Bovine neonatal encephalomyelitis associated with a *Neospora* sp. protozoan

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Recently, a protozoan resembling the canine parasite *Neospora caninum* (1,2) has been reported in several countries in association with bovine abortion and neonatal disease (3). In particular, among drylot dairy cattle in California and elsewhere, this organism seems to be associated with a significant number of abortions (4–6). Such protozoa have only rarely been identified as a cause of abortion in beef cattle (7), or as a cause of bovine congenital encephalitic disease (8–12). This report describes a case of congenital encephalomyelitis in a beef calf in western Canada involving organisms resembling *N. caninum*.

In February 1991, a live three-day-old Salers calf was submitted to the Airdrie Regional Veterinary Laboratory, Airdrie, Alberta, from a herd in which no other losses had occurred and out of a dam who appeared normal. The calf was small, weighing 19.5 kg compared to an average weight for Salers calves of 40.0 kg, with a reduced skeletal muscle mass. It was moribund. The eyes were deviated ventromedially, and there was a palpebral but no menace reflex. The forelimbs were held in extension and could be flexed with manual pressure; there was no withdrawal reflex. The hind limbs were held in flexion and periodically paddled uncontrollably. The withdrawal reflex was intact. The calf was killed with an intravenous injection of barbiturate and immediately necropsied.

There was an "S" shaped lateral deviation of the vertebral column between the eighth thoracic and third lumbar vertebrae (scoliosis). The spinal cord in this area was malformed with the cranial portion being markedly reduced in diameter and ventromedially flattened. Caudal to the scoliosis, the spinal cord gradually resumed its normal shape and size. Tissues, including samples from the central nervous system (CNS) and major thoracic and abdominal organs, were fixed in 10% neutral buffered formalin and processed for standard paraffin embedding, prior to sectioning and staining with hematoxylin and eosin. Lesions were confined to the CNS and were characterized by perivascular cuffing and randomly distributed areas of status spongiosus and glial proliferation in both grey and white matter throughout the brain and spinal cord. Lesions were more severe within the medulla oblongata and malformed section of the thoracic cord, in which variable numbers of macrophages, ceroid-laden "gitter cells", lymphocytes, and plasma cells were found within perivascular spaces often extending into the adjacent neuropil. In addition, locally extensive areas of malacia were present in the ventral horns and ventral funiculi of the grossly affected spinal cord. Less severe foci of inflammation were scattered throughout the meninges. Slightly ovoid protozoan cysts (approximately 40 μm in diameter) characterized by a thick (approximately 2 μm) eosinophilic wall and containing up to 15 crescent-shaped bradyzoites were occasionally seen in the spinal cord. The cysts were inconsistently associated with inflammation and usually unassociated with a host cell, although one cyst was clearly intraneuronal. The neuron was angular with the cyst occupying most of the visible cytoplasm (Figure 1a).

A 30 g portion of cerebral cortex was collected, placed in a plastic bag, and stored in a freezer at −52°C. The tissue was stored for approximately four months at the Airdrie Veterinary Laboratory and then transported to Saskatoon on dry ice, ensuring that the tissues remained frozen. There a portion of the central nervous tissue was thawed to room temperature and homogenized in a blender. The homogenate was resuspended in Percoll (Pharmacia LKB, Uppsala, Sweden) by mixing two parts of isotonic Percoll stock solution and one part homogenate, and centrifuged in a swing-out rotor at 1160 *g* for 20 minutes at room temperature. The sediment was washed twice in sterile saline and inoculated subcutaneously into four-week-old CRL:CD1(ICR)BR mice (Charles River Canada, St. Constant, Quebec). Mice were sacrificed 28 days postinoculation. Light and electron microscopical examination of the lungs demonstrated typical apicomplexan organelles in tachyzoites.

