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Retrovirus-induced spongiform myeloencephalopathy in mice: Regional distribution of infected target cells and neuronal loss occurring in the absence of viral expression in neurons

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ABSTRACT **The Cas-Br-E murine leukemia virus (MuLV) induces a spongiform myeloencephalopathy resulting in a progressive hindlimb paralysis. We have used *in situ* hybridization with a Cas-Br-E MuLV-specific probe to study viral expression in the central nervous system. Infected cells were concentrated in regions where spongiform lesions and gliosis are detected (lumbosacral spinal cord, brainstem, deep cerebellar regions), suggesting a causative link between the level of virus expression and the degree of pathological changes in this disease. However, viral expression was not in itself sufficient to cause disease, since significant viral expression was observed in regions that did not exhibit pathological changes (cerebellar cortex, hippocampus, corpus callosum, peripheral nervous system). In both diseased and nondiseased regions, endothelial and glial cells were identified as the main target cells. Neurons in diseased regions did not show viral expression. The regional distribution of the spongiform changes appears to be laid down very early following infection, since expression could be detected at 10 days postinfection in regions that become diseased. These results indicate that nonneuronal cells have distinct properties in various regions of the central nervous system and suggest an indirect mechanism of neuronal loss consequent to viral expression in nonneuronal cells.**

The Cas-Br-E murine leukemia virus (MuLV), isolated from a population of paralyzed wild mice in Lake Casitas, CA, was found to be the etiologic agent of a progressive hindlimb paralysis (for review, see refs. 1 and 2). Transmission of the disease in the wild is vertical. Experimentally, it can be transmitted by an intraperitoneal injection of 10^4–10^5 plaque-forming units of the retrovirus into neonatal (≤48 hr old) susceptible mice such as NIH/Swiss, CFW/D, SWR/J, or C3H (1, 2). Histopathologically, the neurological disease is characterized as a spongiform myeloencephalopathy with intense gliosis, predominantly involving the anterior horns of the lumbosacral spinal cord, the brainstem, and deep cerebellar nuclei (3–7). The viral determinant of this spongiform degeneration has been mapped within the *env* sequences encoding the glycoprotein gp70 (8, 9).

To develop a rational hypothesis regarding the pathogenesis of the disease, it is necessary to define the regional distribution and cell type(s) supporting virus replication. Previous studies of central nervous system (CNS) target cells of the virus relied on electron microscopy (EM) for virus particle detection (4, 10–13) or on immunocytochemistry with antibodies against MuLV proteins to detect infected cells (5, 14). Neither the antisera employed nor the EM assessment of MuLV particles was specific for Cas-Br-E MuLV, and endogenous MuLV activated during the disease process may have been detected. Thus, the identity of viruses or viral proteins detected in these studies and, consequently, the identity of the target cells remain unclear.

In the present study, we have overcome this problem by using *in situ* hybridization with a cRNA probe specific for Cas-Br-E MuLV sequences. We found that degeneration of neurons proceeds in the absence of detectable viral expression in these cells and that the target nonneuronal cells can be distinguished regionally by their susceptibility to Cas-Br-E MuLV infection.

MATERIALS AND METHODS

Mice. All mice (CFW/D) presented were inoculated intraperitoneally with 0.15 ml of filtered virus suspension (1–5 × 10^5 plaque-forming units per ml) as newborns (<48 hr old). The Cas-Br-E MuLV pBR-NE-8 MuLV clone has been described (15). Six paralyzed mice were sacrificed 71–105 days postinoculation: four were analyzed in detail and two were surveyed for regional distribution of infection and gliosis. Ten nonparalyzed mice were examined: two were killed at 10 days and eight at 30 days postinoculation.

Preparation of Tissue. Fixation of tissues and subsequent processing were performed as described (16). Neurons were identified by their large nuclei with prominent nucleoli and abundant cytoplasm. Nonneuronal cells were identified using the description of McCarthy and Leblond (17). For each paralyzed mouse, 20 sections of brain and 100 sections of spinal cord were processed for *in situ* hybridization. Similar numbers of sections from each animal were processed for pathology and immunocytochemistry. The results shown are representative.

