

免疫初乳の連続給与による新生子牛の牛ロタウイルス感染 の防御

誌名	Japanese journal of veterinary science
ISSN	00215295
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巻/号	51巻2号
掲載ページ	p. 300-308
発行年月	1989年4月

Protection against Bovine Rotaviruses in Newborn Calves by Continuous Feeding of Immune Colostrum

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(Received 8 July 1988/Accepted 6 November 1988)

ABSTRACT. Three pregnant cows were inoculated intramuscularly with inactivated vaccine to bovine rotavirus (BRV) serotype 1 (BRV-1) and serotype 2 (BRV-2). Serum neutralizing antibody (NA) titers against both serotypes increased significantly after immunization. NA titers of colostrum obtained from immunized cows against BRV-1 and BRV-2 were 29286 and 38109, respectively, which were significantly higher than those from non-immunized control cows. Nine and 6 colostrum deprived calves were orally challenged with BRV-1 and BRV-2, respectively, and monitored for clinical manifestation and viral shedding. Five calves of them, 3 with BRV-1 and 2 with BRV-2, received 2 l of milk replacer supplemented with 10% immune colostrum 2 hr before challenge and twice daily for the first 5 days after challenge. Other 10 calves, 6 with BRV-1 and 4 with BRV-2, were fed only milk replacer as controls. All control calves developed severe diarrhea and shed a large amount of BRV in feces, beginning from 24 to 48 hr after challenge inoculation. On the contrary, all calves but one fed colostrum supplement remained clinically healthy after challenge, and BRV was not detected in their feces during feeding immune colostrum. The possibility that continuous feeding of immune colostrum is capable of preventing newborn calves from diarrhea associated with BRV and viral shedding was suggested.—**KEY WORDS:** bovine rotavirus, experimental infection, immune colostrum, passive protection.

Jpn. J. Vet. Sci. 51(2): 300–308, 1989

Bovine rotavirus (BRV) is a major causative agent of neonatal calf diarrhea throughout the world [2, 3, 5, 6, 11, 17]. Recent studies have indicated that there are at least 2 serotypes of BRV, which are distinguishable by a cross neutralization test *in vitro* and a cross protection experiment *in vivo* [16, 18, 25, 30]. Two approaches have been used in attempts to protect newborn calves from BRV infection; one is direct vaccination of newborn calves to elicit active immunity [1, 7, 29], and the other is immunization of pregnant dams to expect providing passive immunity to their suckling calves via immune colostrum and milk [4, 15, 21, 24, 26, 27]. The effects of active immunization of newborn calves with live virus vaccine have been suggested to be questionable, because colostral BRV anti-

body ingested by calves, which is derived from natural infection of dams, may neutralize vaccine virus, and BRV infection in calves occurs usually too early to develop active immunity [1, 6, 7]. Vaccination of pregnant cows has been attempted by using various viral preparations and routes of administration. Several workers have reported that cows immunized with experimental vaccines produced sufficient amount of antibody in mammary secretions to protect calves passively from BRV-associated diarrhea [4, 21, 23, 24, 26, 27]. However, these studies were carried out on only one serotype of BRV.

In the present study, we investigated immune responses in sera and mammary secretions from pregnant cows immunized with mixed vaccine prepared from two

strains of BRV that belong to different serotypes, and the efficacy of continuous feeding of the immune colostrum on experimental infection of newborn calves with those viruses.

MATERIALS AND METHODS

Cell culture: MA104 cell cultures, an established cell line derived from fetal rhesus monkey kidney, were used for the preparation of experimental vaccine, a neutralization test and a virus isolation. The growth medium was Eagle's minimum essential medium (EMEM) supplemented with 10% calf serum, 10% tryptose phosphate broth (Difco), 0.15% sodium bicarbonate and antibiotics (100 U/ml of penicillin and 100 $\mu\text{g/ml}$ of streptomycin). The maintenance medium was EMEM supplemented with 0.11% bovine serum albumin and the same concentrations of sodium bicarbonate and antibiotics as the growth medium.