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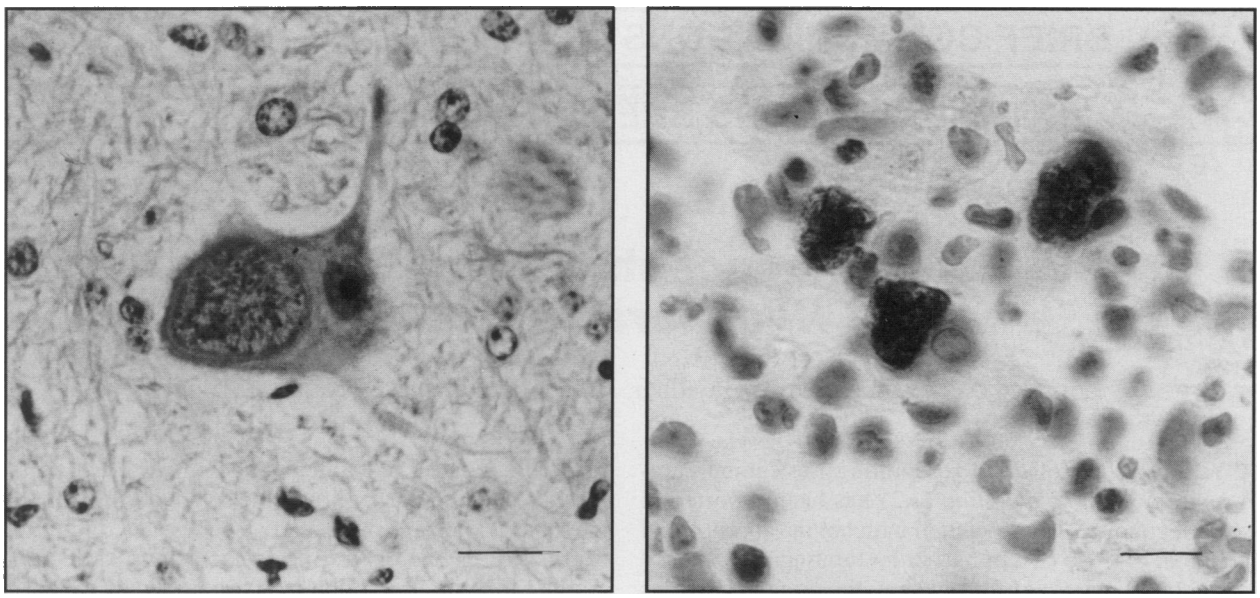

Figure 1. a) Spinal cord tissue from three-day-old Saler calf with congenital encephalomyelitis showing a neuron with cytoplasmic protozoan cyst moderately stained with antisera to *Neospora caninum*. b) Lung tissue from mouse experimentally innoculated with bovine protozoa showing protozoan tachyzoites intensely stained with antisera to *Neospora caninum*. Avidin biotin complex immunoperoxidase stain, hematoxylin counterstain, formalin-fixed, paraffin-embedded tissue sections. (bar = 25 μm).

Serial sections of formalin-fixed, paraffin-embedded, tissues from the affected calf and from experimentally-infected mice were tested immunohistochemically with rabbit polyclonal antisera to *N. caninum* (13), *Toxoplasma gondii* (Biogenex Laboratories, San Ramon, California, USA), and bovine *Sarcocystis* spp. (Dr. M. Jeffrey, Central Veterinary Laboratory, Weybridge, England), using a modification of the avidin-biotin complex technique (14). Positive controls for *N. caninum* immunohistochemical staining were sections of cerebrum from a dog from LaRonge, Saskatchewan. The dog died in 1987 with central nervous system disease, retrospectively diagnosed as being associated with *N. caninum* (1,2). Positive controls for *T. gondii* immunohistochemical staining were sections from a neonatal kitten with disseminated cysts and tachyzoites. Positive controls for staining for *Sarcocystis* spp. were sections of bovine heart muscle containing large numbers of typical *S. cruzi* and *S. hirsuta* cysts. When antisera were used at low dilutions, antisera to *N. caninum* and *T. gondii* were cross-reactive to the heterologous as well as to homologous organisms. Serial dilution of antisera were titrated on tissues containing homologous and heterologous organisms to determine dilutions at which there was dark staining of the homologous organism and no apparent cross-reactive immunostaining. The dilutions of antisera used were: *N. caninum* 1/10,000, 1/20,000, *T. gondii* 1/1,600, 1/3,200, *Sarcocystis* 1/2,000 and 1/4,000. Negative controls for immunohistochemical stains were serial sections of case and control tissues stained with an irrelevant rabbit antiserum at similar dilutions.