***In Situ* Hybridization.** Hybridization of paraffin-embedded or frozen-sectioned tissues was done as described (16, 18), using an ^{35}S-labeled RNA probe (19) corresponding to the Cas-Br-E MuLV-specific 1.05-kilobase *Taq* I–*Bam*HI *env* fragment (20).

Immunocytochemistry. Detection of glial fibrillary acidic protein (GFAP)-positive astrocytes was carried out using a rabbit polyclonal antiserum raised to bovine GFAP (DAKO, Carpinteria, CA), as described (16).

Double Label Immunocytochemistry–*in Situ* Hybridization. Immunocytochemistry was performed in the presence of RNase-free tRNA (1 mg/ml) and RNasin (800 units/ml) (Promega), with a 1:1000 dilution of anti-neurofilament antibody SMI 32 (Sternberger-Meyer Immunocytochemicals, Jarretsville, MD). The secondary antibody was a 1:100 dilution of anti-mouse IgG conjugated to horseradish peroxidase. With the exceptions noted above, all solutions and manipulations were as described for anti-GFAP immunostaining.

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Abbreviations: MuLV, murine leukemia virus; GFAP, glial fibrillary acidic protein; CNS, central nervous system; PNS, peripheral nervous system.

Following immunocytochemistry, slides were processed immediately for *in situ* hybridization.

RESULTS

Specificity of the Probe Used for *in Situ* Hybridization. To avoid detection of endogenous retroviral sequences, which could be expressed normally in CNS cells or which could be induced during disease progression, we used a probe specific for the inoculated virus (Cas-Br-E MuLV), corresponding to a fragment from the *env* region of pNE-8. This probe had previously been shown to be specific for this virus by Southern analysis, failing to hybridize with several other endogenous or exogenous retroviral MuLV sequences under stringent conditions (20).

The specificity of ^{35}S-labeled antisense and sense RNA probes under conditions of *in situ* hybridization was first assessed on fibroblasts in culture. With the antisense probe, a strong hybridization signal was observed in fibroblasts infected with Cas-Br-E MuLV (Fig. 1*B*) but not in uninfected fibroblasts (Fig. 1*A*) or in fibroblasts infected with the ecotropic endogenous BALB/c N-tropic (N-Cl-35) MuLV (Fig. 1*C*). This antisense probe also failed to hybridize to normal brain or spinal cord from uninoculated mice (data not shown). As a control, the sense probe failed to hybridize to Cas-Br-E MuLV-infected brain and spinal cord (data not shown), which exhibited a strong hybridization signal with the antisense probe (see below), indicating that the silver grains observed resulted from the detection of viral RNA and not from viral DNA.

Detection of Infected Cells in CNS Regions Exhibiting Spongiform Degeneration and Gliosis. In this disease, severe spongiform degeneration and gliosis occur preferentially in three CNS areas: the anterior horns and, to a lesser extent, the anterior and lateral funiculi of the lumbosacral spinal cord, the brainstem, and the deep cerebellar regions. Cells harboring high levels of viral RNA were detected in these three affected regions. In these areas, the intensity of the cytopathic process was related to the number of infected cells, with more infected cells observed in regions exhibiting pronounced spongiform lesions (Fig. 2; see also ref. 16). Interestingly, the infected cells were generally not clustered but appeared as individual cells among several noninfected cells (see Fig. 5). Their number was low.

In the lumbar spinal cord, infected cells were detected in the anterior horn, where the most prominent spongiform changes, gliosis, and loss of neurons occur. They were also detected in the intermediate horn as well as in anterior and lateral funiculi, where demyelination and gliosis occur (Fig. 2*C*). Often, a corona of infected cells could be detected at the gray/white matter junction of the anterior and lateral areas of the cord. In the thoracic and cervical cord, more-limited spongiform lesions and gliosis were detected than in the lumbosacral region. Here a lesser number of infected cells were detected (data not shown), but in the same regions described for the lumbar cord. In the brainstem, infected cells were found in the same regions of spongiform changes and gliosis, or of gliosis alone, mainly in the tegmentum, in the pontine nuclei, and in the reticular formation (Fig. 2 *A* and *B*). In deep cerebellar regions and posterior colliculi, infected cells were also found in the same areas as the spongiform changes and gliosis or gliosis alone (Fig. 2*A*). Therefore, a close spatial correlation appears to exist between the spongiform changes and the presence of infected cells, as well as between gliosis and the presence of infected cells.

