Bovine Milk Immunoglobulins for Passive Immunity to Infantile Rotavirus Gastroenteritis

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Pregnant cows were successfully hyperimmunized with all four human rotavirus serotypes, resulting in a 100-fold increase in neutralizing milk antibody titers over those of controls. Milk antibodies were isolated batchwise from 1,000 kg of pooled milk for the first 10 lactation days, yielding 10 kg of freeze-dried milk immunoglobulin concentrate consisting of 50% bovine milk immunoglobulins. Milk immunoglobulin concentrate showed neutralizing activities against all four human rotavirus serotypes that were 100 times higher than those in pooled human milk samples and 10 times higher than those in a commercial pooled immunoglobulin preparation from pooled human blood serum. In vitro neutralization tests showed that milk immunoglobulin concentrate had powerful antiviral activity, even against very high doses of infectious rotaviruses. Because the technology of the milk immunoglobulin concentrate ensures that it is innocuous and can be used for oral application, it is proposed that milk immunoglobulin concentrate be used to induce passive immunity to infantile rotavirus gastroenteritis.

Rotaviruses, which are members of the family *Reoviridae* (14), account for about half of the pediatric hospital admissions of children with acute diarrhea in Western Europe and the United States during the winter (7, 15, 19). Clinically, rotavirus gastroenteritis is characterized by profuse diarrhea, fever, and vomiting, leading to moderate or severe dehydration (5).

One of the most important problems with rotavirus infections is their prevention. This involves infants in the first year of life. Active immunization offers one approach to this problem (34), but we decided to investigate the alternative approach of passive immunization.

It has been shown from results of numerous studies in animals (3, 4, 24, 27, 31) that the presence of antibody in the lumen of the small intestine is of major importance for protection against rotavirus diarrhea. Antibodies in serum, however, do not prevent rotavirus infection (18, 20, 30, 36). Passive immunity may be provided by breast-feeding, but antibody titers are generally low in breast milk (6), and many infants are no longer breast-fed when they are most susceptible to rotavirus infection. Alternatively, passive immunity may be provided by the artificial feeding of antibody-containing preparations. In this study we describe the successful immunization of cows with the four human rotavirus serotypes and the isolation of bovine milk immunoglobulins with potent antiviral activity that is suitable for inducing passive immunity to infantile rotavirus gastroenteritis.

MATERIALS AND METHODS

Viruses. Human rotavirus strains KU and S-2 (33) were obtained from S. Urasawa, Sapporo Medical College, Sapporo, Japan. Human rotavirus strains Ito and Hochi (26) were obtained from Y. Inaba, National Institute of Animal Health, Tsukuba, Ibaraki, Japan. Human rotavirus Wa (37) and simian rotavirus SA11, which belongs to the third serotype defined for human rotaviruses (37), were obtained from P. A. Offit, Children's Hospital of Philadelphia.

Immunization of cows. Pregnant German Brown and Holstein-Frisian cows from our experimental dairy farm (Nestlé Experimental Dairy Farm, Albführen, Federal Republic of Germany) were hyperimmunized with the cell-free culture supernatant of MA-104 cells (macacus Rhesus monkey kidney cells) that were infected with the indicated rotavirus strains. Immunization started 6 weeks before the calculated date of delivery with a subcutaneous injection near the lymph nodes (10 ml of virus and 10 ml of incomplete Freund adjuvant; Difco Laboratories, Detroit, Mich.). For the next 2 weeks cows were immunized weekly by intracisternal infusion through the 4 teat channels (25 ml of virus, four times). Three weeks before delivery cows were immunized subcutaneously into the retromammary lymph nodes [20 ml of virus and 20 ml of 2% Al(OH)$_3$]. At 2 weeks before delivery cows were given an intramuscular injection deep into the hip (10 ml of virus and 10 ml of incomplete Freund adjuvant), followed by a slow intravenous injection [20 ml of virus and 20 ml of Al(OH)$_3$] 1 week before delivery. Antigen was administered to each cow by all the routes indicated and on the same time schedule. Each cow received only one rotavirus strain.

Neutralization test. MA-104 cells, which were grown in 96-well microtiter plates, were inoculated with 10^2 50% tissue culture infective doses (TCID$_{50}$) of the indicated rotavirus strain after incubation with a twofold dilution series of serum, milk, or 10% (wt/vol) suspension of milk immunoglobulin concentrate in H$_2$O. Cells were incubated for 1 day to 1 week, depending on the virus strain, and then fixed with absolute ethanol. Cells were then reacted with a rabbit hyperimmune serum sample that was directed against single-shelled rotavirus particles, followed by a peroxidase-coupled goat antibody to rabbit immunoglobulin G (IgG). Intracellular viral antigen was revealed by 9-aminocarbazole deposition, as described by Gerna et al. (13).

