JOURNAL OF VIROLOGY, Nov. 1995, p. 7300–7303 Vol. 69, No. 11
0022-538X/95/$04.00+0

Inhibition of Murine Retrovirus-Induced Neurodegeneration in the Spinal Cord by Explant Culture

RICHARD A. BESSEN,[1]* WILLIAM P. LYNCH,[2] AND JOHN L. PORTIS[1]

Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840,[1] and Department of Pathology, BWH, LMRC 514, Harvard Medical School, Boston, Massachusetts 02115[2]

Received 3 May 1995/Accepted 2 August 1995

The neurovirulent chimeric mouse ecotropic retrovirus FrCas^E causes a rapid neurodegenerative disease of the central nervous system (CNS) characterized by the appearance of spongiform lesions in motor areas 10 days after neonatal inoculation. To study the details of the pathogenic process, we examined the ability of an ex vivo spinal cord model to recapitulate disease. Organotypic spinal cord slice cultures were established from IRW mice 7 days after neonatal inoculation. This corresponds to a time when virus expression in the CNS is first detectable but spongiform changes have yet to evolve. Infectivity associated with these cultures peaked at 7 days in vitro and persisted at this level for 6 weeks. FrCas^E infection of the spinal cord slices was primarily found associated with microglial cells. Infection of neurons, astrocytes, oligodendroglia, and endothelial cells was not observed; however, significant astrogliosis was found. Despite the presence of extensive microglial infection in close association with spinal motor neurons in organotypic cultures, no virus-specific spongiform degenerative changes were observed. These results suggest that removal of motor neurons from the developing CNS, despite maintaining the local cytoarchitectural relationships, prevents the virus from eliciting its pathological effects. Possible reasons for the interruption of lesion development are discussed.

Retrovirus infections of the central nervous system (CNS) can cause neurological diseases in animals and humans. The chimeric ecotropic murine retrovirus FrCas^E causes a rapid degeneration of the CNS characterized by a noninflammatory spongiform encephalomyelopathy (27). Pathology is initially detectable approximately at 10 days postinoculation (dpi) and is extensive by 15 dpi. Spongiform lesions are observed primarily in brain regions that control motor function, including the ventral horns of the spinal cord, brain stem nuclei, deep cerebellar nuclei, and the motor cortex (8, 22). The onset of clinical disease is characterized by tremors and by hind limb paralysis that begins 15 days after neonatal inoculation and rapidly progresses to death.

Following intraperitoneal inoculation of neonates at postnatal day 0 (P0), the primary site of FrCas^E infection is the spleen, but virus is found in the brain associated with the vasculature by 6 days postinfection. Virus then spreads to the brain parenchyma and is detected in microglial cells and neurons that continue to divide during early postnatal development (8). The neurons that degenerate are not infected, while virally infected neurons do not exhibit cytopathological changes (22). However, FrCas^E-infected microglia are in close proximity to degenerating neuronal processes, suggesting that spongiform lesions are an indirect consequence of microglial infection. Retroviral infection of brain microglial cells has also been implicated in the pathogenesis of neurological disease induced by related murine retroviruses (3, 4, 15) as well as by human immunodeficiency virus in AIDS-associated neurological diseases (14, 20). Recently, it has been demonstrated that implantation of FrCas^E-infected microglia into developmentally resistant mice results in the appearance of focal spongiosis (24).

To gain further insights into the molecular details of lesion induction by neurovirulent murine retroviruses, we developed an in vitro model using organotypic CNS slice cultures. Previous attempts to investigate this disease in dissociated embryonic motor neuron cultures were unsuccessful (1). The advantages of organotypic slice cultures compared with dissociated CNS cultures are that cellular heterogeneity and tissue cytoarchitecture are preserved and slices can remain viable for several months (12). The anatomical and functional resemblance between slice cultures and in vivo tissue has proven useful in several investigations into the mechanisms of neuronal degeneration in amyotrophic lateral sclerosis (28), excitotoxicity (34–36), ischemia/hypoxia (30), and hypoglycemia (33).