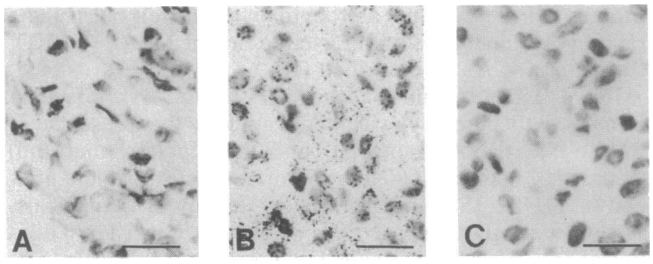

FIG. 1. Specificity of the Cas-Br-E MuLV *env* probe in *in situ* hybridization with NIH 3T3 fibroblasts that were uninfected (*A*) or infected with Cas-Br-E MuLV (*B*) or an endogenous BALB/c ecotropic N-tropic (N-Cl-35) MuLV (*C*). Only the Cas-Br-E MuLV-infected fibroblasts hybridized with the probe. Exposure time was 4 days. (Bar = 50 μm.)

Detection of Infected Cells in Nondiseased Areas of the CNS. The regional distribution of the spongiform lesions induced in Cas-Br-E MuLV-inoculated mice is rather specific. Indeed, lesions are rarely seen in several regions of the CNS, such as the cerebral cortex or the cerebellar cortex, even in mice showing signs of very advanced disease. To determine whether infected cells were present in these areas, *in situ* hybridization was also performed for these regions. Numerous infected cells were detected in several regions of the CNS showing no or minimal signs of cytopathic lesions: all layers of the cerebellar cortex, the corpus callosum, the granule cell layer and fimbria of the hippocampus, and tissue subjacent to the ependyma, arachnoid, and pia mater (Figs. 2*A* and 3).

The histological appearance of the cerebellar cortex of Cas-Br-E MuLV-inoculated paralyzed mice remained normal. However, both its granular and molecular layers contained infected cells (Fig. 3*A*). Infected cells were also detected in white-matter tracts of afferent and efferent fibers that pass in the center of the lobules (Fig. 2*A*). The corpus callosum (Fig. 3*B*) and hippocampus (data not shown) retained a normal architecture in diseased mice, but they also harbored infected cells. Similarly, the CNS tissue subjacent to ependymal cells showed no evidence of spongiform disease, despite the fact that ependymal cells exhibited a strong hybridization signal and were observed as clusters (data not shown). However, a modest hypertrophy of subependymal-plate astrocytes was seen in close contact with the ependymal cell layer. Finally, the arachnoid and pia mater were often intensely labeled in diseased mice. In addition to the above described regions, where groups of infected cells were observed, occasional isolated positive cells were observed throughout the CNS in both gray and white matter (Fig. 2*A*).

Together, these results indicate that infection of CNS cells with Cas-Br-E MuLV and, presumably, production of Cas-Br-E MuLV are not by themselves sufficient to cause spongiform degeneration. Neurons and/or glial cells in selective areas of the CNS seem to be resistant to the pathogenic potential of this virus.

Detection of Infected Cells in the Peripheral Nervous System (PNS). The effect of Cas-Br-E MuLV on the PNS has not previously been reported. The lumbar dorsal root ganglia of diseased mice showing spongiform lesions in the lumbar anterior horns exhibited no discernible signs of neuronal loss. However, satellite cells surrounding the neuronal cell bodies as well as Schwann cells were found to express viral RNA (Fig. 4). This result indicates that virus infection of the nervous system is not limited to the CNS but also extends to the PNS without apparent cytopathic effect.

