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Viral Antigen Distribution in the Central Nervous System of Cattle Persistently Infected with Bovine Viral Diarrhea Virus

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Abstract. Distribution of viral antigens in the central nervous system of 25 cattle with a persistent bovine viral diarrhea virus (BVDV) infection was studied. Using a polyclonal antiserum produced in pigs and the direct immunofluorescence and immunoperoxidase technique, BVDV antigen was located exclusively in neurons. Predilection sites for viral persistence were cerebral cortex and hippocampus; in other areas of brain and spinal cord, viral antigens were in single neurons or small groups of neurons. There was no morphological evidence of cellular alteration due to viral persistence. Perivascular lymphocytic infiltrations were in affected nervous tissue. It is concluded that the central nervous system is an important location for persistence of BVDV.

The occurrence of clinically inapparent and persistent forms of bovine viral diarrhea virus (BVDV) infection has been reported.[3,10,12,13,20–23,29,33] Cattle persistently infected with BVDV may appear healthy or may fail to thrive. They are permanently viremic and lack detectable levels of neutralizing antibodies to BVDV. Persistent BVDV infection can be induced experimentally by infecting the pregnant seronegative dam or the fetus directly with non-cytopathic strains of BVDV before gestation day 125.[9,11,22,24] Furthermore, the offspring of persistently infected cows frequently are born with persistent BVDV infection.[8,20,23,33] BVDV, currently classified as a member of the *Pestivirus* genus of the non-arbo togaviruses,[34] has a marked tropism for lymphoid and certain epithelial tissues, especially the mucosae of the digestive tract.[2,4–7] However, in cattle persistently infected with non-cytopathic BVDV, established by fetal infection during the first 4 months of gestation, BVDV may replicate in certain non-lymphoid and non-epithelial tissues including the central nervous system (CNS).[11,13,20,21,24,25]

In the present study we have examined the distribution of BVDV antigen in the CNS of persistently infected cattle applying immunofluorescence and immunoperoxidase techniques.

Materials and Methods

A total of 25 Holstein-Friesian cattle persistently infected with bovine viral diarrhea virus (BVDV) were used in this immunohistological study; animals were detected in epidemiological studies. Most had growth retardation and sporadic diarrhea. Neurological signs were not recorded. At the time of euthanasia the animals were between 5 and 34 months old, with a mean of 20 months. Over the life-span the animals were viremic; that is, non-cytopathic BVDV was repeatedly demonstrated on peripheral blood leucocytes using techniques described elsewhere.[27] During an observation time of many months the animals failed to produce neutralizing antibodies applying the microtiter neutralization test and BVD test virus strain A 1138/69.[14,16,27]

Animals were euthanized, a complete necropsy done, and the following tissues, among others, collected for immunohistological and virological examination: a total of 18 locations of the brain and spinal cord, trigeminal ganglia, pituitary gland, and eyes. Tissue samples were snap-frozen in liquid nitrogen and stored at −70 C until cryosectioning. Adjacent samples of the same locations were fixed in cold ethanol for 24 hr and then embedded in paraffin according to the method of Sainte-Marie.[30] In addition, the same locations were fixed in 5% neutral buffered formalin for 48 hr and then embedded in paraffin. Cryostat and paraffin sections of ethanol and formalin-fixed tissues were used for immunofluorescence and immunoperoxidase techniques. Hematoxylin and eosin (HE) stains were prepared from formalin-fixed tissues.

A polyclonal antibody against pestivirus was raised in pigs as described elsewhere.[16] Briefly, experimental pigs were immunized with BVDV strain OSLOSS/2498 and subsequently challenged with virulent hog cholera virus, strain Alfort. Neutralizing titers of the hyperimmune sera ranged between 1:2,000 and 1:6,000 when measured against the homologous BVDV strain.

