Bovine Viral Diarrhea Virus Replication in Bovine Follicular Epithelial Cells Derived from Persistently Infected Heifers

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ABSTRACT. Bovine follicle fluid and oocytes surrounded by follicular epithelial (FE) cells were collected from ovaries of two heifers persistently infected with bovine viral diarrhea virus (BVDV). BVDV was present in the follicle fluid at a higher titer than in serum. The oocytes were matured *in vitro* under culture conditions of 39°C in humidified air containing 5% CO_2. *In vitro* fertilization was performed after 24 hr in culture (the day of insemination was defined as day l), and culture was continued through day 10. BVDV was present in the culture medium at titers of $10^{2.25}$ to $10^{3.25}$ TC_lD_{50}/0.1 m*l*. The virus was also detected in FE cells collected on day 10. Viral antigen was demonstrated in the cytoplasm of FE cells by the indirect immunofluorescence technique. However, no BVDV was detected in the embryos on day 10. These findings suggested that the oocytes or embryos were unlikely to be infected with BVDV, but that the FE cells were infected with BVDV and supported virus replication in cattle persistently infected with BVDV. — KEY WORDS: bovine viral diarrhea virus, follicular epithelial cell, follicular fluid, *in vitro* fertilization, persistent infection.

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Half of all calves persistently infected with bovine viral diarrhea virus (BVDV) develop either mucosal disease or chronic diarrhea and die in one to two years, while the other half appear to be healthy, can in no way be distinguished from normal calves and live to breeding age [3]. These calves carry and shed the virus throughout their lives and become a source of virus that infects other cattle in the field [9, 14, 18]. It is well known that persistently infected cows can give birth to persistently infected calves, and the vertical transmission has been demonstrated to occur early in gestation, before about day 90 [6, 10, 15]. However, there have been few reports on vertical infection before placentation. It has been reported that when embryos collected from persistently infected heifers were transplanted into normal heifers, normal calves were born after normal pregnancy [2, 5, 20], and in our laboratory it was demonstrated that BVDV had no effect on either *in vitro* oocyte maturation or *in vitro* development of zona-intact embryos, when oocytes with follicular epithelial (FE) cells were artificially exposed to BVDV [19]. The objectives of this study were to determine whether BVDV actually replicates in FE cells derived from persistently infected heifers and to investigate whether embryos produced *in vitro* from the oocytes of such heifers are infected with BVDV.

MATERIALS AND METHODS

Cattle: Two heifers persistently infected with nonpathogenic (NCP) BVDV were used in this trial. BVDV was isolated from the serum of both animals three times in three months. The first heifer was 32 months old and had developed normally in all respects. The second heifer was 28 months old and its growth was somewhat stunted. Two normal heifers from which neither BVDV nor antibody was detected were prepared as controls.

In vitro maturation, fertilization, and culture: Oocytes surrounded by follicular epithelial (FE) cells were collected from small antral follicles (diameter < 5 mm) on the ovaries of the four heifers with a syringe containing Dulbecco's modified phosphate balanced solution. The oocytes together with the FE cells were washed by transferring them through six changes of wash medium consisting of medium 199 supplemented with 0.2% bovine serum albumin and antibiotics and maintained in the maturation medium for 24 hr at 39°C in humidified air at 5% CO_2. The mature oocytes with the FE cells were coincubated with spermatozoa for about 5 hr according to the procedure reported by Tsuboi and Imada [l9]. After co-incubation, the oocytes with the FE cells were transferred to the developmental medium. The maturation and developmental medium was medium 199 supplemented with 5% fetal bovine serum (FBS) without antibody against BVDV. The maturation and development culture was performed in a 0.l-m*l* drop covered with mineral oil (Squibb Co., Ltd.) in a small Petri dish (35 × 10 mm: Nunc Co., Ltd.). The day of *in vitro* insemination was defined as day l, and embryo development was observed up to day 10. In addition, the "small-follicle fluids" remaining after the oocytes with FE cells had been collected as material for the *in vitro* fertilization (IVF) technique, was centrifuged at 3,000 rpm for 15 min. "Large-follicle fluid" was collected from some large follicles (diameter > 6 mm), whose oocytes with FE cells are unsuitable for the IVF technique, and centrifuged by the same method as the small-follicle fluid. These follicle fluids were fractionated into supernatant, i.e., small-follicle fluid and large-follicle fluid, and sediment, i.e., FE cells.