Viruses: Lincoln (serotype 1) and KK-3 (serotype 2) strains of BRV were kindly supplied by Dr. Y. Murakami (Department of Exotic Disease, National Institute of Animal Health, Kodaira, Tokyo). Virus titration was performed by the methods described by Murakami *et al.* [16]. Briefly, serial 10-fold dilutions of viruses were made, and 4 tubes of MA104 cell culture were inoculated with 0.1 ml of each dilution. Viruses were treated with 10 $\mu\text{g/ml}$ of trypsin (type I, Sigma) for 30 min at 37°C previously. After adsorption for 60 min at 37°C, the cultures were washed once with Earle's balanced salt solution (EBSS), and received 0.5 ml of the maintenance medium supplemented with 1 $\mu\text{g/ml}$ of trypsin. The cultures were incubated in a roller drum for 7 days at 37°C, examined for any cytopathic effect (CPE) and the infective titers were expressed as median tissue culture infective doses (TCID₅₀)/ml.

Preparation of experimental vaccine: Confluent monolayers of MA104 cells grown in Roux bottles were inoculated with either Lincoln or KK-3 strain, which was pre-treated with 10 $\mu\text{g/ml}$ of trypsin, and incubated in the presence of 1 $\mu\text{g/ml}$ of trypsin at 37°C. Infected cells and culture fluids were harvested when about 75% of cells showed CPE, freeze-thawed once, and clarified by low-speed centrifugation. The harvested fluids were concentrated by addition of 10% polyethylene glycol 6000. The precipitates were resuspended in phosphate buffered saline (PBS) to approximately one twentieth of the original volumes, which contained Lincoln and KK-3 strains at titers 10^{9.3} and 10^{7.8} TCID₅₀/ml, respectively. Infectious viruses were inactivated with an equal volume of 0.4% β -propiolactone (Sigma) for 2 hr at 37°C, and subsequently for 18 hr at 4°C. The antigen solutions were dialyzed against 4 changes of a large volume of PBS, mixed in an equal volume, and used as an experimental vaccine.

Cows and immunization: Six pregnant Holstein cows, 3 to 5 years old, were used. Three of them were inoculated intramuscularly with 20 ml of the mixed vaccine at 50 to 57 days before calving. DEAE-dextran (Pharmacia) was used as an adjuvant in a concentration of 50 mg/ml. Four weeks after the initial immunization, the second booster injection was given by the same manners. The remaining 3 cows served as non-immunized controls.

Serum samples were collected from the immunized cows at the time of immunization and calving, and from the control cows at the corresponding times of sampling in the immunized cows.

Colostrum and milk samples were collected from all cows on day of calving and 7 days later, and treated with rennin to obtain clear wheys. Serum and whey samples were heat-inactivated for 30 min at 56°C, and tested for neutralizing antibody (NA).

Neutralization test: A modification of the plaque reduction neutralization test described by Matsuno *et al.* [10] was used for determination of NA titers to both BRV-1 and BRV-2. The viruses were treated with 10 $\mu\text{g/ml}$ of trypsin and diluted to give 100 plaque forming units/0.1 ml. Serial 4-fold dilutions of serum or whey samples were mixed with an equal volume of virus suspensions and incubated for 90 min at 37°C. Two dishes of MA104 cell cultures were inoculated with 0.1 ml of each virus-sample mixture. After adsorption for 1 hr at 37°C, the cultures were washed once with EBSS, received overlay medium consisting of EMEM supplemented with 0.8% Agar Noble (Difco), 2 $\mu\text{g/ml}$ of trypsin, 50 $\mu\text{g/ml}$ of DEAE-dextran and 0.0007% neutral red, and incubated at 37°C. Plaque numbers were counted on day 6 of incubation for Lincoln strain, and day 9 for KK-3 strain. NA titers were expressed as the reciprocal of the calculated dilution of samples that caused an 80% reduction in plaque count. Data obtained in the neutralization test were transformed to logarithm and analyzed by using the 2-independent t test

Passive protection of calves: Fifteen colostrum deprived Holstein calves were separated from cows within 2 hr after birth, reared individually in isolation rooms, and used for the passive protection experiments. Nine calves were challenge-exposed orally to 2 ml of Lincoln strain (BRV-1) titering