For the conjugation with fluorescein isothiocyanate and horseradish peroxidase (PO), respectively, the IgG fraction of the hyperimmune serum was isolated by ammonium sulfate precipitation according to standard techniques. After desalting by dialysis and sephadex G 25 chromatography the purified IgG fraction was conjugated with fluorescein isothiocyanate (Isomer 1, BDH Chemicals, England). Further purification of the conjugated antibody was done by ion ex-

change chromatography on DEAE cellulose ss (Serva, Feinbiochemica, Heidelberg, West Germany). The chromatographic fractions used in the direct immunofluorescence method were eluted with 0.05 M and 0.1 M phosphate buffer. To increase contrast, sections were stained for 4 min in Evans blue (diluted 1/10,000 in phosphate buffered saline [PBS]).

Purified IgG from the anti-pestivirus hyperimmune serum was conjugated with horseradish PO Type VII (Sigma Chemicals, Munich, West Germany). After determination of the optimal working dilution by titration, the PO-labeled antiserum was used in the direct immunoperoxidase method. On deparaffinized 4-μm tissue sections of ethanol and formalin-fixed tissues, endogenous PO was inhibited by pre-incubating with methanol-H_2O_2, followed by three rinses in PBS. Sections were then incubated for 15 min in a blocking solution consisting of normal swine serum diluted 1/5 PBS for 30 min at 37 C with the PO-labeled swine IgG anti-pestivirus at a 1/20 dilution in PBS. The substrate for PO, 3,3-diaminobenzidinetetrahydrochloride (DAB, Sigma Chemical Co., Munich, West Germany) was prepared immediately before use and consisted of 0.05% DAB in PBS with 0.015% H_2O_2. After rinsing, the slides were counterstained with hematoxylin, washed with tap water, and then mounted with Immunomount (Shandon Co.).

Tissues that had been fixed in formalin for several days were treated for 60 min with 0.25% trypsin (Fluka Co., Buchs, Switzerland), pH 7.6, at 37 C.

The number of BVDV antigen-containing neurons in different central nervous system (CNS) areas were evaluated as: (+) single neurons, (++) 50% of neurons, and (+++) >90% of neurons immunostaining. The evaluation of BVDV antigen distribution was based on detailed descriptions of the microscopic anatomy of the bovine CNS.[1,15,31,35]

Results

Applying both direct immunofluorescence (IF) and immunoperoxidase (IP) techniques, various types of neurons in the central nervous system (CNS) were the target cells in persistent bovine viral diarrhea virus (BVDV) infections. This was indicated by IF, but IP staining on ethanol and formalin-fixed tissues more precisely showed that only neurons contained BVDV antigen, whereas the other cellular elements such as oligodendrocytes, astrocytes, microgliocytes, ependymal, and vascular cells were not stained specifically. In neurons, viral antigen was present either in a limited, often cap-shaped area in the cytoplasm at one side of the nucleus or in the entire perikaryon, while the nucleus invariably did not contain antigen (Fig. 1). Depending on the location and type of neuron, viral antigen could also be detected in dendrites and axons. Occasionally, antigen-containing cellular processes could be followed over long distances as they passed through the neuropil.

Cerebrum

For the immunohistological evaluation, tissue samples were taken from the frontal, temporal, parietal, and occipital cerebral cortex. Except for the molecular layer, neurons of all other layers (i.e., the superficial and deep granular cell layer, the superficial and deep pyramidal cell layer, and the polymorphous cell layer) contained viral antigen. In the most severe cases more than 90% of the neurons were stained. A characteristic staining pattern was seen in the superficial pyramidal cell layer, where in infected neurons the entire perikaryon and the main dendrite contained antigen. Thus, BVDV antigen-positive neurons accentuated the pyramid-shaped cells of this layer with the apical dendrite as top of the pyramid directed to the pia mater and the base to the deeper layers of the cerebral cortex (Figs. 2, 3). Viral antigen was not seen in the leptomeninges.