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TABLE 1. Effect of vaccination on bovine serum and milk
antibody titer

Rotavirus strain (serotype)	Antibody titer in:			
	Serum		Milk[a]	
	ELISA	NT[b]	ELISA	NT
KU (1)[c]	200,000	12,000	100,000	7,500
S-2 (2)	100,000	3,200	25,000	2,000
SA11 (3)	200,000	6,400	200,000	4,500
Hochi (4)	400,000	7,500	100,000	4,500
E. coli	1,600	320	1,600	60
Preimmune[d]	800	220	800	90

[a] Pooled milk from the first 8 days after calving.
[b] Neutralization titer (NT) against 10^2 $TCID_{50}$ of the rotavirus strain used for immunization (SA11 rotavirus in the case of E. coli and preimmune controls), as measured by the neutralization test described by Gerna et al. (13).
[c] Mean value for three cows.
[d] Mean value for all 15 cows used in this study.

Neutralization titers are expressed as the reciprocal of the highest dilution of the test sample that exhibited complete inhibition of virus replication.

Alternatively, 10^7 $TCID_{50}$ of rotavirus SA11 was titrated as described by Sato et al. (26) in 10-fold dilution steps in the presence of a fixed milk immunoglobulin concentrate dilution. Residual virus infectivity was determined after a 5-day incubation in tube cell cultures on a roller drum. Virus infectivity was expressed as the reciprocal of the highest dilution of the test sample exhibiting detachment of the cell monolayer in 50% of the tubes.

Preparation of antirotavirus milk immunoglobulin concentrate. Milk obtained during the first 10 days after calving of rotavirus-hyperimmunized cows was immediately cooled to 4°C and kept at this temperature until further treatment. In the pooled milk samples we could not detect rotavirus by infectivity titrations, enzyme-linked immunosorbent assay (ELISA), electron microscopy, or RNA gel electrophoresis. Pooled milk samples were skimmed at 40°C in a pilot plant milk separator and then immediately cooled to 10°C before intermediate storage at −25°C. Milk immunoglobulin concentrate was isolated batchwise from pools of about 1,000 kg of milk. The thawed milk was once again centrifuged before pasteurization in a plate heat exchanger at 62.5°C for 30 min. After cooling to 37°C, casein was precipitated by milk renneting with commercially available rennin (Standard Labextrakt; Winkler AG, Konolfingen, Switzerland). To obtain good curd contraction, the coagulated milk was heated to 56°C for 10 min. Casein separation from lactoserum was carried out in a solid bowl scroll centrifuge (Westfalia, Oelde, Federal Republic of Germany). For final clarification the lactoserum (about 900 kg) was again centrifuged in a clarifier centrifuge (Alfa-Laval AB, Lund, Sweden) and filtered through a depth filter. In a reverse osmosis system (DDS module 35-18; De Danske Sukkerfabrikker, Nakskov, Denmark) the bulk of lactose and mineral salts were removed from the lactoserum by diafiltration , and then by ultrafiltration the resulting whey protein solution was finally concentrated to about 100 kg. This milk immunoglobulin concentrate solution, which contained about 10% dry matter with 7 to 8% total protein and 3 to 4% immunoglobulins, was then submitted to sterile filtration through a membrane filter (pore size, 0.45 μm; Millipore AG, Kloten, Switzerland). Before the final step of freeze-drying, the volume of the sterile milk immunoglobulin concentrate solution was further reduced to about 50 kg by evaporation at

a maximum of 40°C and under reduced pressure in a mechanically agitated thin film evaporator (Luwa SA, Zurich, Switzerland), which was equipped to run under sterile conditions.

Pooled milk (1,000 kg) from the first 10 lactation days after the calving of rotavirus-hyperimmunized cows resulted in an average of 10 kg of dried antirotavirus milk immunoglobulin concentrate containing about 80% protein, 10% lactose, 3% mineral salts, 3% non-protein nitrogen compounds, and 4% residual humidity. Proteins are composed of 45% immunoglobulins, 10% α-lactalbumin, 35% β-lactoglobulin, 5% serum albumin, and 5% minor proteins. Immunoglobulins, in turn, are composed of 75% IgG1, 3% IgG2, 17% IgA, and 6% IgM. Although IgA is the major immunoglobulin in most external secretions of the bovine species, it is only a minor component of colostrum and transition milk (16).