10^{8.2}TCID₅₀/ml, and 6 calves to 100 ml of KK-3 strain (BRV-2) titering 10^{5.7} TCID₅₀/ml between 4 and 12 hr after birth. Five of them, 3 to BRV-1 and 2 to BRV-2, received 2 l of milk replacer supplemented with 10% immune colostrum pool obtained from 2 immunized cows 2 hr before challenge and twice daily for the first 5 days after challenge. NA titers of colostrum pool to Lincoln and KK-3 strains were 25000 and 35000, respectively. Remaining 10 calves, 6 to BRV-1 and 4 to BRV-2, were fed only milk replacer as controls. Two control calves exposed to BRV-2 were given daily intramuscular injection with gentamicin (Schering). All calves were examined for clinical signs twice daily for the first 5 days and once daily for the subsequent 9 days after challenge. Feces were rated as diarrheal when they were muddy or liquid consistency. Fecal samples were collected at the same times of clinical examinations, and tested for BRV and *Escherichia coli* (*E. coli*).

Virus isolation: Fecal samples were examined for BRV by an immunofluorescence assay in MA104 cells [17, 19]. Confluent monolayers of MA104 cells grown in Leighton tubes containing coverslips were inoculated with 0.2 ml of serial 10-fold dilutions of 10% fecal suspensions that had been treated previously with an equal volume of trypsin (20 $\mu\text{g/ml}$) for 30 min at 37°C. After adsorption for 1 hr at 37°C, the cells were

Table 1. Neutralizing antibody titers to BRV-1 (Lincoln strain) and BRV-2 (KK-3 strain) in mammary secretions and serum of rotavirus-immunized or control cows

Group of cows	Virus	Mammary secretion ^{a)}		Serum ^{a)}		
		At time of parturition	At post-parturition day 7	At time of first immunization	At time of second immunization	At time of parturition
Immunized	Lincoln	29286 ^A	93	251	2929 ^A	1166 ^A
Control	—	2512 ^B	32	541	584 ^B	293 ^B
Immunized	KK-3	38109 ^A	117 ^A	417	10471 ^A	2188 ^A
Control	—	2630 ^B	42 ^B	676	708 ^B	347 ^B

a) Geometric mean titers (3 cows each). Means followed by different letters are significantly different at $P < 0.05$.

washed once with EBSS, received 2 ml of the maintenance medium supplemented with 1 μ g/ml of trypsin, and were rolled at 37°C in a roller drum. After 48 hr of incubation, the cells were washed twice with PBS, fixed with cold acetone, and stained with anti-BRV fluorescent conjugate. The conjugate was prepared from hyperimmune serums of guinea pigs immunized with Lincoln strain purified by a CsCl density gradient ultracentrifugation. Fluorescent foci were counted, and virus titers were expressed as fluorescent focus forming unit (FFU) /0.01 g of feces.

Examination of E. coli: Samples were cultured on DHL agar, and 5 colonies of *E. coli* were selected randomly and subcultured on Minca-Isovitalex agar. *E. coli* isolated

was examined for K99 antigen by a slide agglutination test using the monoclonal antibody specific to K99 antigen (Molecular Genetics) [14].

RESULTS

Antibody responses: Before immunization, there were no differences statistically ($P>0.05$) in serum NA titers to both BRV-1 and BRV-2 between immunized and control cows (Table 1). Serum NA against both viruses increased remarkably in immunized cows, and their titers were significantly higher than those of control cows after immunization ($P<0.05$). NA titers of colostrum to BRV-1 and BRV-2 were 29286 and 38109 in immunized cows on average,

