

Biological Basis of Rabies Virus Neurovirulence in Mice: Comparative Pathogenesis Study Using the Immunoperoxidase Technique

ALAN C. JACKSON

*Departments of Medicine and Microbiology and Immunology, Queen's University, 78 Barrie Street,
Kingston, Ontario K7L 3J7, Canada*

Received 23 July 1990/Accepted 11 October 1990

The CVS strain of fixed rabies virus causes acute, fatal encephalomyelitis in young adult ICR mice. Variant RV194-2, which was selected from CVS virus in cell culture with a neutralizing antiglycoprotein monoclonal antibody, has a single amino acid change in the glycoprotein. The infections caused by CVS virus and RV194-2 virus were compared in mice for 14 days postinoculation of 5×10^7 PFU into the right masseter muscle. All CVS virus-infected mice died (mean time to death, 7.9 days), compared with a mortality rate of 8.5% for RV194-2 virus-infected mice. RV194-2 virus spread to the ipsilateral trigeminal ganglion during the first 2 days postinoculation, and both viruses spread to the ipsilateral motor nucleus of the trigeminal nerve in the pons. Both viruses spread centrifugally and caused infection of bilateral trigeminal ganglia on day 3. The viruses spread throughout the central nervous system (CNS) at similar rates, but CVS virus infected many more neurons than did RV194-2 virus. Rabies virus antigen was observed in only occasional CNS neurons after day 6 of RV194-2 virus infection. By this time, CVS virus had caused severe widespread infection. In this model, virulence depends on improved efficiency of viral spread between CNS neurons rather than the rate of spread or topographical distribution of the infection.

Rabies virus is a highly neurotropic virus that causes acute, fatal encephalomyelitis in humans and animals. Experimental rabies virus infection in mice is a convenient model for studying virus-host interactions. Neurovirulence is the relative capacity of a virus to cause disease in the nervous system of a host (6). Rabies virus neurovirulence has been studied experimentally by using the CVS strain of fixed rabies virus in young adult mice. Important information has emerged about both the molecular and biological bases of rabies virus neurovirulence.

Avirulent rabies virus variants selected from CVS virus *in vitro* with neutralizing antiglycoprotein monoclonal antibodies have been useful tools in studying neurovirulence (1-3, 6, 8, 12, 13). Dietzschold et al. (3) selected RV194-2 virus with neutralizing antiglycoprotein monoclonal antibody 194-2, which was raised against the inactivated ERA strain of fixed rabies virus. CVS virus causes fatal rabies in adult mice after intracerebral inoculation. In contrast, RV194-2 virus does not cause clinical disease or death after intracerebral inoculation, and it induces high levels of neutralizing antibody (3). Sequencing studies have demonstrated that the change in pathogenicity corresponds to substitution of glutamine for arginine at position 333 of the rabies virus glycoprotein (3).