Collection of virus samples during in vitro culture and virus isolation: Developmental media were collected at day 3 and day 6. The embryos and FE cells that had formed monolayers were washed 3 times with developmental

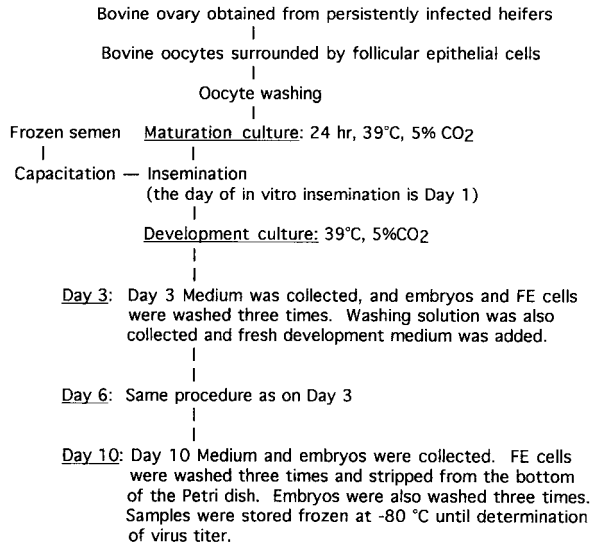

Fig. 1. Methods used for oocyte maturation, fertilization, embryo development and BVD monitoring.

medium, and fresh developmental medium was added to continue the culture. At day 10, the developmental medium and embryos were collected and the FE cells were washed 3 times. The FE cells were transferred to plastic tubes and sonicated for 30 sec in 1 m*l* of a minimum essential medium supplemented with 0.2% bovine serum albumin and antibiotics. The embryos were washed by transferring them through three changes of wash medium, and then transferred to plastic tubes and sonicated the same as the FE cells. The titer of NCP BVDV was determined with an ordinary 50% tissue culture infectious dose ($TCID_{50}$) on microtiter plates. The titer of the NCP BVDV was measured by an interference test in which the cytopathogenic (CP) Nose strain was used as the challenge virus [16]. Bovine fetal muscular (BFM) cell cultures were used for NCP BVDV isolation and titration. The BFM cells and FBS used were confirmed to be free of BVDV. A flowchart of sampling methods is shown in Fig. 1.

Antigen detection by the immunofluorescence technique: Some of the oocytes with FE cells from the persistently

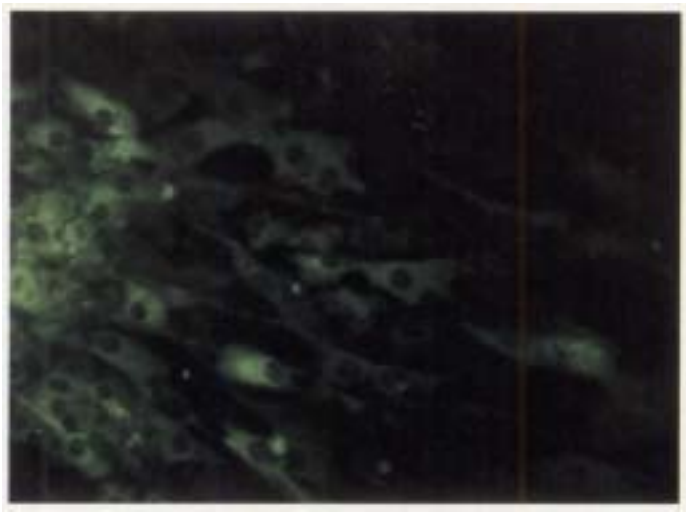

Fig. 2. Immunofluorescence staining of the FE cells of follicles in ovaries derived from persistently infected heifer No. 1(× 120).