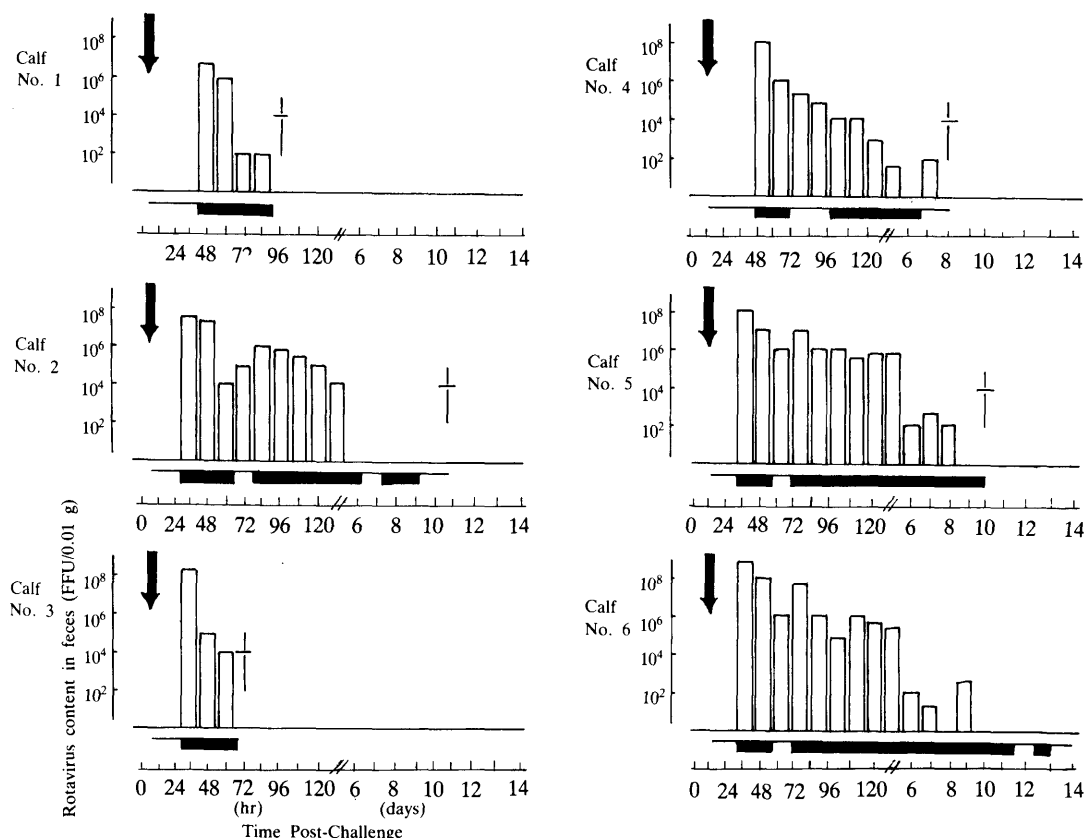


Fig. 1. Clinical response and rotavirus content in feces of control calves after BRV-1 challenge. \blacktriangledown BRV challenge, \blacksquare Diarrhea, \perp Death.

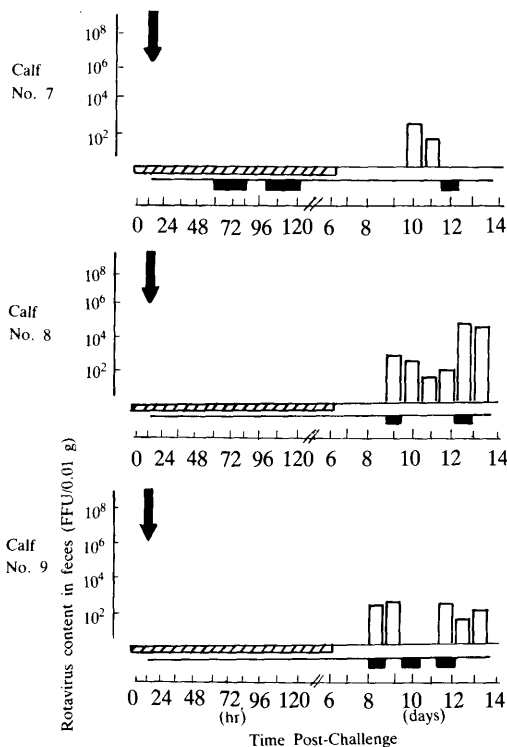


Fig. 2. Clinical response and rotavirus content in feces of calves fed 10% immune colostrum after BRV-1 challenge. \blacktriangledown BRV challenge, \blacksquare Diarrhea, \hatched Supplemental feeding of immune colostrum.

while 2512 and 2630 in controls, respectively (Table 1). Differences between colostrum NA titers of immunized and control cows were statistically significant ($P < 0.05$). NA titers of milk decreased rapidly in both immunized and control cows after calving. However NA titers to BRV-2 in milk obtained 7 days after calving were still significantly higher in immunized than in control cows (117 vs. 42, $P < 0.05$).