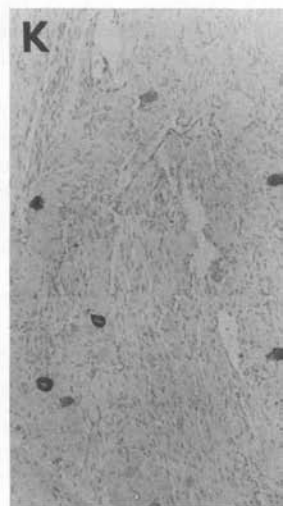
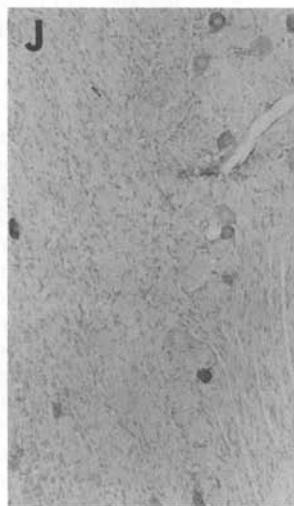
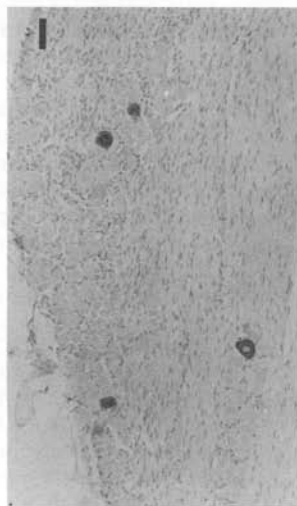
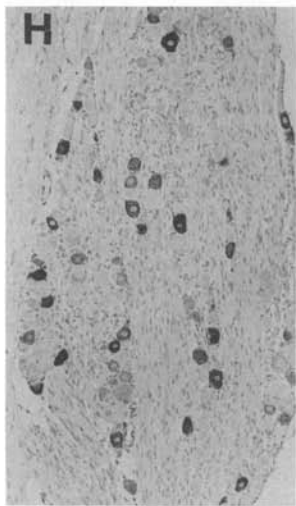
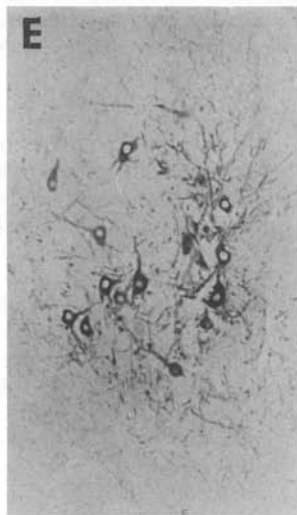
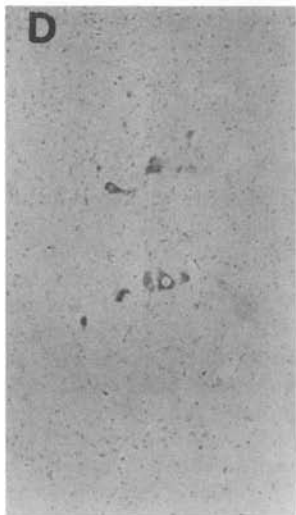
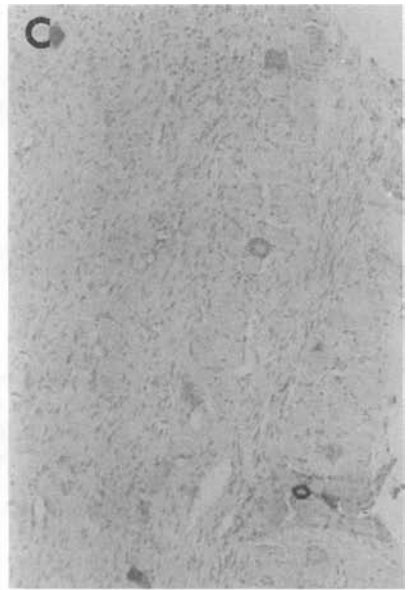
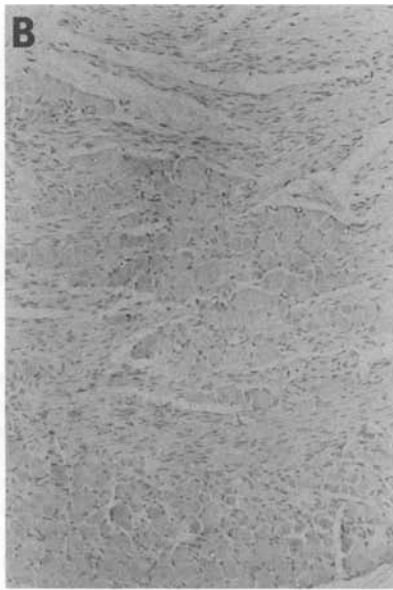
Dietzschold et al. (2) compared CVS virus and RV194-2 virus infections *in vitro* and after intracerebral inoculation of mice. In the present study, the infections caused by strain CVS-11 of fixed rabies virus and RV194-2 virus (obtained from William H. Wunner, Wistar Institute, Philadelphia, Pa.) were compared postinoculation of 5×10^7 PFU in the right masseter muscles of 6-week-old female ICR mice (Charles River Canada Inc., St-Constant, Quebec, Canada). Peripheral inoculation results in a more natural route of entry into the host than the intracerebral route. Variant viruses inoculated peripherally may spread to the central nervous system (CNS) along different pathways, and important biological differences may be determined.

