Induction of Mucosal Disease in Cattle Persistently Infected with Noncytopathic Bovine Viral Diarrhea-Mucosal Disease Virus by Superinfection with Cytopathic Bovine Viral Diarrhea-Mucosal Disease Virus

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ABSTRACT. Three head of cattle persistently infected with noncytopathic bovine viral diarrhea-mucosal disease virus (ncBVD-MDV) were superinfected naturally or experimentally with cytopathic bovine viral diarrhea-mucosal disease virus (cBVD-MDV). In the naturally superinfected case, one animal manifested pyrexia and severe diarrhea, and died without developing antibodies to cBVD-MDV. However, another animal survived with only continual slight anorexia and pyrexia, and developed strong resistance to the superinfected strain. In the experimental cases, induction of MD was unsuccessful in two persistently infected cattle when superinfected with cBVD-MDV antigenically heterologous for persistently infected ncBVD-MDV. They also developed antibodies to the cBVD-MDV strain with which they had been infected. After 6 months, these cattle were infected again with a cBVD-MDV strain different from that used in the previous experiment. One animal infected with this strain, which was antigenically homologous to the persistently infected strain, died after developing MD symptoms without developing antibodies to the infecting strain. It is suggested that the antigenic relationship between the persistent ncBVD-MDV and the superinfected cBVD-MDV was an important factor in developing MD.—**KEY WORDS:** BVD-MDV, induction of MD, persistent infection.

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Persistent bovine viral diarrhea-mucosal disease virus (BVD-MDV) infection of cattle has been reported in the U.S., the U.K., and other countries [1, 6, 11, 12]. At birth and throughout life, these cattle were viremic with noncytopathic BVD-MDV (ncBVD-MDV) and usually lacked neutralizing antibodies to BVD-MDV. Severe-to-fatal BVD-MDV infections were induced in persistently infected cattle by superinfection with cytopathic BVD-MDV (cBVD-MDV) [2, 3, 5]. In our study we took three persistently infected cattle and naturally or experimentally superinfected them with cBVD-MDV, and then carried out clinical, serological and virological studies.

In this report, we describe the results of these studies and discuss some problems with respect to the induction of mucosal-disease (MD) in cattle persistently infected with ncBVD-MDV.

MATERIALS AND METHODS

Cattle: The three head of cattle were Holstein-

Friesians, introduced for use in bovine vaccine experiments in Sept. 1985 (Nos. 154 and 155) or April, 1986 (No. 175).

It was confirmed that they had been continually infected with ncBVD-MDV, and were bred over a long period of time for the experiments.

Viruses: The Nose [9] and T-20 [5] strains of cBVD-MDV and No. 12 strain [10] of ncBVD-MDV were used for the experiments. These viruses were propagated in a bovine testicle (BT) cell culture. The No. 12-43 strain of ncBVD-MDV live vaccine (produced by our laboratories) also was used for the immunological competence test.

Specimens for virus isolation: The specimens taken from the animals for virus isolation were from blood, nasal mucus, urine and feces. Tissues obtained from the dead animals also were used. For fractionation of blood cells, heparinized blood was placed on Ficoll-Paque (Pharmacia Fine Chemicals AB, Sweden) and centrifuged at 2,000 rpm for 70 min. Plasma, mononuclear cells, granulocytes and erythrocytes were collected. These cells were washed five times with RPMI 1640 medium. The nasal mucus, urine and fecal specimens were diluted with Eagle's minimum essential medium (MEM). Tissues from the dead animals were homogenized and clarified by centrifugation at 3,000 rpm for 20

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min. These supernatant fluids were harvested and stored at -70°C until used.

Virus isolation: The isolation and confirmation of ncBVD-MDV was carried out by a method described previously [8, 10]. Interference phenomenon using the Nose strain of cBVD-MDV also was applied for confirmation of viral propagation [9, 15].

Physicochemical properties of isolated viruses: Determination of nucleic acid type and sensitivities to lipid solvents, and a filtration test were carried out as described previously [16].

Serum neutralization (SN) test: Twofold serial dilutions of inactivated serum were prepared. Each dilution was mixed with an equal volume of virus suspension containing 200 TCID₅₀/0.1 ml. The mixture was incubated at 37°C for 1 hr, then 0.1 ml of the mixture of each serum dilution was put into two tubes of BT cell culture to inoculate it. After 1 ml of maintenance medium was added, the cultures were incubated at 37°C for 5–7 days in a roller drum. When ncBVD-MDV was used to neutralize the virus, the test was carried out by the END method [8, 10]. The SN titers were expressed as the reciprocal of the serum dilution corresponding to a 50% endpoint of neutralization.