Responses of calves to challenge with BRV-1: All control calves challenge-exposed to Lincoln strain developed severe diarrhea with incubation periods of 24 to 36 hr, and were depressed and dehydrated. The feces were yellowish liquid initially, and then contained mucoid in many cases. Eventually 5 of them died on postinoculation days (PID) 2.3 to 11. BRV was first detected

in feces obtained from all control cows on PID 1 to 1.5, which coincided with the onset of diarrhea (Fig. 1). Virus shedding persisted until death occurred or for 5 to 9 days after challenge. The maximum virus titers in feces ranged from $10^{6.7}$ to $10^{8.8}$ FFU/0.01g of feces. On the contrary, 2 of 3 calves given immune colostrum remained clinically healthy after challenge exposure. The other showed mild diarrhea, but neither depression nor dehydration was observed (Fig. 2). All calves fed immune colostrum, however, developed mild diarrhea 3 to 7 days after the cessation of colostrum feeding, but all recovered. BRV was not detected in any of their feces during colostrum feeding. However, BRV with the maximum titers of $10^{2.5}$ to $10^{4.8}$ FFU/0.01 g of feces was recovered from their feces 3 to 5 days after the cessation of colostrum feeding, and viral shedding persisted for 2 to 6 days (Fig. 2).

A large number of *E. coli* were isolated from fecal samples and the various organs of the dead calves, but all were negative for K99 antigen.

Responses of calves to challenge with BRV-2: The results of the experiment in calves exposed to BRV-2 were similar to those in calves challenged with BRV-1. All control calves developed profuse diarrhea with depression and dehydration, that began on PID 1.5 to 2. Two of them, including a calf given intramuscular injection with gentamicin, died on PID 4.8 and 10, respectively; one became moribund and was euthanized on PID 3.8; the other recovered (Fig. 3). BRV was first detected in their feces on PID 1.5, and isolated continuously until times of death and euthanasia, or until PID 6 and 8 (Fig. 3). The maximum virus titers of $10^{6.3}$ to $10^{7.2}$ FFU/0.01 g of feces was observed in fecal samples collected on PID 2 to 2.5. On the other hand, 2 calves fed immune colostrum and exposed to BRV-2 remained clinically healthy, and shed no BRV in feces during

colostrum feeding (Fig. 4). Although they showed mild diarrhea and shed virus in feces, with the maximum titers of $10^{4.0}$ to $10^{6.0}$ FFU/0.01 g of feces, 2 to 5 days after the cessation of colostrum feeding, all survived the challenge exposure (Fig. 4).

A large number of *E. coli* were isolated from the various organs of the dead calves, but not from the calf given gentamicin. All *E. coli* isolated were negative for K99 antigen.

DISCUSSION

Before immunization, all cows were seropositive for both BRV-1 and BRV-2. This indicates that both viruses have spread widely among cattle in Japan, as reported by Murakami *et al.* [18]. Cows immunized with BRV-1 and BRV-2 produced high titer serum NA to both viruses. Furthermore, NA titers to BRV-1 and BRV-2 in colostrum were 12- and 14-fold higher in immunized than in control cows, respectively. These seem to indicate that the mixture

of concentrated and inactivated vaccine to BRV-1 and BRV-2 is effective enough to boost antibody levels even in cows exposed previously to natural infection with both viruses. In ruminants, IgG₁ subclass of immunoglobulin is transported selectively from serum to colostrum and milk [20]. It is considered, therefore, that high titer NA in colostrum of immunized cows may result from transfer of serum IgG₁ antibody, not from local production in mammary glands [8, 21, 24].