Table 1. BVDV isolation from serum and follicles of ovaries obtained from persistently infected heifers

	BVDV Titer[a]			
	Infection group		Control group	
	No.1	No.2	No.1	No.2
Serum	4.5	4.25	≤1.5	≤1.5
Small-follicle fluid[b]	5.75	5.5	do[d]	do
Large-follicle fluid[b]	≥7.0	≥7.0	do	do
Follicular epithelial cells[c]	6.25	7.0	do	do

a) log $TCID_{50}$/m*l*.
b) Small-follicle fluid was diluted with Dulbecco's modified phosphate balanced solution to a final volume of 10–15 m*l*. Large-follicle fluid was not diluted, and the volume was 2–3 m*l* because of direct collection.
c) The sediment from small-and large-follicle fluid after centrifugation was resuspended to a volume of 2 m*l*.
d) The same as the above.

Table 2. BVDV titers of media and washing solutions during *in vitro* oocyte maturation and embryo development

	BVDV titer[a)]			
	Infection group		Control group	
	No.1	No.2	No.1	No.2
Maturation medium	2.25	2.5	<0.5	<0.5
Day 3 medium	3.25	3.25	do[b)]	do
3rd washing sol. on day 3	<0.5	<0.5	do	do
Day 6 medium	3.0	2.5	do	do
3rd washing sol. on day 6	<0.5	<0.5	do	do
Day 10 medium	2.75	2.25	do	do
Embryos on day10	<0.5	<0.5	do	do
FE cells on day 10	2.25	1.75	do	do

a) Virus titers are shown as log $TCID_{50}$/0.1 m*l*, but for embryos and FE cells they are shown as log $TCTD_{50}$/all embryos or log $TCID_{50}$/FE cells, respectively.
b) The same as the above.

infected heifers were collected for detection of BVDV antigen after washing. The oocytes with the FE cells were cultured in chamber slides for 4 days. BVDV antigen detection was performed by the indirect immunofluorescence technique [19] after fixing the samples with acetone.

RESULTS

There were 13 and 15 oocytes with FE cells, respectively, in the two heifers persistently infected with BVDV, and 21 and 27, respectively, in the two control heifers. In the control group, development into the 2-cell stage to 8-cell stage was observed on day 3, and development into blastocysts was observed from day 8 to day 10. The oocytes developed into the 2-cell stage to 8-cell stage in the persistently infected group at day 3, the same as the controls. After that, the number of blastomeres increased and the embryos developed to the morula stage, but did not reached the blastocyst stage. The FE cells changed to a clean monolayer state during embryo development, the same as in the controls, and no cytopathic effect was observed in the FE cells.

The results of attempts to isolate BVDV in these trials are shown in Tables 1 and 2. Table 1 shows the BVDV titer of the supernatant and the sediment from the follicle fluid. Table 2 shows the BVDV titers of the culture media, washing solutions, embryos, and FE cells during *in vitro* oocyte maturation and embryo development. No BVDV was isolated from any samples in the control group, whereas high-titer BVDV was isolated from the follicle fluid and FE cells in the persistent infection group (Table 1). The BVDV titers in the medium on day 6 and day 10 increased from $<10^{0.5}TCID_{50}$/0.1 m*l* to $10^{2.25-3.0}TCID_{50}$/0.1 m*l* during 3 days or 4 days of incubation, and BVDV was isolated from the FE cells on day 10, but all control group samples showed titers of $<10^{0.5}TCID_{50}$ /0.1 m*l* (Table 2). Attempts to isolate BVDV from the embryos on day 10 were negative (Table 2).

Viral antigens were detected in the cytoplasm of the FE cells in the persistently infected group (Fig. 2), but not in the control group.

