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An Immunohistochemical Study of Viral Antigen in Aborted Fetuses Naturally Infected by Bovine Viral Diarrhea Virus

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Abstract : The tissue distribution and cellular localization of viral antigen in the brain of aborted fetus with bovine viral diarrhea virus(BVDV) infection was studied. BVDV antigens was detected in spleen, kidney, lung, eyelid as well as brain. In the brain, the virus was recognized in neurons and non-neuronal cells in the cerebellum and cerebrum. Many cells in the superficial layer and occasional Purkinje cells had BVDV antigens. As well, BVDV was also found in the perivascular cells, vascular endothelial cells and smooth muscle cells in the vessels and neuroglial cells in the white matter. This finding suggests that BVD virus favors infect progenitor cells in the brain, notably in the superficial layer of cerebellum, and damage normal development of cerebellum, which leads to cerebellar hypoplasia.

Key words: bovine viral diarrhea virus, fetus, abortion

Introduction

Bovine viral diarrhea virus (BVDV) is a positive-sense single-strand RNA virus, as the other pestiviruses, including border disease virus, and classical swine fever virus. BVDV is one of the most important viral pathogens of cattle and its control and prevention are of worldwide concern.[1] BVDV infection has been associated with enteric disease, mucosal disease,[2,3] diabetes[4] as well as reproductive failure.[5-8] The reproductive effects of BVDV infection include early embryo loss, abortion, and congenital defects.[5,6] Several studies have shown that a variety of organs including lymph nodes, spleen and liver are preferred sites of viral replication in fetuses and adult cattle.[2,3]

Viruses from several genera and families are able to induce apoptosis (programmed cell death) in infected cells. Cells undergoing apoptosis show certain morphological changes (cell shrinkage, pronounced cell surface swelling, and chromatin condensation) and DNA fragmenation.[9] BVDV is an example of viruses that induce apoptosis of infected cells in vitro.[10-12]

Cerebellar hypoplasia is one of the prominent features of neurotropic virus infections, including BVDV infection in the fetus.[6] Apoptotic cell loss in external granular cell layer is one of the important features of normal development of cerebellum.[13] Excessive apoptosis occurs in the progenitor cells in the external granular cell layer of the cerebellum of weaver mice.[14] Taking these into consideration, it is worthwhile to examine the relationship between BVDV infection in the fetus and cerebellar malformation.

The aim of this study is to analyze the cell phenotype of BVD virus infection in the fetal cerebellum aborted by BVDV infection, and to find extra-neuronal tissue for the diagnosis.

Materials and Methods

Case history

BVDV positive fetuses were reviewed from the medical records at the Pennsylvania Veterinary Laboratory, Harrisburg, PA. Fetuses were 4 to 7 months of gestation. Diagnosis of BVDV was based on the presence of BVDV antigens by immunohistochemistry with BVDV antisera. The fetuses had been examined, and selected tissues including cerebrum, cerebellum, pons, intestines, liver, kidney, adrenal gland, lung, heart, skeletal muscle and other tissues were fixed in 10% buffered formalin and processed for paraffin embedding. Five micron sections were stained with hematoxylin and eosin. Selected sections were used for immunohistochemical staining of BVDV.

Immunohistochemistry

Monoclonal antisera to BVDV (15.c.5, ascites) was used in this study. It was supplied by Dr. Edward Dubovi, New York State College of Veterinary Medicine, Veterinary Diagnostic Laboratory, Cornell University, Ithaca, N.Y. Immunohistochemical staining was done with semiautomated

capillary system (Microprobe® staining system, Fisher Biotech, Fisher Scientific, St. Louis, MO.) using the Mouse Histostain Plus Kit (Zymed Laboratory Inc., San Francisco, CA). In brief, deparaffinized sections were blocked with 3% hydrogen peroxide in distilled water for 15 min., and then treated with 0.05% proteasec in phosphate buffered saline (PBS). After washing with PBS, sections were reacted sequentially with normal blocking sera and primary antisera (diluted in 1:1000) for 60 min. Then, biotinylated secondary antisera and streptavidin-peroxidase were applied according to manufacturers recommendations. All of the reactions were done in a humid chamber at 36°C. For the negative control, normal mouse serum(Zymed) substituted for primary antiserum. After finishing color development, sections were counterstained with hematoxylin and mounted with Clearmount.