RESULTS

Effect of vaccination on antibody titer in milk and serum. Vaccination with human rotaviruses raised the neutralizing antibody titer in serum of pregnant cows from a mean of 220 at 8 weeks before calving (the start of vaccination) to a mean of 7,300 on the day of calving. No increase in rotavirus antibody titer was seen in control cows that were immunized with enteropathogenic Escherichia coli strains (Table 1). Immunization with any of the four human rotavirus serotypes increased the neutralizing antibody titers in the pooled milk of the first 8 lactation days about 100-fold when compared with those in control cows (Table 1).

Very high neutralizing antibody titers were found in the colostral milk from the first two milkings of cows that were immunized with any of the four human rotavirus serotypes. Thereafter, neutralizing antibody titers decreased rapidly but remained significantly higher than those in control cows for an extended period (Fig. 1). This decrease in neutralizing milk antibodies with progressing lactation parallels the decrease of antirotavirus IgG, which is the major immunoglobulin of bovine colostrum and milk (16), which was detected by ELISA in the milk of immunized cows and also by the decrease of total IgG concentration that was observed in cows milk (data not shown).

Antiviral activity of different milk immunoglobulin concentrates. When milk immunoglobulin concentrate was isolated
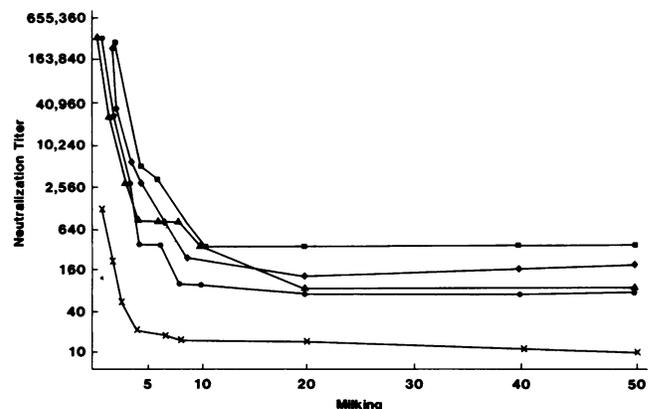

FIG. 1. Neutralizing antibody titers in colostrum and milk of cows immunized with rotavirus KU (▲), S-2 (●) SA11 (■), and Hochi (◆) and control cows (×) during the first 50 milkings (25 days of lactation) after calving. Neutralization titers (13) were determined against the virus that was used for immunization and against SA11 virus for control cows.

TABLE 2. Neutralization of human rotavirus serotypes with milk immunoglobulin concentrate

Sample[a]	No. of batches tested	Origin of milk immunoglobulin concentrate	Neutralization titer[b] to the indicated rotavirus strain (serotype)				
			Wa (1)	S-2 (2)	Ito (3)	Hochi (4)	Mean neutralization titer
MI concentrate							
0	10	Control cows; 40 lactation days	40	20	160	40	65
A	10	Control cows; first-day colostrum	400	100	800	40	335
B	10	Immunized cows; 40 lactation days	400	400	3,200	400	1,100
C	10	Immunized cows; 10 lactation days	4,700	2,000	12,800	3,600	5,800
C'	2	Cows immunized with human rotavirus strain Hochi; 10 lactation days	6,400	1,600	12,800	3,200	6,000
C"	2	Cows immunized with simian rotavirus SA 11; 10 lactation days	12,800	6,400	12,800	3,200	8,800
Human breast milk		Pooled colostrum and milk of 30 Swiss women; lactation days 1 to 5	20	30	60	20	40
Human gamma globulin		Sandoglobulins administered intravenously	600	150	600	800	540

[a] MI concentrate, Milk immunoglobulin concentrate.