DISCUSSION

Clarke *et al.* [7] reported isolating BVDV from nearly all of the tissues of three infected animals, one with persistent viremia and two with experimentally induced mucosal disease. However, there is little information on virus isolation from follicles in ovaries collected from persistently infected cattle. In this study, BVDV titers were found to be higher in follicle fluid and follicular epithelial cells than in serum (Table 1). These findings suggest that the follicular fluid or FE cells were not simply contaminated by a serum/blood cell source during collection, but that BVDV was present in the follicular fluid and the FE cells at least from a very early stage of follicular development and that BVDV replicated in the follicles during follicular development.

When oocytes from heifers persistently infected with BVDV were matured, fertilized, and cultured *in vitro*, no BVDV titers were detected in the embryos produced. BVDV did not appear to attach to the zona pellucida of embryos after washing with culture medium. No blastocysts were observed in the persistently infected group during the culture period. However, the *in vitro* embryo development into blastocysts has been reported to be observed continuously after day 8 [17], and development to the morula stage was observed in this study. Thus, these embryos appeared to continue to develop up to the final day in this experiment. The development into the morula stage suggested that the total number of blastomeres of these embryos collected on day 10 became several hundred. In addition, we confirmed that BVDV could be isolated at titers of $10^{0.62}TCID_{50}$/0.1 m*l* from BVDV-infected one hundred BFM cells in 1 m*l* by the same microtiter methods. Thus, the negative result suggest that oocytes or embryos derived from persistently infected cattle may not be infected with

BVDV. These findings are consistent with a report that normal recipients implanted with embryos collected from heifers persistently infected with BVDV gave birth to normal calves [2, 5, 20]. However, BVDV was isolated from the FE cells co-incubated with embryos (Table 2), and viral antigen was detected in the cytoplasm of FE cells (Fig. 1). These observations suggest that BVDV replicates in the FE cells of ovaries derived from persistently infected heifers. This finding, along with the report by Booth *et al*. [4] supports the hypothesis that BVDV has a predilection for epithelial cells in general, as well as the central nervous system and lymphoid tissues [l, 8, 12, 13].

It has been reported that cumulus cells, such as FE cells, have cytoplastic projections that penetrate through the zona pellucida and contact the vitelline membrane [11]. Based on this information, BVDV may have an opportunity to come into direct contact with oocytes by way of FE cells during oogenesis or oocyte maturation *in vivo*. However, our results showed that the zona-intact embryos produced by the IVF procedure did not appear to be infected with BVDV. These findings suggest that before hatching embryo cells may not have acquired susceptibility to BVDV yet.

Thus, methods in which oocytes from persistently infected cattle are matured *in vitro*, fertilized *in vitro*, and the embryos are transplanted to normal healthy recipients, will serve as an effective method of interrupting the vertical transmission of BVDV. Further studies are needed to evaluate the susceptibility of embryonic cells to BVDV at various stages during embryonic development from the 2-cell stage to the hatched blastocyst stage.