Previous studies have reported that the use of an appropriate adjuvant was important to enhance BRV antibodies in serum and mammary secretions of cows [22, 23, 26]. In this study, DEAE-dextran was used as an adjuvant, and immunized cows produced high titer NA to BRV. This suggests that DEAE-dextran is one of effective adjuvants for immunization of cows with BRV.

Saif and Smith [22] have reported that BRV inactivated with β -propiolactone was less effective as an immunogen than BRV

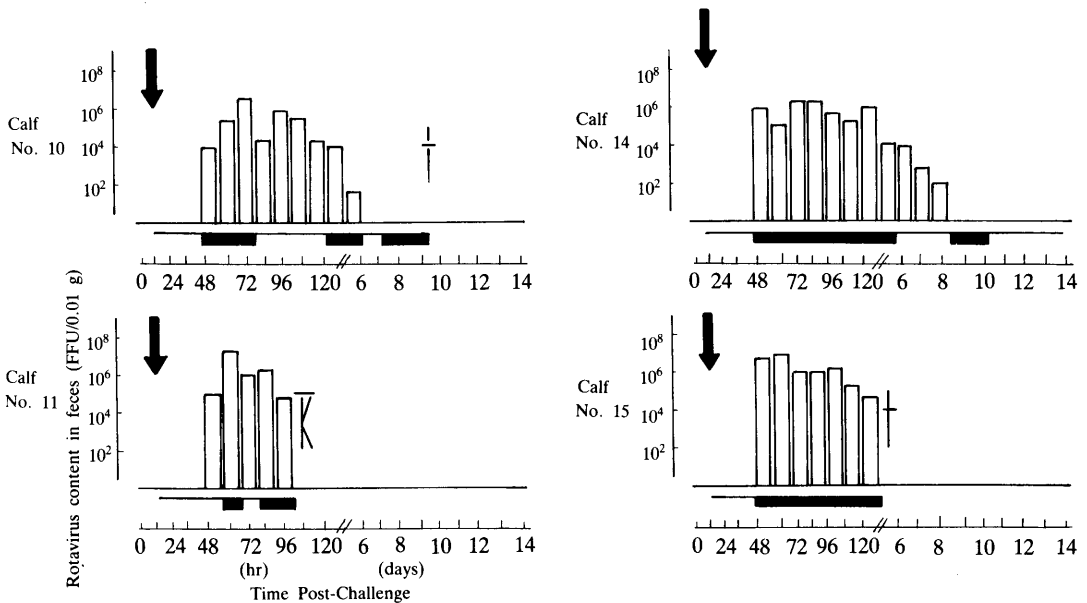


Fig. 3. Clinical response and rotavirus content in feces of control calves after BRV-2 challenge.
 ▼ BRV challenge, ■ Diarrhea, † Death, K Euthanasia.

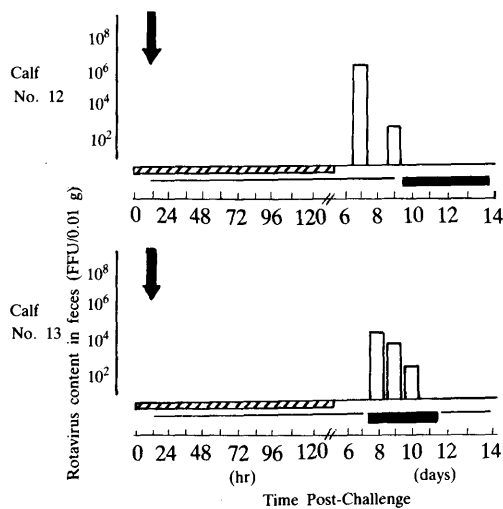


Fig. 4. Clinical response and rotavirus content in feces of calves fed 10% immune colostrum after BRV-2 challenge.