In the present study, organotypic spinal cord cultures were used to analyze FrCas^E infection and pathology and to relate the findings obtained to FrCas^E infection in vivo. FrCas^E infects microglia and brain capillary endothelial cells in the spinal cord, and extensive spongiform lesions are found in this region. The experimental approach used in this study was to establish organotypic slice cultures (9, 12, 32) at P7 from either uninfected inbred Rocky Mountain White (IRW) mice or mice inoculated intraperitoneally at P0 with FrCas^E. From 7 to 42 days in vitro (DIV), organotypic slice cultures were examined for production of infectious virus, for identification of infected cells, and for FrCas^E-induced pathology. In the mouse CNS at 7 dpi FrCas^E is primarily found in brain capillary endothelial cells, while CNS lesions begin to appear at 10 dpi (8). These times are equivalent to 0 and 3 DIV for slice cultures, respectively.

Viral spread in organotypic slice cultures. To determine the level of FrCas^E infection in slice cultures, viral infectivity in spinal cord organotypic cultures was determined at 0, 7, 21, and 42 DIV. Slice cultures were homogenized in Dulbecco's phosphate-buffered saline (50 μl per tissue slice), serial 10-fold dilutions were made from each homogenate, and viral titers were assayed on *Mus dunni* cells by a focal immunoassay as described previously (7). At 0 DIV the slice cultures had a titer of $10^{2.3}$ focus-forming units per spinal cord slice. By 7 DIV viral titers peaked at greater than $10^{4.0}$ focus-forming units per

* Corresponding author. Phone: (406) 363-9251. Fax: (406) 363-9204.