In an observation group, neurologic signs, including hunching and limb paralysis, and deaths first occurred 6 days postinoculation with CVS virus. The mean date of death was 7.9 days postinoculation, and all (31 of 31) mice died by day 12. Most of the mice demonstrated no neurologic signs postinoculation with RV194-2 virus. Some mice became hunched, and 8.5% (4 of 47) died between days 8 and 10 postinoculation.

Three to six mice were sacrificed at daily intervals for 14 days. After perfusion with buffered 4% paraformaldehyde, brains, spinal cords, and trigeminal ganglia were removed, immersion fixed in the same fixative, dehydrated, and paraffin embedded. Tissue sections were stained for rabies virus antigen by the avidin-biotin-peroxidase method as previously reported (7), with minor modifications. Rabbit antirabies virus serum (obtained from K. M. Charlton, Animal Diseases Research Institute, Nepean, Ontario, Canada) was diluted 1:2,000, biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, Calif.) was diluted 1:200, and Elite avidin-biotinylated horseradish peroxidase complex (Vector Laboratories) was used.

On days 1 and 2 postinoculation with CVS virus, rabies virus antigen was found in the ipsilateral motor nucleus of the trigeminal nerve in the pons (Fig. 1A and D). No infected neurons were observed in trigeminal ganglia on these days (Fig. 1B). Early infection did not occur in the chief sensory nucleus or the nucleus of the spinal tract. Only one of six mice had a single neuron infected in the ipsilateral mesencephalic nucleus on day 2.

Rabies virus antigen was also found in the ipsilateral motor nucleus of the trigeminal nerve on days 1 and 2 postinoculation with RV194-2 virus (Fig. 1E). In addition, infected neurons were present in the ipsilateral trigeminal ganglion in four of six mice on day 1 and all six mice on day 2 (Fig. 1C). One of six mice also had a single neuron infected in the ipsilateral mesencephalic nucleus on day 2.