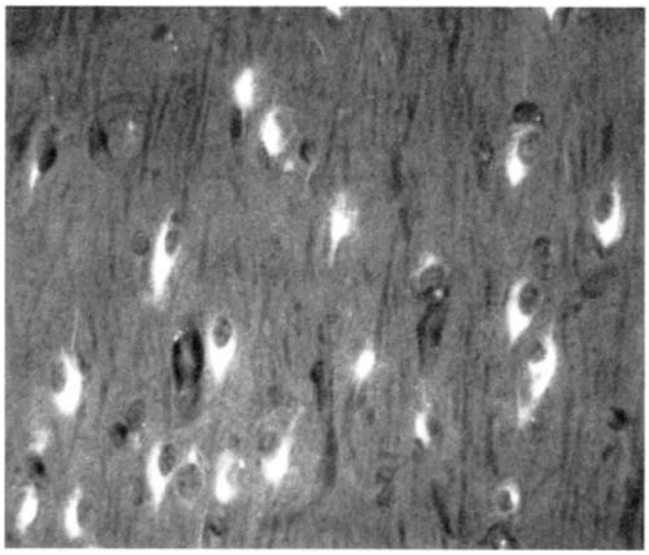

Fig. 1. Occipital cerebral cortex, 12-month-old heifer (13.963). Pyramidal cells show viral antigen-specific fluorescence. FITC-labeled pig anti-bovine viral diarrhea virus.

Hippocampus

Neurons of the layers of polymorphous cells, pyramidal cells, and molecular cells contained BVDV antigen (Figs. 4, 5). In the pyramidal cell layer many of the dendritic and axonal processes showed intense viral-antigen-specific staining.

Diencephalon and Mesencephalon

In areas of the diencephalon such as thalamus, hypothalamus, and nuclei, only individual cells or groups of neurons showed viral-antigen-specific staining (Fig. 6). Similarly, in the corpus quadrigeminus, nucleus ruber and niger of the mesencephalon specific staining was limited to small groups of neurons.

Rhombencephalon

In sections of the pons, anterior and posterior parts of the medulla oblongata, BVDV-antigen was detect-