Identification of CNS Cell Types Harboring Viral RNA. The infected cells in diseased areas were identified by morphological criteria. Morphologically, virtually all infected cells found in areas exhibiting pathology appear to be nonneuronal cells (Fig. 5 *A–C*), the majority of which appeared to be glial cells. Some (10–20%) of these nonneuronal cells were identified as endothelial cells (Fig. 5 *D–E*).

In nondiseased areas, such as the cerebellar cortex, the cells expressing viral RNA predominated in the granular layer but were also seen in the molecular layer. Although, the

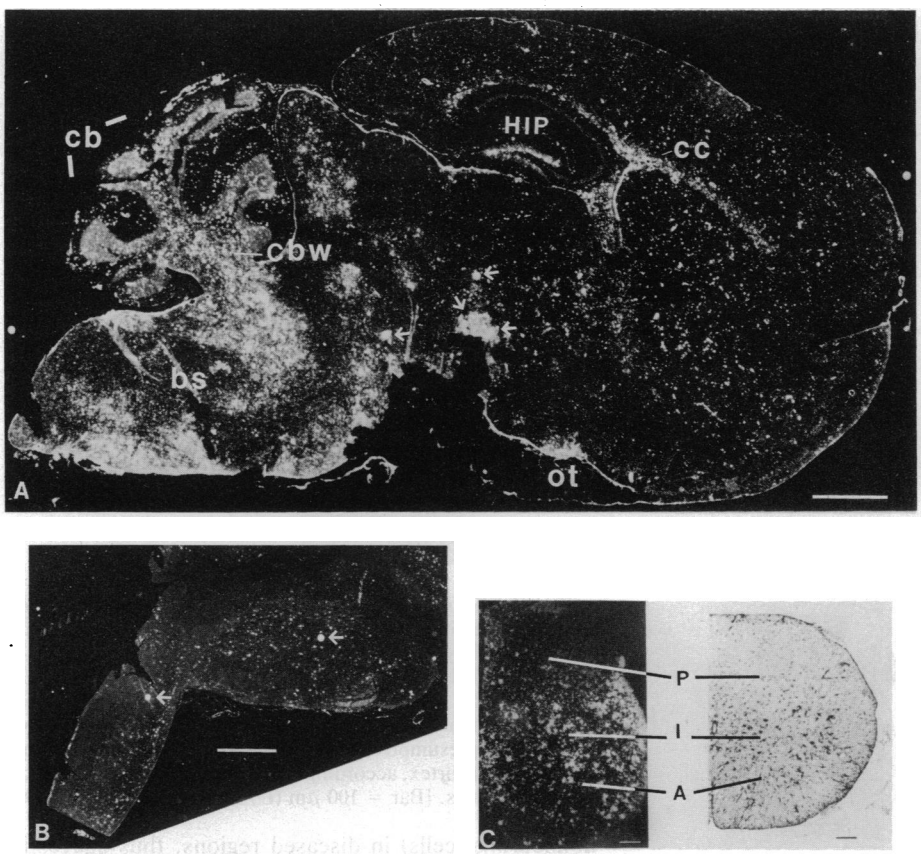

FIG. 2. Regional distribution of infected cells in Cas-Br-E MuLV-infected CNS, shown by sagittal sections of brain (*A*) and brainstem (*B*) and cross-section of lumbar cord (*C Left*) after *in situ* hybridization (dark field). Anti-GFAP immunoreactivity is also shown (*C Right*). HIP, hippocampus; cc, corpus callosum; cb, cerebellum; cbw, cerebellar white matter; bs, brainstem, ot, optic tract; in *C*, anterior (A), intermediate (I), and posterior (P) horns are indicated. Exposure time was 8 days (*A* and *C*) or 4 days (*B*). Some white spots represent artifacts (arrows). [Bar = 1 mm (*A* and *B*) or 100 μm (*C*).]

majority (70–80%) appeared to be endothelial cells, some were also granule cells. In the hippocampus, some neurons and glial cells of the granular layer of dentate gyrus were found to be infected. In the corpus callosum, the majority of *in situ* positive cells had the appearance of endothelial cells and glial cells.