REFERENCES

1. Allan, G. M., McNulty, M. S., Bryson, D., Mackie, D. and Platten, M. 1989. Demonstration of bovine virus diarrhea virus antigen in formalin-fixed, paraffin embedded tissue using a streptavidin/biotin technique. *Res. Vet. Sci*. 46: 416–418.
2. Bak, A., Callesen, H., Meyling, A. and Greve, T. 1992. Calves born after embryo transfer from donors persistently infected with BVD virus. *Vet. Rec*. 13l: 37.
3. Baker, J. C. 1990. Clinical aspects of bovine virus diarrhea virus infection. *Rev. Sci. Tech. Off. Int. Epiz*. 9: 25–4l.
4. Booth, A. J., Stevens, D. A., Collins, M. E. and Brownnlie, J. 1995. Detection of bovine viral diarrhea virus antigen and RNA in oviduct and granulosa cells of persistently infected cattle. *J. Reprod. Fertil*. 105: 17–24.
5. Brock, K. V., Lapin, D. R. and Skrade, D. R. 1997. Embryo transfer from donor cattle persistently infected with bovine viral diarrhea virus. *Theriogenology* 47: 837–844.
6. Brownlie, J., Clarke, M. C., Howard, C. J. and Pocock, D. H. 1987. Pathogenesis and epidemiology of bovine virus diarrhea virus infection of cattle. *Ann. Rech. Vet*. 18: 157–166.
7. Clarke, M. C., Brownlie, J. and Howard, C. J. 1987. Isolation of cytopathic and non-cytopathic bovine viral diarrhoea virus from infected tissues of infected animals. pp. 3–12. *ln*: Pestivirus Infectious of Ruminants (Harkness, J. W. ed.), Commission of the European Communities, Luxembourg.
8. Desport, M., Collins, M. E. and Brownlie, J. 1992. Detection of bovine viral diarrhea virus nucleic acid by *in situ* hybridization. Pestivirus. pp. 137–141. *ln*: Proceeding of the Second Symposium on (Edwards, S. ed.), European Society of Veterinary Virology, Annecy.
9. Duffell, S. J. and Harkness, J. W. 1985. Bovine virus diarrhoea-mucosal disease infection in cattle. *Vet. Rec*. 117: 240–245.
10. Liess, B., Orban, S., Frey, H. R., Trautwein, G., Wiefel, W. and Blindow, H. 1984. Studies on transplacental transmissibility of a bovine virus diarrhea (BVD) vaccine virus in cattle. II. inoculation of pregnant cows without detectable neutralizing antibodies to BVD virus 90–229 days before parturition (5lst to 190th day of gestation). *Zbl. Vet. Med. B* 3l: 669–68l.
11. Motta, P and Van Blerkom, J. 1979. *ln*: The Cellular Basis of Mammalian Reproduction, Urban &Schwarzenberg, Baltimore-Munch.
12. Ohmann, H. B., Jensen, M. H., Sorensen, K. J. and Dalsgaard, K. 198l. Demonstration of bovine viral diarrhea virus antigen in cryostat- and paraffin-sections of bovine tissues by the immunoperoxidase technique. *Acta Pathologica et Microbiologica Scandinavica Sect C* 89: 28l–285.
13. Ohmann, H. B. 1983. Pathogenesis of bovine viral diarrhoea-mucosal disease: distribution and significance of BVDV antigen in disease calves. *Res. Vet. Sci*. 34: 5–10.
14. Roeder, P. L. and Harkness, J. W. 1986. BVD infection: Prospects for control. *Vet. Rec*. 118: 143–147.
15. Roeder, P. L., Jeffrey, M. and Cranwell, M. P. 1986. Pestivirus fetopathogenicity in cattle: Changing sequelae with fetal maturation. *Vet. Rec*. 118: 44–48.
16. Shimizu, M., Satou, K., Nishioka, N., Yoshino, T., Momotani, E. and lshikawa, Y. 1989. Serological characterization of viruses isolated from experimental mucosal disease. *Vet. Microbiol*. 19: 13–2l.
17. Shioya, Y. 1993. Calf production by *in vitro* fertilization of follicular oocytes matured *in vitro*. *JARQ* 26: 287–293.
18. Stöber, M. 1984. Current knowledge of the BVD syndrome of cattle: Agent, immune response, course and spread, control. *Bov. Pract*. 19: 49–60.
19. Tsuboi, T. and lmada, T. 1996. Noncytopathogenic and cytopathogenic bovine viral diarrhea-mucosal disease viruses do not affect *in vitro* embryonic development into the blastocyst stage. *Vet. Microbiol*. 49: 127–134.
20. Wentink, G. H., Aarts, T., Mirck, M. H. and van Exsel, A. C. A. 199l. Calf from a persistently infected heifer born after embryo transfer with normal immunity to BVDV. *Vet. Rec*. 129: 449–450.