Rabbit immune serum: A rabbit was inoculated intravenously with a single dose of 1 ml of virus infected culture. Serum was collected from the rabbit 4 weeks after inoculation, by which time antibody titers had reached a plateau. The serum was then heated to 56°C for 30 min before use.

RESULTS

Cattle persistently infected with ncBVD-MDV and their immunological competence for ncBVD-MDV

vaccine: Three head of cattle were used as assay animals for the ncBVD-MDV vaccine. They were inoculated intramuscularly with a $10^{4.5}$ TCID₅₀/dose of the No. 12–43 strain of ncBVD-MDV live vaccine. SN antibodies to the Nose and No. 12 strains of BVD-MDV were not detected at all after two or three vaccinations. These findings suggested that these cattle were continually infected with ncBVD-MDV.

Three strains of ncBVD-MDV to which the cattle had the fewest antibodies after vaccination were isolated. The virus derived from No. 154 was designated as Uji-1, from No. 155 as Uji-2 and from No. 175 as Uji-4. In these animals, the virus was isolated from blood, nasal mucus and urine at titers of $10^{5.0} \sim 10^{6.5}$ TCID₅₀/ml, but not from feces.

Infective blood constituent titers from animal No. 155 were investigated. In whole blood and plasma, the virus was detected in titers of $10^{6.5}$ TCID₅₀/ml, and the virus was also found in the titers of $10^{4.75} \sim 10^{5.25}$ TCID₅₀/10⁶ cells in the mononuclear cells and granulocytes, but not in the erythrocytes.

A change in infective titers in the peripheral blood and nasal mucus of animal No. 155 from Sept., 1986 to Jan., 1987 was examined. The virus was always recovered in titers of $10^{4.5} \sim 10^{7.0}$ TCID₅₀/ml from both materials.

MD naturally caused by superinfection with cBVD-MDV: Cattle Nos. 154 and 155 did not manifest clinical signs of the MD until Aug., 1986. These cattle were about ten months of age at the time of onset of the disease. They were examined on Aug. 21, 1986. Animal No. 154 died on Aug. 30. Clinical signs were anorexia, pyrexia (41.4°C) and diarrhea. The animal had intermittent watery diarrhea and was anorectic and dehydrated and lost

Table 1. A natural outbreak of MD in cattle persistently infected with ncBVD-MDV

Cattle with PI-ncBVD-MDV		Pre outbreak						Post outbreak							
		Antibodies to						Clinical symptoms	Isolation of virus ^{b)}	Antibodies to					
		Nose	T-20	Uji-						Nose	T-20	Uji-			
No.	Strain				1	2	3	4				1	2	3	4
154 ^{a)}	Uji-1	— ^{d)}	—	—	—	—	—	MD Died (9) ^{c)}	Uji-1 Uji-3 (cBVD-MDV)	—	—	—	—	—	—
155 ^{a)}	Uji-2	—	—	—	—	—	—	Pyrexia Survived	Uji-2	+ ^{d)}	+	+	—	+	+

a) Holstein-Friesian, 10 months old.

b) Used blood and nasal mucus.

c) Days to death after clinical symptoms appeared.

d) SN antibody: —; <1:2, negative, +; $\geq 1:2$, positive.

weight, but was not pyrexemic until the week before death. From most of the organs (cerebrum, cerebellum, lung, liver, spleen, pancreas, kidney, jejunum, ileum, colon, thyroid, thymus, hypophysis, amygdala, mesenteric lymph node, submandibular lymph node) and nasal mucus, the virus which was cytopathic to BT cells was isolated. This virus was designated as Uji-3.

Animal No. 155 became anorectic and pyrexemic (41.1°C) on Aug. 25, but began eating after about 5 days of clinical disease, and remained healthy for more than two years (Table 1).

Fig. 1. Neutralizing titers of antibody in cattle Nos. 154 and 155 to Uji-1 strain (—■—), Uji-2 strain (—●—), Uji-3 strain (---□---), No. 12 strain (—▲—) and Nose strain (---△---).