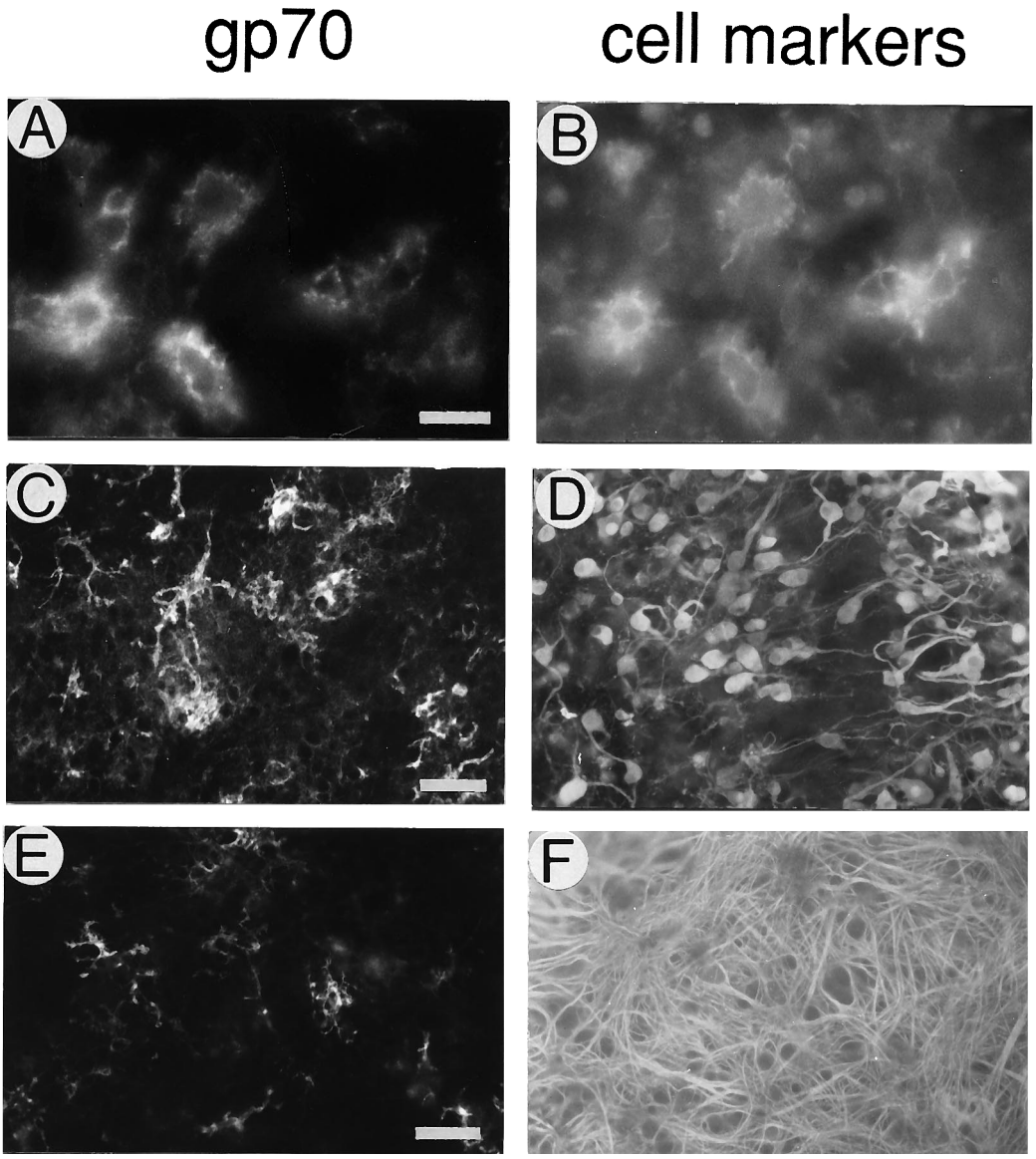

FIG. 1. Identification of virally infected cells in spinal cord organotypic slice cultures. Immunofluorescent staining for viral envelope protein (anti-gp70) shown in panels A, C, and E was performed by using monoclonal antibody 667 (25) followed by anti-mouse antibody conjugated with fluorescein (diluted 1:200; Vector, Burlingame, Calif.). The identical cultures were simultaneously immunostained (cell markers) for Mac-1 microglial cell marker (monoclonal rat antibody from ATCC TIB128) (B), neuron-specific enolase (rabbit polyclonal antibody from Biogenex, San Ramon, Calif.) (D), and glial fibrillary acidic protein (rabbit polyclonal antibody from Dako, Minneapolis, Minn.) (F). Detection of cell-specific markers was performed by incubation with biotinylated secondary antibodies (1:200; Vector) directed to either rabbit (D and F) or rat (B) primary antibodies followed by incubation with avidin D conjugated with rhodamine 600 (Vector). Bar in panel A, 10 μm; bars in panels C and E, 30 μm.

slice, and they plateaued at this peak level until 42 DIV. These results indicate that virus had spread in the CNS slice cultures and that infection was persistent.

Identification of infected cells in slice cultures. To identify virally infected cells in organotypic spinal cord cultures, we used monoclonal antibody 667, which is specific for the $FrCas^E$ viral envelope glycoprotein (25). $FrCas^E$ infection of microglia has been reported to be a prominent feature in vivo, these cells being particularly concentrated in regions that exhibit vacuolar lesions (8, 22). The major cell type infected in spinal cord slices had a very heterogeneous morphology (Fig. 1A, C, and E) and colocalized with the macrophage cell surface marker Mac-1 (Fig. 1B) in 25 to 50% of the infected cells. The majority of cells that expressed viral envelope protein but not the Mac-1 marker also had a cellular morphology consistent with microglia. We observed widespread infection of both microglia having an ameboid morphology (Fig. 1A and B) and microglia having a ramified morphology (Fig. 1C and E). Since virus is primarily associated with the vasculature at the time at which slice cultures were established (8), these results support the infectivity assays described above and indicate that virus had spread to parenchymal microglial cells after explant.