These results indicate that glial, endothelial, meningeal, and ependymal cells are infected by the virus in diseased (spongiform changes and gliosis) and several nondiseased areas of Cas-Br-E MuLV-infected CNS. In nondiseased areas, some neurons are also infected. The precise identification of the glial cell type(s) infected will be presented separately.

Viral Expression in the CNS of Young Phenotypically Normal Mice. As a first approach to understanding the initial phase of CNS infection by Cas-Br-E MuLV, we studied 10- and 30-day-old phenotypically normal mice that had been inoculated at birth. No spongiform degeneration was detected in the brainstem of the 10-day-old mice, and some very limited degeneration was detected in only three of the eight 30-day old mice. Fewer infected cells were detected (Fig. 6 *A* and *B*), but their regional distribution paralleled the one described for paralyzed mice, except that the lumbosacral spinal cord was not studied in the 10-day-old mice. In CNS regions susceptible to disease, these cells were nonneuronal, as seen in paralyzed mice, with a higher proportion of endothelial then glial cells. Limited neuronal infection was observed in the cerebellar cortex and in the granular layer of the hippocampus, as was seen in paralyzed mice. No RNA was detected in neurons showing normal morphology or in those showing signs of degeneration (displacement of the nucleus toward the cell membrane and/or vacuolization). Unique to the 10-day-old mice was the finding of a strong but patchy hybridization signal in neuroblasts of the external granular layer of the cerebellar cortex (Fig. 6*C*) as well as in migrating neurons of the developing molecular layer and in granule cells of the internal granular layer (Fig. 6*D*). These infected neurons were located in regions not destined to show signs of spongiform degeneration.

These results show that specific brain cells are infected very early in the course of infection and indicate that their regional distribution is also determined at the initial stage of the disease. This regional specificity of infection precedes that of the lesions and most likely explains it.

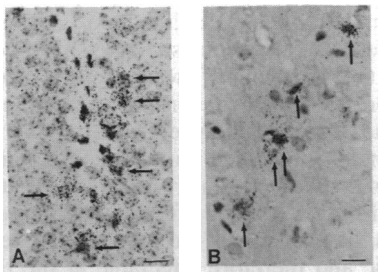

FIG. 3. Distribution of infected cells in nonspongiform regions, hippocampus (*A*) and corpus callosum (*B*), after *in situ* hybridization. Note the absence of spongiform changes. Arrows indicate positive cells. Exposure time was 4 days. (Bar = 100 μm.)

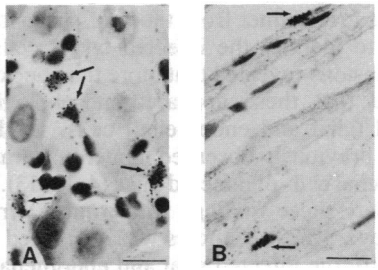

FIG. 4. Distribution of infected cells in the PNS. Viral RNA is detected both in satellite cells of dorsal root ganglia (*A*) and in Schwann cells (*B*). Exposure time was 4 days. (Bar = 50 μm.)