In the serological findings of persistently infected cattle during and after the appearance of clinical signs, cattle Nos. 154 and 155 had no antibodies to any strains of BVD-MDV until No. 154 died. After that, animal No. 155 developed antibodies to Uji-1 and 3 strains at a high titer and against No. 12 and Nose strains of BVD-MDV at a low titer, but no antibodies to the Uji-2 strain (Fig. 1).

Identification and cross neutralization test of the isolated viruses: The physicochemical properties of the isolated viruses, Uji-1, 2, 3 and 4 strains, were coincident with BVD-MDV as previously reported [16]. The Uji-1, 2 and 4 strains, as well as No. 12 strain of ncBVD-MDV, exalted the Miyadera strain of the Newcastle disease virus, and interfered with the Nose and Uji-3 strains of cBVD-MDV.

The antigenic relationships were examined. The results of the cross-neutralization test of Uji-1, 2, 3, 4, No. 12, Nose and T-20 strains with rabbit immune sera are shown in Table 2. From the results, it was concluded that Uji-1, 3 and 4 strains were antigenically closely related, but Uji-2 was different from these isolates. No. 12 and Nose strains were antigenically homologous, but T-20 was somewhat different from all other strains. It was noted that the antiserum to the Nose strain had high SN titers for other strains (Table 2).

Experimental induction of MD: We attempted to experimentally induce MD. Animal No. 155 was inoculated with the T-20 strain of cBVD-MDV which had shown resistance to the naturally occurring Uji-3 strain superinfection, showing mild symptoms, and No. 175 which had probably had no experience of cBVD-MDV superinfection was also inoculated. In animal No. 155, no clinical signs were observed, but No. 175 showed a slight febrile reaction (41.5°C) for a few days after inoculation.

Table 2. Cross-neutralization test among isolates and known BVD-MDV strains

Virus		Rabbit immune sera to						
Strains	Type	Uji-1	Uji-2	Uji-3	Uji-4	No. 12	Nose	T-20
Uji-1	nc ^{a)}	32 ^{c)}	2	64	32	8	128	8
Uji-2	nc	8	16	2	8	32	64	4
Uji-3	c ^{b)}	32	<2	128	128	16	128	4
Uji-4	nc	32	<2	64	128	8	128	16
No. 12	nc	4	<2	2	<2	512	256	16
Nose	c	8	<2	2	<2	256	256	128
T-20	c	8	2	8	<2	64	128	256

a) Noncytopathic type.

b) Cytopathic type.

c) SN antibody titer.

Table 3. Trial for experimental induction of MD by inoculation with T-20 strain of cBVD-MDV in cattle persistently infected with ncBVD-MDV

Cattle with PI-ncBVD-MDV		Pre challenge						After challenge inoculation with T-20 strain ^{b)}							
No.	Strain	Antibodies to						Clinical symptoms	Isolation of virus ^{c)}	Antibodies to					
		Nose	T-20	Uji-						Nose	T-20	Uji-			
				1	2	3	4					1	2	3	4
155 ^{a)}	Uji-2	+	⁺ 4 ^{e)}	+	-	+	+	None Survived	Uji-2	+	⁺ 128 ^{e)}	+	-	+	+
175 ^{a)}	Uji-4	-	-	-	-	-	-	Pyrexia Survived	Uji-4 T-20 ^{d)}	+	⁺ 2048 ^{e)}	-	+	-	-

a) Holstein-Friesian, 28 months old (155) and 12 months old (175).

b) Inoculated with 10^{8.0} TCID₅₀ of virus.

c) Used blood and nasal mucus.

d) Virus detected from 5 to 12 days in blood and from 1 to 11 days in nasal mucus after inoculation.

e) SN titer of antibody to T-20 strain 4 weeks after inoculation.

Table 4. Trial for experimental induction of MD by inoculation with Uji-3 strain of cBVD-MDV in cattle persistently infected with ncBVD-MDV

Cattle with PI-ncBVD-MDV		Pre challenge				After challenge inoculation with Uji-3 strain ^{b)}									
No.	Strain	Antibodies to				Clinical symptoms	Isolation of virus ^{c)}	Antibodies to							
		Nose	T-20	Uji-				Nose	T-20	Uji-					
				1	2					3	4	1	2	3	4
155 ^{a)}	Uji-2	+	+	+	−	+	+	None Survived	Uji-2	+	+	+	−	+	+
														512 ^{f)}	
175 ^{a)}	Uji-4	+	+	−	+	−	−	MD Died (57) ^{d)}	Uji-4 Uji-3 ^{e)}	+	+	−	+	−	−

a) Holstein-Friesian, 34 months old (155) and 18 months old (175).

b) Inoculation with 10^{7.0} TCID₅₀ of virus.

c) Used blood and nasal mucus.

d) Days up to death after inoculation.

e) Virus detected continuously up to death.

f) SN titer of antibody to Uji-3 strain 4 weeks after inoculation.

