THE EFFECT OF INFECTION OF HATCHED BLASTOCYST BOVINE EMBRYOS WITH BORDER DISEASE AND BOVINE VIRAL DIARRHEA VIRUSES

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Abstract. The effect of infection with teratogenic viruses at early stages of pregnancy is not fully understood. This study aimed to look at the effect of infection with teratogenic viruses such as bovine viral diarrhea virus (BVDV) and border disease virus (BDV), on early stage embryos at the hatched blastocyst stage. BVDV and BDV are known to cross the placenta of infected mothers and lead to congenital defects and death of developing fetuses. This study can be a good model for better understanding the effects of other teratogenic viruses such as Rubella virus in humans.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) was first detected in the United States (Olfason *et al*, 1946), and has since been recognized in most countries. In England and Wales, 62% of cattle have serum neutralizing antibody to the virus (Harkness *et al*, 1978). Bovine viral diarrhea virus and mucosal disease (MD) are two clinically dissimilar conditions caused by the same virus, Bovine viral diarrhea is the outcome of an acute infection in susceptible cattle which may occur at any age in postnatal life and usually has an illness of a few days duration (Duffell and Harkness, 1985). However, MD is almost always fatal. It occurs in cattle persistently infected with BVD-MD during fetal life and is characterized by specific immune tolerance to the infecting virus strain and absence of antibody (Duffell and Harkness, 1985). Transplacental transmission is the most common route of transmission of BVDV. Fetal infection with BVDV results in a broad spectrum of abnormalities, ranging from death *in utero* to persistent life-long infection without clinical signs (Brownlie, 1991). Between these two extremes lie the congenital defects. Newborn calves from dams exposed to BVDV at 100 days of pregnancy show 3 forms of intrauterine growth retardation (Muskens *et al*, 2004). However, fetuses infected with BVDV between 3-6 months of gestation show congenital malformations of the brain (Scott *et al*, 1973). A marked depletion in lymphoid tissues has been reported in calves from mothers persistently infected with non-cytopathogenic BVDV (Liebler-Ternorio *et al*, 2004). In the present study we have taken this work further by infecting bovine embryos at a latter stage of their development at the hatched blastocyst stage.

Border disease (BD) is a congenital disease of sheep caused by a virus in the genus Pestivirus. Border disease was first described by Hughes and Kershaw (1959). The transmission of the disease occurs both laterally from one sheep to another and vertically from parent to offspring (Osburn *et al*, 1973). Border disease virus (BDV) and BVDV may cross-infect the ovine and the bovine species and induce congenital defects (Ward, 1971; Terlecki *et al*, 1980). BDV causes an infection in sheep, which becomes clinically manifeste when pregnant ewes are infected (Van Orchist, 1983), thus resulting in abortion or delivery of clinically affected lambs. The clinical signs include abnormal hairy birth coat and

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tremor (Hughes and Kershaw, 1959; Braun *et al*, 2002; Thabti *et al*, 2002) BDV is antigenically related to BVDV, and BDV and BVDV may cross-infect ovine and bovine species and induce congenital defects (Ward, 1971; Gibson *et al*, 1974; Terlecki *et al*, 1980). From this, it is clear that BD can infect and cause congenital defects in bovine fetuses (Gibson *et al*, 1974). In order to investigate if BD virus can infect bovine embryos at early stages at the hatched blastocyst stage, and for the purpose of this study, we looked at the effects of BDV and BVDV on the development of early bovine embryos. To date, and to our knowledge, there is no study that has looked at the effects of BDV on the development of bovine embryos and at the hatched blastocyst stage. This study can be a good model to better understand the effects of early infection with human teratogenic viruses at very early stages of infection.