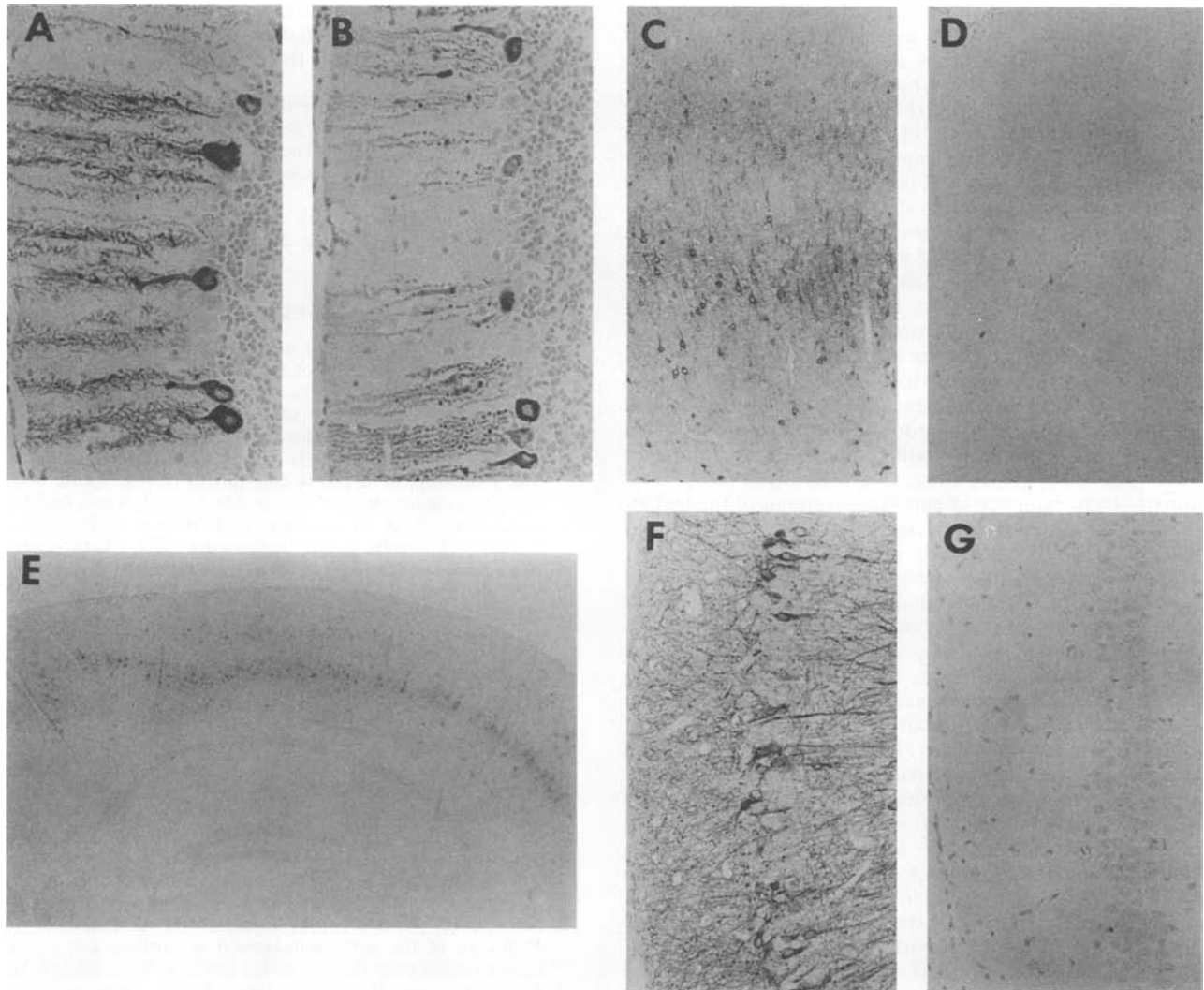


FIG. 2. Rabies virus antigen was present in cerebellar Purkinje cells 5 days postinoculation with CVS (A) and RV194-2 (B) viruses. Note the antigen in the dendritic processes of the Purkinje cells in the molecular layer. Antigen was present in the cerebral cortex 6 days postinoculation with CVS virus (C) and RV194-2 (D). There were many more infected neurons in the cortex in CVS virus infection. Antigen was present in many neurons of the cerebral cortex but not the hippocampus at 4 days postinoculation with CVS virus (E). Antigen was present in the CA1 region of the hippocampus 8 days postinoculation with CVS virus (F) but not postinoculation with RV194-2 virus (G). Immunoperoxidase-hematoxylin staining was used. Magnifications: A and B, $\times 235$; C and D, $\times 55$; E, $\times 20$; F and G, $\times 140$.

On day 3, both viruses had spread into the brain stem tegmentum bilaterally (Fig. 1F and G). At this time, there was also infection of neurons in the trigeminal ganglia bilaterally (Fig. 1H to K), indicating centrifugal spread from the infected brain stem.

Both viruses produced cerebellar infection on day 3 or 4. Infection in deep cerebellar nuclei occurred before infection

of the Purkinje cell layer, which became infected about 1 day later (Fig. 2A and B). Rabies virus antigen was not observed in the cerebellar white matter. RV194-2 virus infected fewer neurons in the deep cerebellar nuclei and fewer Purkinje cells than did CVS virus. Both viruses also spread to the spinal cord at this time. CVS virus infected more spinal cord neurons than did RV194-2 virus.