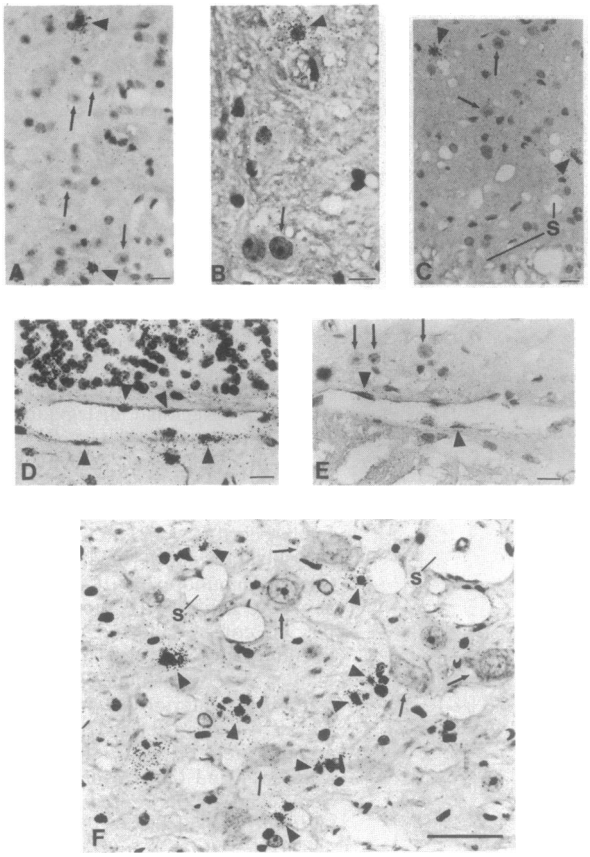

FIG. 5. Identification of cell types infected by the virus. *In situ* hybridization is shown for glial cells (*A–C*) and endothelial cells (*D* and *E*) infected by virus. Double label *in situ* hybridization/immunocytochemistry (for nonphosphorylated neurofilament) is shown on the same section of positive brainstem (*F*). Neurons (arrows) are negative for viral RNA; arrowheads indicate positive cells. Note the spongiform changes (S) in *C* and *F*. Exposure time was 4 days (*A–E*) or 6 days (F). (Bar = 50 μm.)

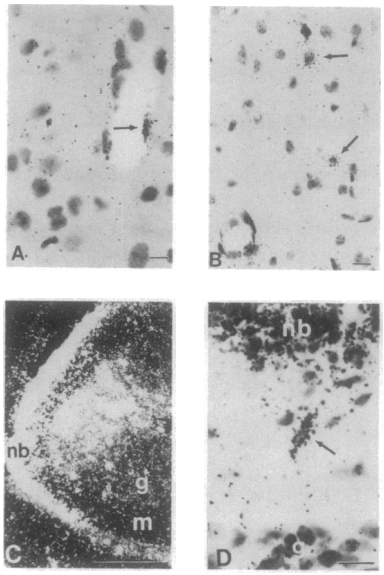

FIG. 6. Regional distribution and identification of Cas-Br-E MuLV-expressing cells in the brain of a young (10-day-old) nondiseased animal by *in situ* hybridization. (*A*) Endothelial cells (brainstem). (*B*) Glial cells (deep cerebellar region). (*C*) Dividing neuroblast (nb) in the external granular layer and neurons in the molecular (m) and internal granular (g) layers of the cerebellar cortex (dark field). (*D*) A presumptive migrating neuron in the molecular layer of the cerebellar cortex, according to the description of Cajal. Exposure time was 6 days. [Bar = 100 μm (*C*) or 50 μm (*A*, *B*, and *D*).]

DISCUSSION

CNS Target Cells of Cas-Br-E MuLV. We have used *in situ* hybridization with a probe specific for Cas-Br-E to study the CNS cells supporting replication of this virus. Infected cells were detected in all regions of the CNS that demonstrate spongiform changes and gliosis or gliosis alone, and their number was greater in the more diseased areas, suggesting a causative link between virus production and the gliosis and spongiform changes. Infected cells were also detected in regions where no or minimal spongiform lesions could be seen, such as the cerebellar cortex, dentate gyrus, corpus callosum, dorsal root ganglia, tissue subjacent to meninges, ependyma, and peripheral nerve. In these regions, the presence of virus was not by itself sufficient to result in cytopathic effects, suggesting that nonneuronal target cells or neurons in these regions are distinct from those of susceptible regions.

Attempts to identify the infected target cells within the spongiform lesions revealed that more than one cell type was infected and that these appeared morphologically to be nonneuronal (glial and endothelial) cells. By EM, several groups have previously observed numerous viral particles in CNS endothelial cells of infected mice (4, 10–13). Our present results validate these findings with a more stringent technique. However, further studies will be needed to unambiguously identify the infected glial and endothelial cells.