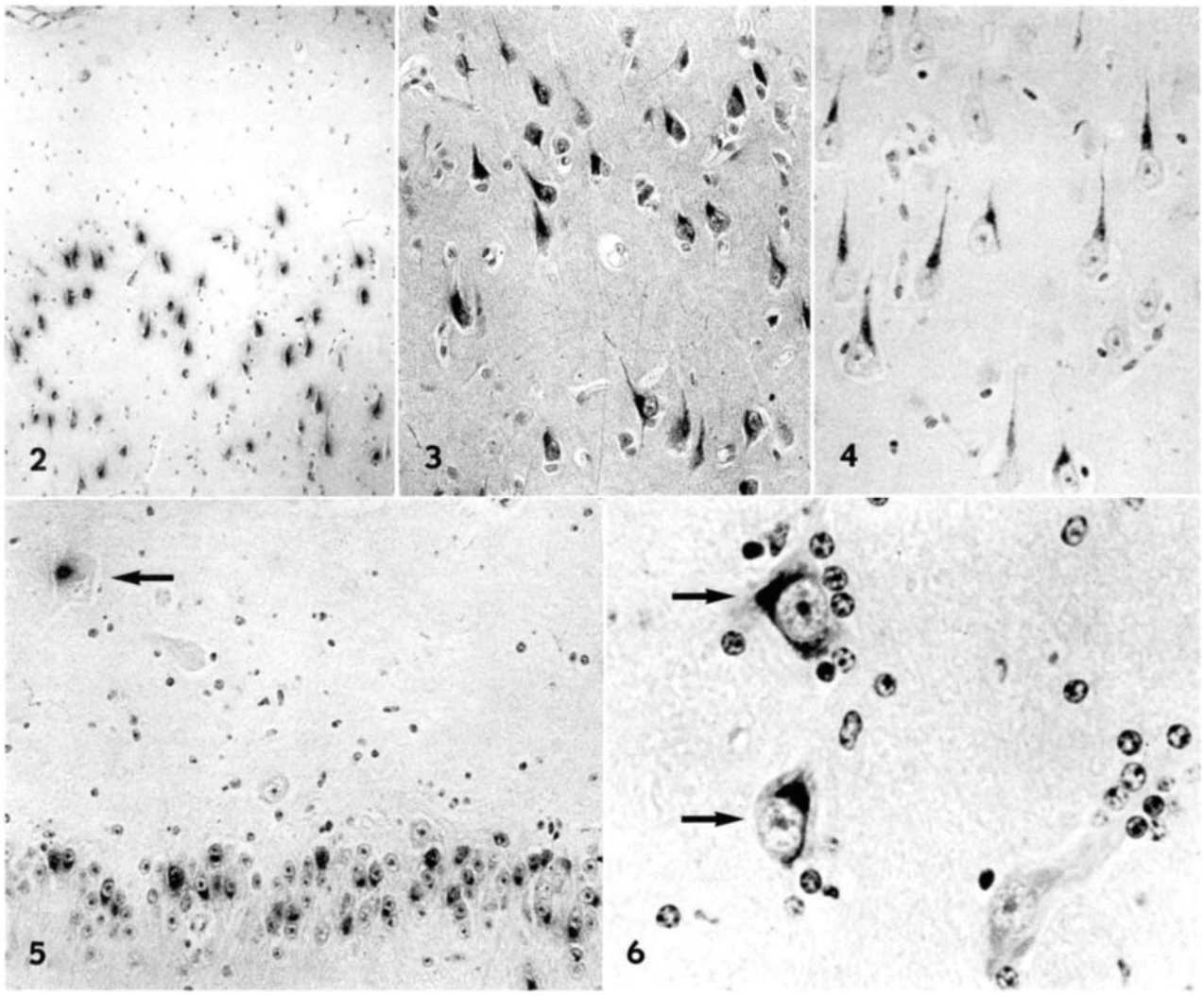

Fig. 2. Parietal cerebral cortex, 21-month-old cow (15.153). Pia mater and molecular layer are negative. Majority of neurons in granular and pyramidal cell layer contain antigen. Pig anti-bovine viral diarrhea virus-peroxidase.

Fig. 3. Parietal cerebral cortex, 21-month-old cow (15.634). Intense antigen-specific staining of perikaryon, cellular processes of neurons. Pig anti-bovine viral diarrhea virus-peroxidase.

Fig. 4. Hippocampus, 12-month-old heifer (15.323). Triangle-shaped and ovoid cells of polymorphous cell layer contain viral antigen. Pig anti-bovine viral diarrhea virus-peroxidase.

Fig. 5. Dentate gyrus of hippocampus, 21-month-old cow (15.158) with antigen-specific staining of many granular cells and a single polymorphous cell (arrow). Pig anti-bovine viral diarrhea virus-peroxidase.

Fig. 6. Thalamus, 15-month-old heifer (15.236). Two neurons show viral antigen-specific staining (arrows), one neuron is antigen-negative. Mild satellitosis. Pig anti-bovine viral diarrhea virus-peroxidase.

able in neurons of the nuclei. Like in other areas of the brain astrocytes, oligodendrocytes, microglial cells, and ependymal cells of the fourth ventricle did not stain specifically.

Cerebellum

The leptomeninges covering the cerebellum, the molecular layer, and the large neurons of the Purkinje cell layer did not stain with the peroxidase (PO) labeled antibody. Furthermore, none of the small nerve cells of the granular cell layer contained BVDV antigen. However, scattered nerve cells, larger than the typical granular cell and having a small rim of cytoplasm, contained antigen. These were the so-called Lugaro cells or horizontal fusiform cells[28] located in the outermost granular layer immediately beneath the Purkinje cells (Fig. 7). Another cell type, selectively staining for BVDV antigen, was the large Golgi cell in the upper half and the small Golgi cell located in the deeper half of the granular layer (Fig. 8). Occasionally, two or three small positive Golgi cells were clustered together.