◆ BRV challenge, ■ Diarrhea, ▨ Supplemental feeding of immune colostrum.

inactivated with binary ethylenimine. In this study, however, immunization of cows with β -propiolactone-inactivated BRV induced high titer NA in serum and colostrum, that is comparable to the result of their experiment with binary ethylenimine. The reason of discrepancy in the results between their and our experiments is unknown. However our result suggests that β -propiolactone is one of effective chemicals to produce inactivated vaccine for BRV.

Many workers have reported that frequent ingestion of colostrum or milk that contain NA can protect suckling animals from enteric viral infections [9, 12, 28]. This immunogenic mechanism was referred as lactogenic immunity. Lactogenic immunity seems to be effective for protection of newborn calves from BRV infection. It is well known that BRV infection occurs frequently in calves older than 4 days in the field [2, 6, 13]. Probably this is due to that antibodies ingested by newborn calves via colostrum persist in the gut lumen for several days, because almost all cows are infected naturally with BRV. Many

attempts have been made to provide newborn calves with passive immunity to BRV infection by continuous feeding of milk supplemented with immune colostrum [4, 21, 24, 27]. Saif *et al.* [21] have shown that continuous feeding of milk with NA titers of more than 678 is necessary for complete protection of calves from experimental BRV infection. On the other hand, Snodgrass *et al.* [27] have reported that continuous feeding of milk with NA titers of 256 reduced development of diarrhea and viral shedding in calves exposed naturally to BRV. In this study, NA titers of the immune colostrum pool used as supplement for milk replacer were 25000 and 35000 to BRV-1 and BRV-2, respectively. During continuous feeding of milk replacer supplemented with 10% of the immune colostrum pool, calves were protected from diarrhea and viral shedding after exposure to either BRV-1 or BRV-2. These agree with the results reported by Saif *et al.* [21]. On the contrary, all control calves challenge-inoculated with either BRV developed severe diarrhea, and shed a large amount of virus in feces. Eventually the majority of them died. These appear to indicate that lactogenic immunity plays a significant role in the protection of calves against BRV infection, and that continuous feeding of milk replacer with NA titer of 2500–3500 provides sufficient protective immunity for calves. However, minimal protective doses of immune colostrum for lactogenic immunity, and the possibility of cross protection between 2 distinct serotypes of BRV by lactogenic immunity were not determined in this study. Further experiments to elucidate these points are expected.

All calves fed immune colostrum began shedding BRV and developed mild diarrhea several days after cessation of immune colostrum. Saif *et al.* [21] have reported similar observation, and mentioned that

limited subclinical infection kept in abeyance by daily feeding of immune colostrum may account for this phenomenon. Another explanation is that BRV was transmitted to calves by animal caretakers after cessation of immune colostrum feeding. In order to elucidate this point, it would be necessary to compare electrophoretic mobilities of genome RNA of viruses extracted from feces with those of challenge viruses.

ACKNOWLEDGEMENTS. We thank Mr. T. Sakurai and Ms. Y. Furuto for capable technical assistance.

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要 約

免疫初乳の連続給与による新生子牛の牛ロタウイルス感染の防御：恒光 裕・清水実嗣¹⁾・平井綱雄・米道裕弥・工藤卓二・森 清一・尾上貞雄（北海道立新得畜産試験場，¹⁾農林水産省家畜衛生試験場北海道支場）——妊娠末期の乳牛に、牛ロタウイルス（BRV）1型と2型を筋肉内に接種した。血清中の中和抗体価は、両型とも有意に上昇し、初乳中の中和抗体価は、両型とも非免疫対照群と比較して有意に高値を示した。次に新生子牛15頭にBRV 1型あるいは2型を経口投与して観察した。5頭は免疫初乳群として、免疫初乳を10%混合した代用乳2 lをBRV攻撃の2時間前、次いで攻撃後1日2回、5日間給与した。他の10頭は対照群とした。対照群の全例がBRV攻撃24~48時間後より激しい下痢を呈し、糞便からBRVが検出された。一方免疫初乳群では、初乳給与中には5例中4例が下痢を呈さず、糞便からBRVは全く検出されなかった。これらの成績から、免疫初乳の連続給与により、BRV 1型ならびに2型による子牛下痢症を予防できる可能性が示唆された。