DNA fragmentation was detected by *in situ* nick end-labeling, as described in the manufacturers instructions (Intergen, Purchase, NY). In brief, the paraffin sections were deparaffinized, rehydrated, and washed in PBS. The sections were treated with 0.02% proteinase K (Sigma) for 15 min at room temperature, blocked with 3% hydrogen peroxide in PBS for 10 min, and washed with PBS. Tissue was incubated with equilibration buffer for 5 min and reacted with TdT enzyme for 60 min at 37°C. Then, the reaction was stopped in stop buffer for 15 min. Finally, sections were reacted with peroxidase-labeled anti-digoxigenin antibody for 60 min. Positive cells were visualized using a diaminobenzidine substrate kit and counterstained with hematoxylin.

Results

Table 1 describes the case history and gross lesions of fetuses naturally aborted by the infection of BVDV. The cerebellar hypoplasia was prominent in the late abortion (6 to 7 months of gestation), but was not evident in early abortion.

Table 1. Case description of bovine viral diarrhea virus associated abortion

Case	Breed	Stage of gestation (mo)	Lesions
1	Holstein	4	mild placentitis
2	Holstein	5	placentitis
3	Holstein	6	necrotizing placentitis
4	Brown Swiss	6	endocarditis, cerebellar hypoplasia
5	Holstein	7	encephalitis, adrenalitis, nephritis
6	Holstein	7	encephalitis
7	Holstein	not known	cerebellar hypoplasia, hydrocephalus

By immunohistochemisty, the neurons in the cerebral cortex showed cytoplasmic localization of viral antigens in perikarya and neuronal processes(Fig. 1). Some cells in the choroid plexus were also positive for BVDV. The perivascular cuffing was occasionally found in the cerebrum, but viral antigens were consistently localized in the perivascular cells, and vascular endothelial cells (Fig. 1).

In the cerebellum which shows cerebellar disorganization (Fig. 2), virus antigens were localized in occasional Purkinje cells, some basket cells and especially in the small granular cells lining the superficial layer (Fig. 3). The virus loaded cells were occasionally lined with Bergmann glial process. In the white matter, the virus was found in a variety of neuroglial cells (Fig. 3). By TUNEL reaction, moderate number of TUNEL-positive cells were recognized in the superficial layer of cerebellum (Fig. 4).

In non-neural organs, oval shape BVDV positive cells were found in liver, spleen, lung, heart, kidney, adrenal gland, esophagus, placenta and eyelid. In the walls of arteries of all organs examined, BVDV was consistently found in the smooth muscle cells. Occasional vascular endothelial cells were positive for BVDV in the fetus. More importantly, BVDV was identified in squamous epithelium of the skin of eyelid, to less extent of esophagus. The immunoreactivity of BVDV in eyelid sample was most prominent and comparable to that of brain sections.

In the control slides of adjacent serial sections treated with normal mouse sera, no immunoreaction was identified. As well, bovine tissues with non-BVDV infection were negative when BVDV antisera was applied with the same protocol.

Discussion

In the present study, we found that BVDV favors brain cells including neurons, especially in proliferating cells in the superficial layer of cerebellum.

During cerebellar development, small granular cells in the superficial layer are important to organize the granular cell layer because the progenitor cells in the superficial cell layer are selected to either migrate to the former region or to be eliminated through apoptosis.[13] Apoptosis, in other words programmed cell death, is one of normal physiological cell selection mechanism in developing brain. In BVDV infection, we suppose that a large number of progenitor cells are

infected with BVDV, and the affected cells undergo apoptosis, so that many more cells are affected than in the physiological apoptosis of normal cerebellar development. Cerebellar hypoplaia has been associated with the decreased cell number because of loss of a large number of cells. We postulate that BVDV infection in the fetus has an intimate relationship of cerebellar hypoplasia through the apoptosis of progenitor cells in the external granular cell layer during development, although cerebellar development is completed at the early stage of gestation. This finding may be supported by the finding that BVDV induces apoptosis of infected cells in tissue cultures.[12] We also found eyelid as a site of tissue sampling for virus diagnosis in BVDV infections. It is an alternative extra-neuronal site for viral diagnosis as well as lymphatic tissues including spleen. It may be useful to screen for BVDV infection in newborn calf. We suppose that tissues of ectodermal origin are preferable sites for virus infection.