MATERIALS AND METHODS

Preparation of primary fetal bovine and ovine cell cultures

The skin of 2-3 month old ovine and 3-5 month old bovine fetuses was disinfected with alcohol and incised using sterile scissors. Organ and skeletal muscle were removed aseptically with sterile forceps and placed in Petri dishes containing sterile phosphate buffered saline (PBS). It was then transferred to a second Petri dish containing sterile PBS, washed thoroughly and placed in clean Petri dish where it was minced with sterile scissors. The fragments were transferred to a sterile flask and washed, first with 10 ml of warm PBS and then with 10 ml of warm 10% trypsin EDTA Gibco, UK) in PBS. The supernatant was discarded. Another 10 ml of trypsin solution was added, the flask was agitated for 10 minutes at 37ºC and the supernatant poured into a glass bottle containing 2 ml heat inactivated fetal calf serum (FCS, Sera Lab, UK), cooled on ice. The trypsinization procedure was repeated twice and approximately 30 ml of cell suspension was collected. The collected cell suspension was distributed between two centrifuge tubes (Inverin, Ireland) and spun at 1,000*g* for 10 minutes. The supernatant was poured off and the pellet was suspended in 30 ml of Dulbecco's growth medium. The cell suspension was counted using a hemocytometer (Hawksley, England). The cell suspension was diluted in Dulbecco's growth medium (General Material and Methods) to give a concentration of $1X10^7$ cells/ml. Fifteen ml aliquots were placed in 75cm^2 plastic tissue culture flasks (Sterilin, UK) and incubated at 37ºC in 5% CO_2. The medium was changed every two days. Primary cells were frozen in liquid nitrogen using the standard methods. In order to obtain a high recovery rate of primary cells after freezing in liquid nitrogen, we optimized the standard protocol, by thawing of cells rapidly to 37ºC in a waterbath. The vials were dried and their necks were swabbed with alcohol; the cells were removed using a 1 ml sterile pipette and placed in 20 ml sterile centrifuge tubes (Inverin Ireland) containing 20 ml serum free medium [Minimum essential medium (MEM), Gibco, UK]. The cells were pelleted by centrifugation at 2,000*g* for 6 minutes, the supernatant was discarded, another 20 ml of serum free medium (MEM) was added, and the centrifugation procedure repeated. The cells were then suspended in 1 ml of serum containing medium (Dulbecco's growth medium) and placed in 25 cm^2 tissue culture flasks (Flow Laboratories, UK) containing 5 ml Dulbecco's growth medium. The flasks were incubated at 37ºC in 5% CO_2 until confluent.

Microtiter assay

One hundred microliters of fetal lamb muscle cell suspension containing $2 X 10^4$cells was added to each well of a 96 well microtiter tissue culture plate (Costar, USA). The plates were incubated at 37ºC in 5% CO_2 until the cell monolayer was just subconfluent and ready for infection. One hundred microliters of virus sample was then added to the first well, mixed and 10 µl was transferred from the first well to the second well, and so on until 100 µl from the last well was discarded. Cells in the last row of the microtiter plate were left as an uninfected control. The microtiter plates were incubated at 37ºC in 5% CO_2 until the cytopathic effect (cpe) was apparent. The virus titer was taken as the highest dilution in which the cells still showed cpe.

Immunogold silver staining

Fixing of cells. Cells were grown on cover slips placed in 35 cm^2 tissue culture dishes (Sterilin, UK) and fixed with 1% glutaraldehyde (BDH Chemicals, UK) in PBS. The cover slips were then treated with ethanolamine pH 7.4 for 30 minutes, with ethanolamine pH 8.6 for 2 hours, washed and stored overnight in sterile PBS at 4ºC.