For spinal cord cultures, attempts to colocalize $FrCas^E$ envelope protein (Fig. 1C and E) with cell-specific markers for neurons (Fig. 1D; anti-neuron-specific enolase antiserum), astrocytes (Fig. 1F; anti-glial fibrillary acidic protein [GFAP] antiserum), and oligodendroglia (data not shown) by using anti-2′, 3′-cyclic nucleotide 3′-phosphohydrolase antibody (kindly

provided by Bruce Trapp, The Johns Hopkins University, Baltimore, Md.) failed to show evidence of infection of these cell types. Detection of viral infection in neurons, astrocytes, and oligodendroglia from the spinal cord tissue of FrCas^E-infected mice also has been unsuccessful (22).

GFAP-immunoreactive astrocytes formed an extensive network of intertwined cell bodies and processes that ensheathed the entire slice culture by 21 DIV (Fig. 1F). This cellular morphology and this tissue distribution are characteristic of activated astrocytes often observed after CNS injury but not found in the normal CNS in vivo. The resistance of astrocytes to FrCas^E infection was surprising, since these cells undergo proliferation in slice cultures (10, 19) and therefore should be susceptible to infection by oncoretroviruses, which require cell division to replicate (29). It is noteworthy that infection of astrocytes also has not been observed in FrCas^E-infected mice (22). However, Lynch et al. have demonstrated that astrocytes in primary mixed glial cultures are infected by FrCas^E (21), while other investigators using the neurovirulent *ts*1 Moloney murine leukemia virus demonstrated infection of astrocyte-enriched cultures (31). This apparent lack of permissiveness of astrocytes residing in CNS tissue suggests that viral infection and/or expression in astrocytes may be restricted by other cells in the CNS, a situation maintained in slice cultures because of preservation of tissue cytoarchitecture.

At 21 DIV we could not detect infection of endothelial cells in spinal cord slice cultures by using antibodies to either factor VIII-related antigen or Sca-1 as endothelial cell markers (data not shown). This was a surprising result, since brain endothelial cell infection is prominent in vivo (22) and should be present when organotypic cultures are established at 7 dpi. However, cells expressing endothelial markers at 21 DIV were very sparse in spinal cord slices from either infected or uninfected mice, suggesting that the cultures may become depleted of these cells as a function of time.

Absence of virus-specific spongiform lesions in slice cultures. Once we established that FrCas^E could replicate to high levels in slice cultures and infect similar cells in vitro and in vivo, we examined organotypic cultures for virus-induced spongiform change. In vivo, FrCas^E infection had induced widespread spongiform change in the ventral horns of the lumbar spinal cord by 15 dpi (Fig. 2B). Examination of spinal cord slice cultures revealed many large motor neurons in the ventral horns (Fig. 2A), but we were unable to detect retrovirus-induced spongiform lesions at 21 to 35 DIV (28 to 42 dpi). Abnormalities in neuronal morphology were observed in spinal cord cultures. For example, some neurons had eccentric nuclei and shrunken perikarya. However, these changes were also seen in uninfected cultures at the same frequency.

In view of the extensive nature of FrCas^E infection found in the slice cultures, we were surprised at the absence of lesions related to motor neurons. The age of the spinal cord cultures greatly exceeded the point at which lesions are observed in vivo. For example, after 35 DIV (42 dpi) no pathology was observed in the slice cultures, and yet in vivo, lesions, first detectable at 10 dpi, are extensive by 15 dpi (8). In organotypic slice cultures we found widespread infection of microglial cells, and we therefore expected to observe retrovirus-induced pathology, since it has been demonstrated that brain implantation of FrCas^E-infected microglia results in focal spongiform lesions (24). It is possible that even in the presence of infected microglial cells the slice culture system insufficiently mimics the CNS in vivo. For instance, one component of the in vivo infection, brain capillary endothelial cells, was missing in the slice cultures at 21 DIV. However, the induction of spongiform lesions by implantation of FrCas^E-infected microglial cells (>98% purity) indicates that lesions can be induced in the absence of endothelial cell infection.

