loss in productivity. We believe that some of the activities indicated by these studies are important candidates for those biological replacements.

Acknowledgments

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