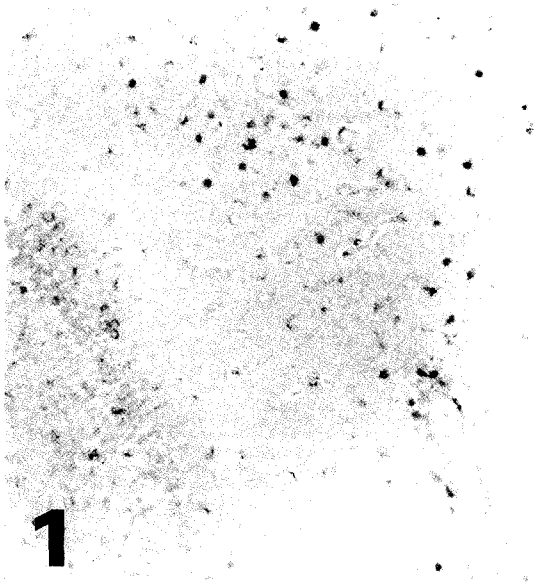

Fig. 1. Cerebrum of bovine fetus(case 4), showing immunostaining of BVDV in neurons neuroglial cells and perivascular cells. Counterstained with hematoxylin. Magnification, X132.

Fig. 2. Cerebellum of bovine fetus(case 5), H-E staining. X66.

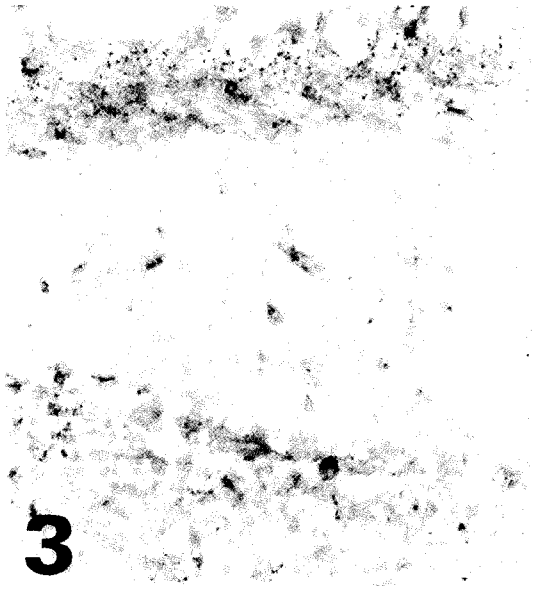

Fig. 3. Immunostaining for BVD virus in external granular cell layer. Counterstained with hematoxylin. Magnification, X132.

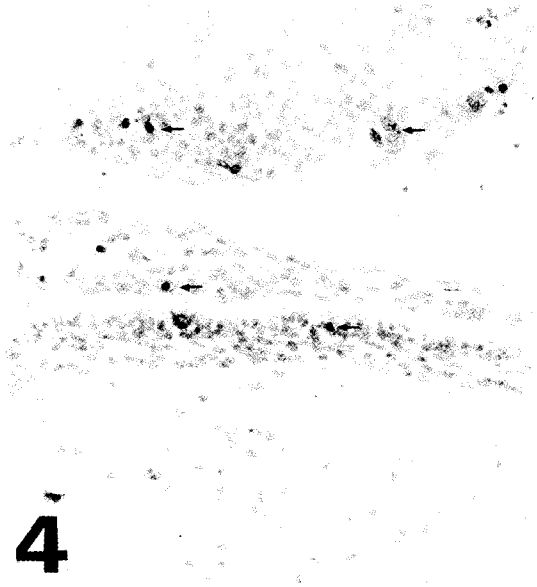

Fig. 4. TUNEL reaction of cerebellum. Some TUNEL-positive cells were seen in the external granular cell layer. Counterstained with hematoxylin. X132.

The supposition is supported in the present study, where both cerebral cortex and eyelid skin, which share same embryological origin, show viral infection by immunohistochemistry.

Taken all into consideration, we suggest that cerebellar hypoplasia in BVDV infection is associated with the early cell damage (probably apoptosis) in the superficial cell layer of cerebellum, and that eyelid may be serve as an extraneuronal site for BVDV diagnosis.

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