Tissues from the affected calf containing obvious protozoan cysts were stained moderately positive (Figure 1a) with antiserum to *N. caninum* but were unstained with antiserum to *T. gondii* and *Sarcocystis* spp., or with irrelevant rabbit antiserum at antisera dilutions that produced intense, noncross-reactive, immunostaining in corresponding positive control tissues. There was no staining of structures other than obvious cysts. While immunostaining of cysts in the affected calf was only of moderate intensity, tachyzoites in tissues from a mouse experimentally infected with the bovine protozoa displayed dark, intense staining with *N. caninum* antiserum (Figure 1b) and were unstained with the *T. gondii* and *Sarcocystis* antisera.

The clinical, histological, and immunohistochemical findings in this case are consistent with previous descriptions of *Neospora*-associated neonatal encephalomyelitis in calves (8–10). There were severe tissue lesions and several readily apparent large cysts in the present case, in contrast with the findings in bovine abortions associated with *Neospora* sp. in which, usually, only a rare, small tissue-cyst or small cluster of tachyzoites has been found (6). On light microscopy, the feature of this protozoan that is similar to that of *N. caninum* is the thick cyst wall without septa.

The diagnosis of *Neospora* sp. in most clinical cases has been aided by immunohistochemical staining with polyclonal antisera. However, just as *Neospora* has been shown to share morphological features with similar protozoa, particularly *T. gondii*, antisera to other protozoa including *T. gondii* and *Hammondia hammondi* have been shown to be at least partially cross-reactive to *Neospora sp.* in cattle (10). In the present study, antisera to *N. caninum* and *T. gondii* were partially cross-reactive to *T. gondii* and to *N. caninum*; however specific staining could be achieved by using high dilutions of antisera. In the immunohistochemical staining in the present case, when the polyclonal antisera were diluted to yield specific immunostaining, only the antisera to *N. caninum* stained the tissues of the affected calf; however, this staining was much less intense than the staining of *N. caninum* in infected canine tissues. This finding is similar to that of previous studies (10), in which the protozoan organism in bovine tissues was reported to have

differing immunohistochemical staining properties compared to *N. caninum* in canine tissues. However, in the present case, it was shown that the bovine parasite innoculated into mice produced tachzoites with intense immunohistochemical staining identical to that of *N. caninum* in canine tissues. This finding, and recent *in vitro* studies (15), suggests that the apparent antigenic differences between the organism in cattle and in dogs may relate to the stage or growth conditions of the parasite rather than to differences in the organism *per se*.

To our knowledge, this was the first known instance of infection of cattle with the neosporan protozoa in Canada. Subsequently, this organism has been similarly identified in several cases of bovine abortion in western Canada, primarily from British Columbia (16). In cases in which protozoan cysts are associated with abortion or neonatal congenital encephalomyelitis, multiple blocks of tissue should be tested immunohistochemically in attempts to determine the prevalence of this organism in Canadian livestock. This is, in addition, the first report in which these protozoa has been successfully isolated from frozen tissues. In neonatal calves with similar gross lesions or in abortions in which *Neospora* is suspected, storage of tissues in low temperature freezers (−50°C or colder) and shipment on dry ice may facilitate isolation, propagation, and more accurate identification of the organism. CVJ

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