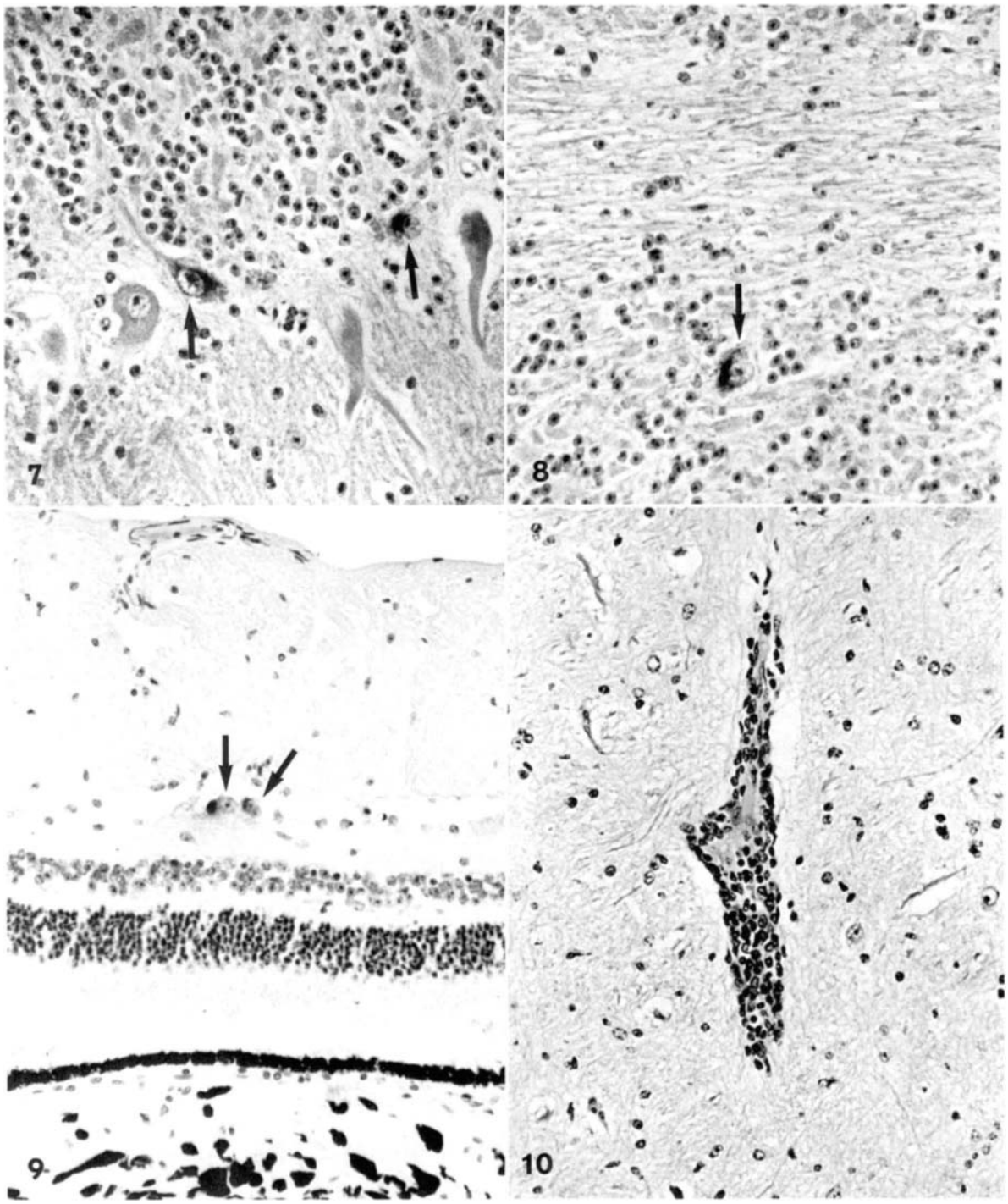

Fig. 7. Cerebellum, 18-month-old heifer (15.903). Two viral antigen-positive Lugaro cells (arrows). Pig anti-bovine viral diarrhea virus-peroxidase.