[b] Reciprocal of milk immunoglobulin concentrate (0.1 g of lyophilized milk immunoglobulin concentrate per 1 ml of H_2O), gamma globulin (0.1 g of lyophilized gamma globulin per 1 ml H_2O), and breast milk dilution totally inhibiting the replication of 10^2 $TCID_{50}$ of indicated rotavirus in the neutralization test described by Gerna et al. (13).

from the pooled milk of the first 40 lactation days of control cows, we found a mean neutralization titer of 65 against human rotaviruses (Table 2, MI concentrate 0), which is comparable to the neutralizing activity of pooled human breast milk (Table 2). A fivefold higher neutralization titer was obtained when milk immunoglobulin concentrate was prepared from the first day colostrum of control cows (Table 2, MI concentrate A). Milk immunoglobulin concentrate prepared from the pooled milk of the first 40 lactation days of immunized cows showed a mean neutralization titer of 1,100 (Table 2, MI concentrate B), which was comparable to the neutralization activity of commercial human pooled immunoglobulins from blood serum (Table 2). Milk immunoglobulin concentrate isolated from the pooled milk of the first 10 lactation days of immunized cows gave a neutralization titer of 5,800 (Table 2, MI concentrate C), which was 10-fold higher than the activity of human pooled immunoglobulins. For milk immunoglobulin concentrate B and C (Table 2) preparations, milk from cows immunized with each of the different human rotavirus serotypes was included in the pooled milk. However, milk from cows immunized either with a single human rotavirus strain (Table 2, MI concentrate C') or with simian rotavirus SA11 alone (Table 2, MI concentrate C") also yielded milk immunoglobulin concentrate preparations with good neutralizing activities against all human rotavirus serotypes.

Quantitative aspects of virus neutralization by milk immunoglobulin concentrate. The milk immunoglobulin concentrate C preparation (0.1 g of lyophilized powder per ml of H_2O) was tested against different doses of rotavirus SA11, which corresponds to serotype 3 of human rotavirus (37), in a β-type neutralization test. We found a linear relationship between milk immunoglobulin concentrate C neutralization titer and the infectivity titer of input rotavirus SA11 in the test (data not shown), ranging from a neutralization titer of 1,000,000 against 1 $TCID_{50}$ to a neutralization titer of 100 against 10^6 $TCID_{50}$ in the test. Then, 10^7 $TCID_{50}$ of input rotavirus was titrated in an α-type neutralization test in the presence of different milk immunoglobulin concentrate dilutions. Here, milk immunoglobulin concentrate C showed powerful antiviral activity: 99.99 and 99% of 10^7 $TCID_{50}$ of input virus were neutralized by 1:1,000 and 1:10,000 milk immunoglobulin concentrate dilutions, respectively (Fig. 2).

A kinetic analysis of the neutralization process showed that 10^3 $TCID_{50}$ of rotavirus SA11 were instantaneously neutralized by a 1:1,000 dilution of milk immunoglobulin concentrate C (100 μg of milk immunoglobulin concentrate per ml) and within 10 min by a 1:10,000 dilution (10 μg of milk immunoglobulin concentrate per ml), whereas corresponding dilutions of milk immunoglobulin concentrate from control cows neutralized the input virus only slowly and partially or not at all (Fig. 3).

DISCUSSION

It has been shown that large amounts of human rotavirus neutralizing antibodies can be isolated from colostrum and transition milk of hyperimmunized cows.

For several reasons cows are ideal animals for the production of antirotavirus antibodies. Rotaviruses are common pathogens for cows (35), and therefore, bovine blood serum and milk show appreciable natural neutralizing antibody titers against rotaviruses (1, 36) (Fig. 1 and Table 1). Bovine serum (29) and milk (38) cross neutralize human rotaviruses. Snodgrass et al. (29) showed that Scottish cows have elevated preimmune titers in serum to human rotavirus serotypes 1, 2, and 3 but not to serotype 4. We found in our German cows similar preimmune titers in serum against all four human rotavirus serotypes (H. Brüssow, unpublished
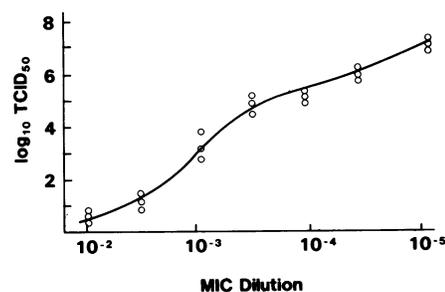