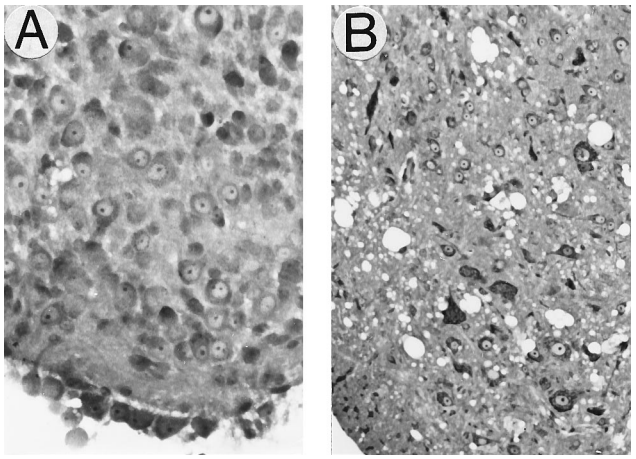

FIG. 2. Morphology of FrCas^E-infected CNS tissues. Spinal cord organotypic slice cultures at 21 DIV (A) were prepared for light microscopy by using JB-4 embedding compound (Polysciences, Warrington, Pa.) and stained with Lee's methylene blue and basic fuchsin. The lack of spongiform lesions in the slice cultures contrasts with the intense spongiform lesions observed in the spinal cord of a mouse at 15 dpi (B) (hematoxylin and eosin staining).

The lack of pathology in vitro could be related to the developmental state of the CNS. Previous studies have demonstrated that FrCas^E-induced spongiform lesions and neurological disease are dependent on postnatal development of the CNS (23). Establishment of organotypic brain cultures on P7 could restrict or delay development of the CNS. An important time for spinal motor neuron development is between P7 and P14, when maturation is activity dependent and requires intact descending and spinal segmental afferents (16, 17), both of which are interrupted in spinal cord slice cultures. We believe that this could be important for manifestation of lesions, since several studies have demonstrated that deafferentation of a target site results in attenuation of the neurotoxic effects of exogenously applied kainic acid and quinolinic acid (5, 6, 18, 26).

Another host factor that may have prevented the formation of spongiform lesions in FrCas^E-infected spinal cord slice cultures was the intense astrogliosis which occurred simultaneously with viral replication and spread to microglia. Injuries to the CNS often lead to activation of astrocytes, which increases their metabolic activity, including expression of GFAP. Reactive astrocytes also upregulate their expression of cytokines and trophic factors, some which are neuroprotective (11). It has been reported that FrCas^E infection of microglia in association with a brain stab wound, which induced a focal gliosis, also failed to induce spongiosis in vivo (24). The possibility that astrogliosis in the slice cultures may have been neuroprotective is supported by the observation of astrocyte-induced rescue of neurons from neurotoxic injury in vitro (13). Currently, we are testing inhibitors of cell division in order to block astrogliosis in organotypic slice cultures.

We thank D. Tumas, K. Hasenkrug, D. Lodmell, and S. Robertson for critical reading of the manuscript. Thanks to F. McAtee for supplying virus stocks and to B. Evans and G. Hettrick for photographic assistance.

This work was supported in part by a National Institutes of Health Intramural Research Training Award fellowship (R.A.B.).