Although neurons die as a consequence of infection with Cas-Br-E MuLV, neurons themselves did not appear to be infected at a detectable level (1/50th the level detected in nonneuronal cells) in diseased regions, thus suggesting an indirect mechanism of cell loss. These results contrast with some previous EM studies reporting the presence of virus particles in neurons (5, 10). However, our results confirm other EM investigations indicating that the detection of virus in neurons of mice infected with the Cas-Br-E MuLV is a rare event (4, 11, 13) and clarify this issue with a more specific detection technique. Production of virus particles in neurons of diseased areas may simply result from the induction of endogenous retroviral replication as a consequence of the disease process (21, 22). However, in some nondiseased areas, some neurons seem to be productively infected, as suggested by the detection of viral RNA in cerebellar granule cells and their progenitors of 10-day-old mice and in the granular layers of the hippocampus and of the cerebellum of 30-day-old and of diseased mice. The presence of infected neurons being restricted to regions where neuronal proliferation continues after birth, this result suggests that virus infection requires cell division.

Regional Distribution of CNS Infected Cells. Infected target cells appear to have a specific distribution in the CNS. Since the infected target cells in diseased regions seem to be nonneuronal (glial and endothelial cells), this result indicates that nonneuronal cells have distinct properties according to their location within the CNS. In support of this contention, regional differences between astrocytes have been reported (23). One of the properties of the nonneuronal cells analyzed here—the propensity to be productively infected by the Cas-Br-E MuLV—most likely reflects a regional difference in the complement of host-cell transcription factors promoting expression of the viral promoter. Alternatively, this selective regional distribution of viral infection within the CNS could reflect the mitotic activity of the cells in these regions, being either mitotic and therefore infectable at the time of virus challenge, or in other regions, being postmitotic and therefore uninfectable. In regions susceptible to disease, a regional difference in the expression of the cell receptor for the viral gp70 is unlikely to explain the observed regional distribution

of infection, since the distribution of the lesions can be modified by exchanging only the long terminal repeat of the virus (24).

The regional specificity of infected target cells is determined very early following viral infection. In 10- or 30-day-old animals, infected cells are principally detected in regions that will exhibit virus replication in clinically diseased animals. It is likely that the cells being infected are dividing at the time of infection. Our results suggest that the virus gains entry to the CNS very early following infection of the animal, probably via endothelial cell infection, as suggested also from the kinetics of detection of viral particles in endothelial cells (4, 7, 11). However, the possibility that infection of the CNS also occurs by infiltration of infected immune-system cells (25, 26) or by free virus passaging an immature blood–brain barrier (27) cannot be discounted. It has been suggested (13) that neuronal loss in this disease is a consequence of the local breakdown of the blood–brain barrier, occurring as a result of endothelial cell infection. This model seems unlikely in light of our demonstration that despite the high proportion of infected endothelial cells in the adult cerebellar cortex, no lesions are discernible in this area.

A Model of CNS Pathogenesis by Cas-Br-E MuLV. Our results indicate that the neuronal loss seen in Cas-Br-E MuLV-induced spongiform myeloencephalopathy is indirect and is a consequence of infection of nonneuronal cells. Because gp70 had been found to harbor the viral determinant of spongiform degeneration, it was proposed (9) that this disease is a receptor disease of the neuron, with gp70 competing with a trophic factor or directly interfering with normal neuronal functions by binding directly to a receptor (9). This model implies that the nonneuronal cells that we identified as the main infected target cells provide sufficient virions (or free gp70) to bind to this neuronal receptor. Alternatively, infected nonneuronal cells may play a specific role: their receptor could be occupied by gp70, leading to decreased secretion of a factor essential for neuronal survival or to an increased production of a factor that is toxic at high levels for neurons. Alternatively, gp70, if it is produced, may not act through a receptor but could induce disease in a totally different way. Nor can we exclude that an undetectable level of infection in neurons is sufficient to lead to their degeneration. Further studies should determine whether infection of nonneuronal cells is a necessary and sufficient step to induce spongiform degeneration.

Note. While this manuscript was under review, a report appeared claiming abortive Cas-Br-E MuLV replication almost exclusively in neurons (28), in contrast to our results. This work was done with nonspecific antibodies able to detect not only Cas-Br-E MuLV proteins but also proteins encoded by several endogenous MuLV sequences: this nonspecificity of reagents may explain the discrepancies between their observations and ours.

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