Cell were examed for the presence of BVD and BD viral antigen by immunogold silver staining. The immunogold silver staining was carried out as described by Holgate *et al* (1983). The purpose of conducting immunogold silver staining was to determine if prepared primary fetal lamb and calf cells were persistently infected or contaminated with BVD and BD viruses. The primary antibody was anti-BVD antiserum raised in a goat and provided by Dr Michael Gunn (Veterinary Research Laboratory, Abbotstown, Dublin) The secondary antibody was colloidal gold conjugated rabbit antigoat serum (Janssen Life Science, Belgium). Both antigen-antibody complexes were detected using silver intensifier (Janssen Life Science, Belgium). Fixed cells were hydrated with distilled water, treated with Lugol's iodine (1% iodine, 2% potassium iodine) for 5 minutes, decolorized by 5% sodium thiosulphate for 4 minutes, and digested at 37ºC with trypsin [0.1% trypsin (Sigma, USA) in 0.1 % calcium chloride (BDH Chemicals, UK)] for 3 minutes. The trypsin was rinsed out with 2% sucrose, and the cells were incubated with 5% normal rabbit serum (Dako, Denmark) in BSA Tris (General Materials and Methods) for 20 minutes at room temperature. The primary antibody (anti BVD antiserum raised in a goat) diluted to 1/80 in 1% normal rabbit serum in BSA Tris was applied for 30 minutes, followed by washing with agitation for 1 hour in six changes of PBS buffer. The gold conjugated secondary antibody (rabbit antigoat) diluted to 1/40 in 0.5% Tween 20 in BSA-Tris was added for 30 minutes, followed by washing with agitation for 30 minutes in 3 changes of PBS. The cells were post fixed in 2% glutaraldehyde (BDH Chemicals, UK) in PBS for 15 minutes, washed for 30 minutes in 3 changes of PBS and incubated with silver enhancement solution for about 8 minutes. The coverslips were washed with distilled water, stained with hematoxylin, dehydrated through an ascending alcohol series, cleaned in inhibisol and mounted in DPX (BDH Chemicals, UK).

Culturing of bovine embryos

Bovine embryos. Bovine embryos, washing medium (containing medium 199, 10% early estrus cow serum and 100 µg/ml streptomycin) and culture medium (containing medium 199 20% early estrus cow serum, 100 µg/ml streptomycin) were provided by Professor Ian Gordon, Agriculture Department, University College, Dublin. The bovine embryos were transferred to our laboratory at an early stage (prior to the blastocyst stage). The embryos were cultured and allowed to develop in our laboratory into the hatched blastocyst stage as described below.

Setting up the microdrop culture. Using a Gilson pipette with a sterile tip, six separated drops of 50 µl washing medium were placed on the bottom of a tissue culture dish 60 X 15 mm.

Culture medium. One drop of 400 µl culture medium was placed on the bottom of a 35 X 10 mm tissue culture dish (Flow Laboratories, UK) using a Gilson pipette. This drop was covered completely with 10 ml of mineral oil (Sigma, USA). The washing and culture media were placed in an incubator (38ºC, 5% CO_2) for at least 3 hours for temperature and CO_2 equilibration of washing and culturing medium.

Virus infection of bovine embryos. Using a stereomicroscope (Nikon, Japan) and a micropipette, six hatched blastocyst bovine embryos were placed in 1 ml of medium containing l X 10^6 TCID of BVD NADL and another six embryos were placed in 1 ml of medium containing 1 X 10^4 TCID of BD/Wey. After virus absorption for one and half hours the embryos were washed by passing them through five droplets of washing medium. They were then placed in 400 µl culture medium covered with 10 ml of mineral oil. The medium was replaced after 12 hours, and this medium and the medium from the final wash were stored at 70ºC for viral assay. This procedure was repeated every 12 hours for six days. Six mock-infected control embryos were manipulated and exposed simultaneously to the same number of washing and media changes.

The experiment was repeated twice each time using six embryos for control and infected groups. The morphological appearances of the infected and control embryos were compared.

Photography. Cells and embryos were photographed using a phase contrast inverted microscope (Nikon Japan) and XP1 400 (Ilford, UK) film.

RESULTS

Growth of primary fetal lamb and calf cells

The following six different types of fetal lamb and fetal calf cells were grown after five days incubation: fetal lamb kidney (FLK), fetal lamb muscle (FLM), fetal lamb testis (FLT), and fetal calf muscle (FCM), fetal calf testis (FCT), and fetal calf kidney (FCK).

Immunogold silver staining

Since the cells were prepared from lamb and calf fetuses, it was necessary to determine first if they were persistently infected or contaminated by BVD or BD viruses. All fetal lamb and calf cells examined were consistently negative. Positive control fetal lamb muscle infected with BVD/NADL and BD/Wey showed cytoplasmic staining for virus specific antigen.

Effect of BVD/NADL and BD/Wey infection on fetal lamb and calf cells

Each of the six different types of cells prepared was successfully infected with BVD/NADL and BD/Wey.