REFERENCES

1. **Andrews, J., and R. Andrews.** 1976. Comparative neuropathology of motor neuron diseases, p. 181–216. *In* J. M. Andrews, R. T. Johnson, and M. A. B. Brazier (ed.), Amyotrophic lateral sclerosis. Academic Press, New York.
2. **Andrews, J. M., and M. B. Gardner.** 1974. Lower motor neuron degeneration associated with type C RNA virus infection in mice: neuropathological features. J. Neuropathol. Exp. Neurol. **33:**285–307.
3. **Baszler, T. V., and J. F. Zachary.** 1990. Murine retroviral-induced spongiform neuronal degeneration parallels resident microglial cell infection: ultrastructural findings. Lab. Invest. **63:**612–623.
4. **Baszler, T. V., and J. F. Zachary.** 1991. Murine retroviral neurovirulence correlates with an enhanced ability of virus to infect selectively, replicate in, and activate resident microglial cells. Am. J. Pathol. **138:**655–671.
5. **Biziere, K., and J. T. Coyle.** 1978. Influence of cortical-striatal afferents on striatal kainic acid neurotoxicity. Neurosci. Lett. **8:**303–310.
6. **Coyle, J. T., S. J. Bird, R. H. Evans, R. L. Gulley, J. V. Nadler, W. J. Nicklas, and J. W. Olney.** 1981. Excitatory amino acid neurotoxins: selectivity, specificity, and mechanisms of action. Neurosci. Res. Program Bull. **19:**1–427.
7. **Czub, M., S. Czub, F. J. McAtee, and J. L. Portis.** 1991. Age-dependent resistance to murine retrovirus-induced spongiform neurodegeneration results from central nervous system-specific restriction of virus replication. J. Virol. **65:**2539–2544.
8. **Czub, S., W. P. Lynch, M. Czub, and J. L. Portis.** 1994. Kinetic analysis of spongiform neurodegenerative disease induced by a highly virulent murine retrovirus. Lab. Invest. **70:**711–723.
9. **Delfs, J., J. Friend, S. Ishimoto, and D. Saroff.** 1989. Ventral and dorsal horn acetylcholinesterase neurons are maintained in organotypic cultures of postnatal rat spinal cord explants. Brain Res. **488:**31–42.
10. **del Rio, J. A., B. Heimrich, E. Soriano, H. Schwegler, and M. Frotscher.** 1991. Proliferation and differentiation of glial fibrillary acidic protein-immunoreactive glial cells in organotypic slice cultures of rat hippocampus. Neuroscience **43:**335–347.
11. **Eddleston, M., and L. Mucke.** 1993. Molecular profile of reactive astrocytes: implications for their role in neurologic disease. Neuroscience **54:**15–36.
12. **Gahwiler, B. H.** 1981. Organotypic monolayer cultures of nervous tissue. J. Neurosci. Methods **4:**329–342.
13. **Giulian, D., K. Vaca, and M. Corpuz.** 1993. Brain glia release factors with opposing actions upon neuronal survival. J. Neurosci. **13:**29–37.
14. **Giulian, D., E. Wendt, K. Vaca, and C. Noonan.** 1993. The envelope glycoprotein of HIV-1 stimulates monocyte release of neurotoxins. Proc. Natl. Acad. Sci. USA **90:**2769–2773.
15. **Gravel, C., D. G. Kay, and P. Jolicoeur.** 1993. Identification of the infected target cell type in spongiform myeloencephalopathy induced by the neurotropic Cas-Br-E murine leukemia virus. J. Virol. **67:**6648–6658.
16. **Kalb, R. G., and S. Hockfield.** 1988. Molecular evidence for early activity-dependent development of hamster motor neurons. J. Neurosci. **8:**2350–2360.
17. **Kalb, R. G., and S. Hockfield.** 1990. Induction of a neuronal proteoglycan by the NMDA receptor in the developing spinal cord. Science **250:**294–296.
18. **Kohler, C., R. Schwarcz, and K. Fuxe.** 1979. Intrahippocampal injections of ibotenic acid provide histological evidence for a neurotoxic mechanism different from kainic acid. Neurosci. Lett. **15:**223–228.
19. **Kusaka, J., A. Hirano, M. B. Bornstein, and C. S. Raine.** 1985. Basal lamina formation by astrocytes in organotypic cultures of mouse spinal cord tissue. J. Neuropathol. Exp. Neurol. **44:**295–303.