Fig. 8. Cerebellum, 18-month-old heifer (15.904). A single small Golgi cell in the deep granular layer contains bovine viral diarrhea virus (BVDV) antigen (arrow). Pig anti-BVDV-peroxidase.

Fig. 9. Eye, 21-month-old cow (15.166). Two antigen-positive neurons in the ganglion cell layer of the retina (arrows). Pig anti-bovine viral diarrhea virus-peroxidase.

Fig. 10. Corpus quadrigeminus, 18-month-old heifer. Perivascular lymphocytic infiltration. HE.

Table 1. Distribution of bovine viral diarrhea virus antigen in the central nervous system, pituitary gland, and eyes.

Topography	Number of Antigen-containing Neurons*
Cerebral cortex, frontal	+++
temporal	+++
parietal	+++
occipital	+++
Olfactory lobe	+++
Olfactory bulb	−
Hippocampus	+++
Thalamus, hypothalamus	++
Optic chiasm	−
Corpus quadrigeminus	+
Pons	+
Medulla, anterior and posterior	+
Ganglion N. trigeminus	−
Cerebellum	+
Spinal cord	+
Pituitary	−
Eyes	+

* + = single neurons immunostaining. ++ = 50% of neurons immunostaining. +++ = 90% of neurons immunostaining.

Spinal cord

Segments of the cervical, thoracic, and lumbar spinal cord were examined with the IP method. In the majority of cases, single neurons in the dorsal and ventral column contained BVDV antigen.

Ganglion of trigeminus nerve

When applying the IP method, neurons and nerve fibers did not stain specifically.

Pituitary gland

BVDV antigen was not detectable in the neuro- or adenohypophysis.

Eyes

BVDV antigen-specific staining was limited to scattered cells in the layer of neurons (Fig. 9), whereas all other cellular elements of the different layers did not stain.

From the topography of neurons persistently infected with BVDV predilection sites for virus persistence are the cerebral cortex and the hippocampus, whereas in the other areas viral antigen is present in single neurons or small groups of neurons only (Table 1).

Paraffin sections stained with hematoxylin and eosin (HE) and others with Luxol fast blue were examined for the presence of morphological abnormalities and evidence of a cellular immune response. The only significant lesions were occasional perivascular infiltrations in the white matter of the brain, composed of lymphocytes and few macrophages. BVDV antigen was not detected in these infiltrations (Fig. 10).

Focal satellitosis around degenerating neurons, as described by others,[13] was seen in the cerebral cortex of some animals. Satellitosis occurred around infected and non-infected neurons and is also present in control brains of cattle. Thus, its significance remains uncertain.

Discussion

Persistent bovine viral diarrhea virus (BVDV) infections may occur if the bovine fetus is transplacentally infected with non-cytopathic BVDV at a time when he is still immunologically immature; that is, before day 125 of gestation. The results of the present immunohistological study indicate that in the central nervous system (CNS) of persistently infected young and adult cattle, neurons are the most important target cells. Thus, it appears that in the CNS, BVDV has a selective tropism for the cell population of neurons. It could be assumed that only these permissive target cells have specific virus receptor sites accessible on their cytoplasmic membrane.[17]

Our findings confirm and expand earlier reports on viremic BVDV-infected cattle in which BVDV antigen in neurons of the cerebral cortex[13,20,24,25] and hippocampus[25] was demonstrated by immunofluorescence techniques. While the predilection sites for virus persistence obviously are the cerebral cortex and the hippocampus, in other areas viral antigen can be detected in single neurons or small groups of neurons only.