Development of an assay system for both BVD and BD viruses

Using a microtiter assay technique, we compared all the fetal bovine cells (FCT, FCK, FCM) and fetal ovine cells (FLM, FLK, FLT), for their efficiency for assaying BVD and BD viruses. Of the six cell types examined, fetal lamb muscle was found to be the best cell type for assaying BVD and BD viruses. These muscle cells showed clear cpe with BVD/NADL and BD/Wey at 48 hours post-infection. Also, fetal lamb muscle showed a higher recovery rate following freezing in liquid nitrogen compared to other cell types. After assaying of BVDV/NADL and BD/Wey on fetal lamb muscle, the titer of BVD/NADL was $1x10^6$ and BD/Wey was $1X10^4$.

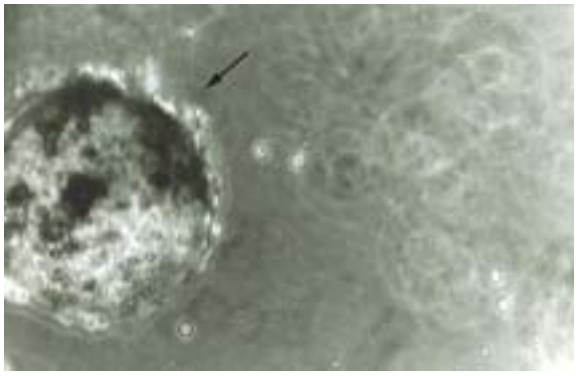

Fig 1–Hatched blastocyst bovine embryos infected with BVD/NADL. No changes in morphology were visible at 5 days postinfection. (Original magnification: 200X).

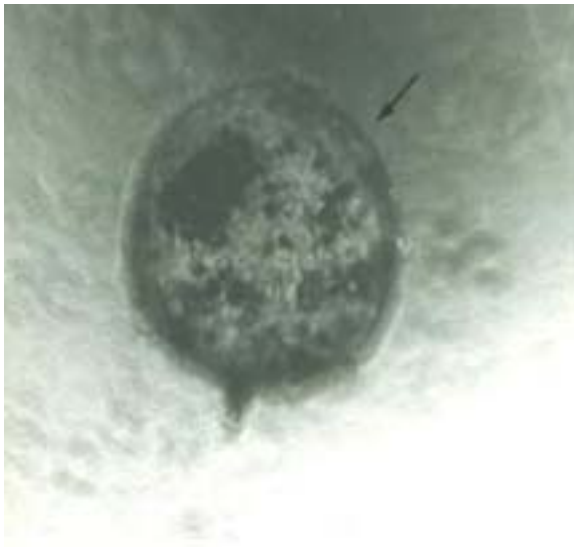

Fig 2–Hatched blastocyst bovine embryo infected with BD/Wey. No changes in morphology were visible at 5 day postinfection. (Original magnification: 200X).

Effect of BVD/NADL and BD/Wey on bovine embryos

Following infection of hatched blastocyst bovine embryos with BVD/NADL and BD/Wey viruses, no differences in development were seen (Figs 1, 2), compared to the uninfected controls (Fig 3). All the samples harvested from bovine embryos infected with either BVD/NADL or BD/Wey (when assayed on fetal lamb muscle using a microtiter assay) were negative. Even when

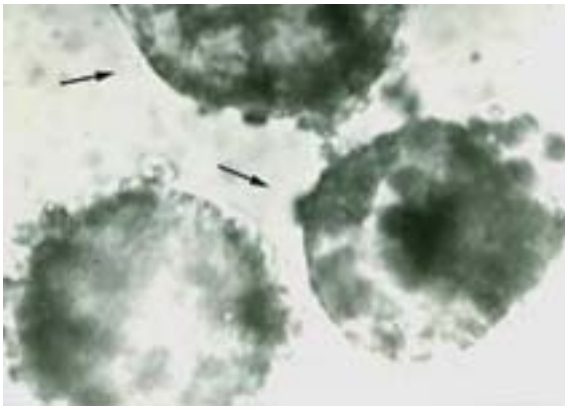

Fig 3–Hatched blastocyst bovine embryos. Uninfected control. (Original magnification: 200X).