They developed antibodies to the challenge strain, and remained healthy during the 5-month observation period (Table 3).

These animals were again superinfected with the Uji-3 strain of cBVD-MDV. No. 155 was healthy with no clinical signs during the 10 months of observation, because of a high antibody level for this strain. At 19 days after infection, animal No. 175 began to show MD symptoms such as anorexia, watery diarrhea, and bloody feces, and died 57 days after inoculation. The virus was recovered continuously from blood, nasal mucus, urine, and feces, and identified serologically as the Uji-3 strain. The SN antibody to the Uji-3 challenge strain in this animal was not detected nor were Uji-1 and Uji-4 after the challenge inoculation (Table 4).

DISCUSSION

Three head of cattle which did not respond to the ncBVD-MDV live vaccine were suspected of being persistently infected with ncBVD-MDV, and viruses were isolated from these cattle. They were continually viremic with ncBVD-MDV, and excreted the virus via nasal mucus and urine. The infective titer of the virus was almost constant in peripheral blood and nasal mucus for several months. For that reason, it seems that the presence of cattle persistently infected with ncBVD-MDV is a major source of infection on a breeding farm.

For the induction of MD, 3 head of cattle persistently infected with ncBVD-MDV and 2 antigenically different strains of cBVD-MDV were used. The MD occurred in one animal naturally and

in one animal under experimental conditions. But MD did not occur in one animal naturally and in 3 animals in duplicated trials. In the animals with MD, the superinfected strain of cBVD-MDV was antigenically identical with the persistently infected ncBVD-MDV strain, and no antibodies to the superinfected strain and some other heterologous BVD-MDV strains were developed after superinfection. However, in the animal without MD, the superinfected cBVD-MDV strain was antigenically different from the persistent ncBVD-MDV strain, and antibodies to the superinfected strain and some other strains were developed after superinfection. The antigenic relationship among some strains was confirmed by a cross-neutralization test with rabbit antiserum.

Several investigators have reported that most of the ncBVD-MDV and cBVD-MDV isolated from cattle with MD were antigenically indistinguishable [7, 13]. For the origin of cBVD-MDV in MD, Howard *et al.* [7] discussed the possibility of mutation *in vivo* from persistently infecting noncytopathic virus to cytopathic virus. It seems most probable that there is a mutation of the persisting noncytopathic virus *in vivo*, mainly because the cytopathic biotype has only been recovered from cases of MD [4] and it has been confirmed by cross-neutralization studies between pairs of biotypes from clinical cases [7]. However, in the present case, although the origin of the Uji-3 strain was not clear, cattle Nos. 154 and 155 when infected naturally became ill simultaneously. Furthermore, the possibility of outside infection cannot be denied absolutely. Cattle resistant to MD have survived for more than two years after exposure without excreting cBVD-MDV or falling ill with MD.

Shimizu *et al.* [14] showed that MD could be induced in persistently ncBVD-MDV infected cattle by introduction of an antigenically different cBVD-MDV. We also introduced 2 strains of antigenically heterologous cBVD-MDV to persistently ncBVD-MDV infected animals. However, MD could not be induced, and the animals survived, developing antibodies to the challenge strain. One reason may be that 2 of the 3 cattle had antibodies to the challenge viruses at the time of the challenge inoculation. On the other hand, Brownlie [4] reported that failure to reproduce a disease experimentally may be due to a lack of homology between the two biotypes. For the induction of MD with an antigenically heterologous strain, it is

supposed that at least two factors relating to the pathogens are necessary. One is the propagation of the virus in the bodies of infected animals, and the other is the mutation of the antigenic variation of a persistent virus or superinfected virus. The etiological factors are still unknown.

From our results, it seems that the antigenic relationship between the persistently infected ncBVD-MDV and the superinfected cBVD-MDV was one of the most important factors in the development of MD.

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