20. **Lipton, S. A.** 1992. Requirement for macrophages in neuronal injury induced by HIV envelope protein gp120. Neuroreport **3:**913–915.
21. **Lynch, W. P., W. J. Brown, G. J. Spangrude, and J. L. Portis.** 1994. Microglial infection by a neurovirulent murine retrovirus results in defective processing of envelope protein and intracellular budding of virus particles. J. Virol. **68:**3401–3409.
22. **Lynch, W. P., S. Czub, F. J. McAtee, S. F. Hayes, and J. L. Portis.** 1991. Murine retrovirus-induced spongiform encephalopathy: productive infection of microglia and cerebellar neurons in accelerated CNS disease. Neuron **7:**365–379.
23. **Lynch, W. P., and J. L. Portis.** 1993. Murine retrovirus-induced spongiform encephalopathy: disease expression is dependent on postnatal development of the central nervous system. J. Virol. **67:**2601–2610.
24. **Lynch, W. P., S. J. Robertson, and J. L. Portis.** 1995. Induction of focal spongiform neurodegeneration in developmentally resistant mice by implantation of murine retrovirus-infected microglia. J. Virol. **69:**1408–1419.
25. **McAtee, F. J., and J. L. Portis.** 1985. Monoclonal antibodies specific for wild mouse neurotropic retrovirus: detection of comparable levels of virus replication in mouse strains susceptible and resistant to paralytic disease. J. Virol. **56:**1018–1022.
26. **McGeer, E. G., P. L. McGeer, and K. Singh.** 1978. Kainate-induced degeneration of neostriatal neurons: dependency upon corticostriatal tract. Brain Res. **139:**381–383.
27. **Portis, J. L., S. Czub, C. F. Garon, and F. J. McAtee.** 1990. Neurodegenerative disease induced by the wild mouse ecotropic retrovirus is markedly accelerated by long terminal repeat and *gag-pol* sequences from nondefective Friend murine leukemia virus. J. Virol. **64:**1648–1656.
28. **Rothstein, J. D., L. Jin, M. Dykes-Hoberg, and R. W. Kuncl.** 1993. Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. Proc. Natl. Acad. Sci. USA **90:**6591–6595.
29. **Rubin, H., and H. M. Temin.** 1959. A radiological study of cell-virus interaction in the Rous sarcoma. Virology **7:**75–91.
30. **Shibata, S., K. Tominaga, Y. Kagami-ishi, and S. Watanabe.** 1992. Neuroprotective effect of protein kinase C inhibitors on oxygen/glucose free-induced decreases in 2-deoxyglucose uptake and CA1 field potentials in rat hippocampal slices. Brain Res. **594:**290–294.
31. **Shikova, E., Y. C. Lin, K. Saha, B. R. Brooks, and P. K. Wong.** 1993. Correlation of specific virus-astrocyte interactions and cytopathic effects induced by *ts*1, a neurovirulent mutant of Moloney murine leukemia virus. J. Virol. **67:**1137–1147.
32. **Stoppini, L., P.-A. Buchs, and D. Muller.** 1991. A simple method for organotypic cultures of nervous tissue. J. Neurosci. Methods **37:**173–182.
33. **Tasker, R. C., J. T. Coyle, and J. J. Vornov.** 1992. The regional vulnerability to hypoglycemia-induced neurotoxicity in organotypic hippocampal culture: protection by early tetrodotoxin or delayed MK-801. J. Neurosci. **12:**4298–4308.
34. **Vornov, J. J., and J. T. Coyle.** 1991. Enhancement of NMDA receptor-mediated neurotoxicity in the hippocampal slice by depolarization and ischemia. Brain Res. **555:**99–106.
35. **Vornov, J. J., and J. T. Coyle.** 1991. Glutamate neurotoxicity and the inhibition of protein synthesis in the hippocampal slice. J. Neurochem. **56:**996–1006.
36. **Whetsell, W. O., and R. Schwarcz.** 1983. The organotypic tissue culture model of corticostriatal system used for examining amino acid neurotoxicity and its antagonism: studies on kainic acid, quinolinic acid and (−) 2-amino-7-phosphonoheptanoic acid. J. Neural Transm. Suppl. **19:**53–63.