Fig 4–Hatched blastocyst bovine embryo infected with BVD/NADL and placed on the top of fetal lamb muscle in cells in microtiter plate. No cytopathic effects were visible in the underlying primary fetal lamb muscle cells. (Original magnification: 40X).

infected, bovine embryos placed on the top of fetal lamb muscle in microtiter plates had no cpe detected for BVD/NADL or BD/Wey (Figs 4, 5). This indicates that there was no viral shedding from infected bovine embryos.

DISCUSSION

Many different types of cells have been used for assaying BVD and BD viruses, such as bovine bone marrow cells (Pritchett and Zee, 1975), bovine fetal lungs (Goldsmith and Barzilai, 1975), fetal lamb kidney (Vantsis *et al*, 1976) and bovine turbinate cells (Bolin *et al*, 1985). A scientific basis for using such cells has not been proposed. In the present work, six different types of fetal bovine and ovine cells were compared in an attempt to develop a more efficient cell system for assaying BVD and BD viruses. Of the six cell types examined, fetal lamb muscle cells were the best cell line in comparison to the other five ovine and bovine cell lines for assaying both BVD and BD viruses. Laude and Gelfi (1979) reported both BVD and BD viruses produced clear cytopathic effects (cpe) in the infected fetal lamb muscle cell line. The application of the microtiter technique for assaying BVD and BD viruses as used in the present study was also recommended by Roberts *et al* (1988), who demonstrated that such a technique was simple, fast, and equivalent to the standard plaque assay technique.

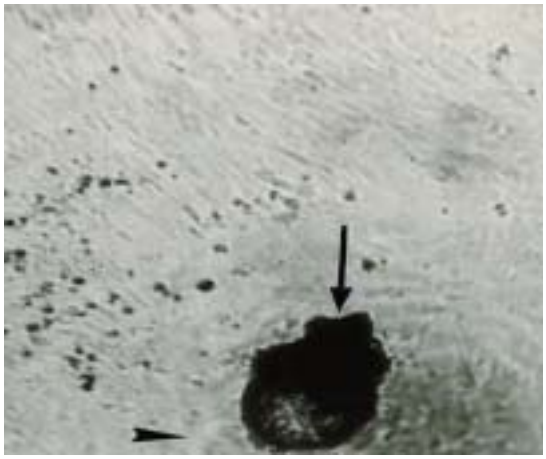

Fig 5–Hatched blastocyst bovine embryo infected with BD/Wey placed on the top of fetal lamb muscle cells in microtiter plate. No cytopathic effects were visible in the underlying primary fetal lamb muscle cells. (Original magnification: 40X).

The achievement in the present study of 80% recovery rate of primary fetal bovine and ovine cells following freezing in liquid nitrogen has not previously been reported.

The use of heat inactivated fetal calf serum in the culture medium for preparing primary cells was necessary because of the possibility of serum contamination by BVD virus (Nuttall *et al*, 1977; Vilcek, 2001).

Nuttall *et al* (1977) and Vilcek (2001), using immunofluorescence, reported the presence of non-cytopathic BVD virus in calf testis and calf kidney cell cultures. Such viruses were not demonstrated in primary cultured cell preparations in the present study using the immunogold silver staining protocol described previously by Holgate *et al* (1983).

The reason for using hatched bovine embryos in this study was to eliminate the protective role of the zona pellucida.

The present work indicates that neither BVD nor BD viruses can infect bovine embryos at the hatched blastocyst stage *in vitro.* This finding is in agreement with the results of Potter *et al* (1984) who found that there was no uptake of BVD virus by preimplantation bovine embryos (not at the hatched blastocyst stage). For titration of virus from such embryos, these authors used bovine turbinate cells in microtiter plates.

In the present study, the possibility remains that either the virus was not released and remained in the embryonic cells, or that the virus was present but remained below detectable levels. It is not possible to answer this question in the present study because of the short term culture system. Longer term culture periods are necessary. This may involve the transfer of infected blastocyst bovine embryos to suitable pseudo-pregnant mothers.

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