FIG. 2. Decrease of the infectivity of a fixed amount of 10^7 $TCID_{50}$ input rotavirus SA11 after incubation for 1 h at 37°C with milk immunoglobulin concentrate C (0.1 g/ml) at the indicated dilution. Virus titers were determined by the test tube titration test (26). MIC, Milk immunoglobulin concentrate.
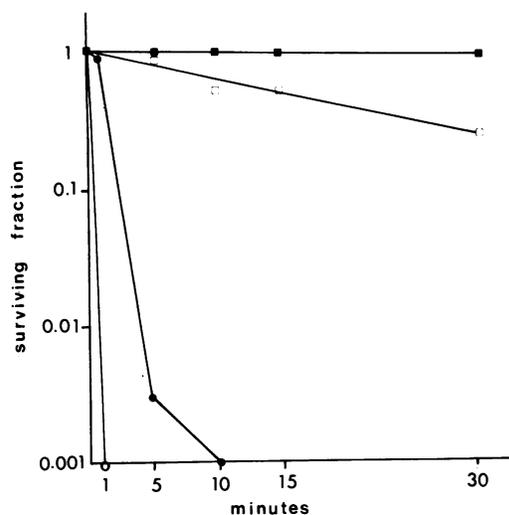
FIG. 3. Decrease in the infectivity of input rotavirus SA11 after incubation for various times with milk immunoglobulin concentrate C (0.1 g/ml) at 1:1,000 (O) and 1:10,000 (●) dilutions and after incubation with milk immunoglobulin concentrate of control cows at 1:1,000 (□) and 1:10,000 (■) dilutions.

data). We observed a comparable stimulation of heterologous antibodies to all four human rotavirus serotypes after immunization with a simian rotavirus or a serotype 4 human rotavirus (Table 2), whereas Snodgrass et al. (29) obtained stimulation of heterologous antibodies to human rotavirus serotypes 1, 2, and 3 but not to serotype 4 after immunization with a bovine rotavirus. One possible explanation for this difference may be that our German cows were primed also by natural infection to serotype 4 human rotavirus. We favor an alternative hypothesis that the bovine immune system recognizes a minor neutralizing antigen that is common to different rotavirus serotypes (H. Brüssow, I. Walther, V. Fryder, J. Sidoti, submitted for publication). Whatever the final explanation, this particular reactivity of the bovine immune system is of practical importance. There is no need to immunize cows with fastidiously growing human rotavirus strains. Actually, it is sufficient to immunize cows with simian rotavirus SA11, which can be grown easily in cell culture, to obtain high-titered milk antibodies that neutralize all four human rotavirus strains. Large quantities of antirotavirus milk antibodies can be isolated from colostrum and milk of vaccinated cows (25, 28). A total of 3 to 4 kg of milk immunoglobulin concentrate consisting of 45% immunoglobulins was obtained from the pooled milk of the first 10 lactation days of a single cow. Because of the technology used for the preparation of milk immunoglobulin concentrates, risks do not differ relative to the use of any other product based on pasteurized cows milk.

Finally, bovine secretory antibodies are remarkably resistant to proteolytic digestion in the intestine of a heterologous host. More than 10% of the orally administered antiviral activity was recovered from the stools of milk immunoglobulin concentrate-treated infants, resulting in stool neutralization titers which were higher than those found in pooled human breast milk (H. Hilpert, H. Brüssow, C. Mietens, J. Sidoti, L. Lerner, and H. Werchau, J. Infect. Dis., in press).

It has been shown that either human pooled immunoglobulins or bovine milk antibodies from rotavirus-immunized cows could prevent rotavirus gastroenteritis when added to the food of infants (2, 10, 11). Thus, milk immunoglobulin

concentrate may offer an attractive means of prophylaxis of rotavirus gastroenteritis in infants at risk, e.g., nurseries for premature babies, day-care centers, hospitalized infants, and household contacts (9). In industrialized countries, at least, a considerable proportion of rotavirus diarrhea treated in hospitals may be nosocomially acquired (8, 23, 32).

It may even be beneficial to treat hospitalized infants suffering from acute rotavirus gastroenteritis with milk immunoglobulin concentrate because, as has been shown here, milk immunoglobulin concentrate neutralizes very high doses of infectious rotavirus. When given orally to patients infected with rotavirus, it may reduce the number of infectious rotavirus isolates that are excreted into the feces and thus also reduce the risk for surrounding patients. Indeed, for rotavirus gastroenteritis outbreaks, high secondary attack rates have been reported (12, 21, 22). If a propagation of the infection process takes place along the intestine of patients infected with rotavirus, as has been hypothesized for calves (17), milk immunoglobulin concentrate might interfere with this propagation and thus also be directly beneficial for the patient infected with rotavirus. In fact, in a clinical test for the therapeutic use of milk immunoglobulin concentrate now under way, a shortening of virus excretion time was observed in milk immunoglobulin concentrate treated infants when compared with that in matched controls (Hilpert et al., in press).

ACKNOWLEDGMENTS

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