In the cerebellum the large neuronal population of Purkinje cells does not express viral antigens, whereas neurons scattered in the granular cell layer, i.e., Lugaro and small and large Golgi cells contain BVDV antigen. One possible explanation may be the presence of Purkinje cells in the still immature cerebellum at the time of infection of the fetus. The time sequence of cerebellar development in the bovine fetus has been thoroughly studied.[19] While the external and internal granular cell layer and a narrow molecular layer are differentiated at day 60, the first immature Purkinje cells do not appear until day 90 of gestation. It is not before day 180 that the differentiation of Purkinje cells is complete. Thus, at the time of infection prior to day 120 of gestation no (or only very few) Purkinje cells may be available for viral infection.

Purkinje cells may also have a receptor for BVDV. In one of our cases (a 2-week-old viremic Holstein-Friesian calf with moderate cerebellar hypoplasia associated with porencephalic cysts) many Purkinje cells contained BVDV antigen, both in the perikaryon and dendrites (unpublished observation). In that case, it may be assumed that BVDV infection occurred in a

later phase of cerebellar development when sufficient numbers of Purkinje cells were available.

BVDV infection of neurons does not cause obvious morphological alterations or cellular destruction. Whether electron microscopy would reveal more subtle cellular changes is unknown. No information is yet available whether BVDV interferes with the function of neurons without causing morphological alterations. This could be tested by demonstrating a decrease in enzymes specific for neurons, i.e., enolase and the neurotransmitter enzymes choline acetyltransferase and acetylcholine esterase. BVDV may shorten the lifespan of neurons, an obviously important pathogenic pathway in this non-renewing cell population in the CNS.[26]

In addition, the localization of BVDV antigen in the cerebral cortex, including the olfactory lobe, hippocampus, and other areas of the brain belonging to the limbic system (i.e., dentate gyrus, mamillary bodies) suggests that behavioral alterations might be expected in cattle persistently infected with BVDV. Recently, we observed a case of persistent BVDV infection in a 2-year-old Holstein-Friesian cow which showed definite behavioral alterations.[32] The behavioral findings in this heifer correlated well with the demonstration of BVDV antigen in high numbers of neurons belonging to the limbic system.

Primary infection of the CNS with BVDV most likely occurs across the blood-brain barrier. This assumption is supported by experimental studies in mice in which after intravenous inoculation of vast quantities of togavirus an almost immediate virus replication in the CNS was observed.[18] It is conceivable that in persistently BVDV-infected cattle viremia is of sufficient magnitude and duration to allow CNS invasion across the blood-brain barrier and possibly the blood-cerebrospinal fluid barrier.

At present, how the virus spreads within the CNS cannot be explained, i.e., how the vast number of neurons in the cerebral cortex and hippocampus are infected. In experimental mice certain togaviruses have been demonstrated within intercellular gaps of the neuropil, suggesting that these viruses may move through narrow gaps between cells and cellular processes.[18] However, there are several other possibilities of how virus spreads in the CNS to reach susceptible cells (i.e., cell-to-cell transport, transit via glial processes, or transport along extensive axonal and dendritic ramifications of neurons).[18] In none of our cases of persistent BVDV infection of the brain were antigen-containing ependymal cells seen. Thus, the spread of BVDV through cerebrospinal fluid pathways appears unlikely.

The failure of virus clearance by immunological mechanisms from the CNS in persistent BVD may be explained by the general fact that the CNS is to some extent protected from immune defenses and thus is a good site for persistent infections.[26] Furthermore, virus clearance may not be possible in a state of immunological tolerance acquired during ontogenesis in which neutralizing antibodies and immune cells are not produced against a specific BVDV strain.

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