FIG. 1. Rabies virus antigen was present in the ipsilateral motor nucleus of the trigeminal nerve in the pons 2 days postinoculation into the masseter muscle (A). Antigen was not found in the ipsilateral trigeminal ganglion 1 day postinoculation with CVS virus (B), but it was found in multiple neurons postinoculation with RV194-2 virus (C). Antigen was present in the ipsilateral motor nucleus of the trigeminal nerve 1 day postinoculation with CVS virus (D) and RV194-2 virus (E). Antigen was present in the brain stem bilaterally 3 days postinoculation with CVS virus (F) and RV194-2 virus (G). Antigen was found in the left (H and J) and right (I and K) trigeminal ganglia 3 days postinoculation into the right masseter muscle with CVS virus (H and I) and RV194-2 (J and K). The trigeminal ganglia were infected bilaterally, indicating centrifugal spread. Immunoperoxidase-hematoxylin staining was used. Magnifications: A, $\times 45$; B and C, $\times 110$; D and E, $\times 85$; F and G, $\times 20$; H to K, $\times 90$.

CVS virus produced widespread infection of the cerebral cortex on day 4 (Fig. 2E), and RV194-2 virus infected relatively few cortical neurons at this time. On subsequent days, the cortical involvement by CVS virus was also greater than that by RV194-2 virus (Fig. 2C and D). CVS virus produced extensive infection of the cerebral cortex before involvement of the hippocampus, which did not usually become infected until day 6 (Fig. 2E). There were very few infected neurons after day 6 in RV194-2 virus infection (Fig. 2F and G). No differences in the distribution of rabies virus antigen were noted in RV194-2 virus infection between mice that were hunched and mice without neurologic signs.

This study provides strong evidence that rabies virus spreads by a motor pathway from the masseter muscle to the ipsilateral motor nucleus of the trigeminal nerve. This pathway was taken by both CVS virus and the variant, RV194-2 virus. The entry of these viruses at motor end plates (11) and their transport in axons of motor neurons must proceed in a similar fashion. After inoculating rabbits in the masseter muscle with street rabies virus, Goodpasture (5) also found histopathologic evidence of early involvement of the ipsilateral motor nucleus of the trigeminal nerve. Martin and Dolivo (10) inoculated herpesvirus suis into the masseter muscles of rats and found early infection of the ipsilateral motor nucleus of the trigeminal nerve. Hence, this motor pathway is also important for other neurotropic viruses.

RV194-2 virus caused early infection of neurons in the ipsilateral trigeminal ganglion, but CVS virus did not. In contrast, both CVS virus and avirulent variant AvO1, which has the same amino acid substitution at position 333 of the glycoprotein as RV194-2 virus (12), spread to the ipsilateral trigeminal ganglion after inoculation into the anterior chambers of the eyes of rats (8). Similarly, both CVS virus and AvO1 virus spread to local dorsal root ganglia after intramuscular inoculation into the forelimbs of mice (1). It is unclear whether the lack of spread of CVS virus along the sensory trigeminal pathway in this study is related to the site of inoculation, the species or strain of the host, the biologic characteristics of the variant virus, or the viral dose administered. Unlike AvO1 virus (1), RV194-2 virus infected the contralateral trigeminal ganglion, indicating that it retained the ability to spread centrifugally from the infected brain stem.

RV194-2 virus maintained its ability to spread to distant sites in the CNS. It infected many fewer neurons in the CNS than did CVS virus. A significant temporal delay in the involvement of various CNS sites was not observed with RV194-2 virus compared with CVS virus, indicating that spread did not proceed at a slower rate. Infection developed in the brain stem at the same time for both viruses, and centrifugal spread to the trigeminal ganglia occurred at similar rates. This contrasts with the observations of Dietzschold et al. (2) after intracerebral inoculation. The transport mechanisms of these two viruses are probably not significantly different. There is strong *in vitro* (9, 15) and *in vivo* evidence that rabies virus spreads by fast axonal transport in both the peripheral nervous system (14) and the CNS (4).

Although the spread of CVS virus and RV194-2 virus was similar between the masseter muscle and the motor nucleus of the trigeminal nerve in the pons, spread was less efficient for RV194-2 virus than CVS virus between CNS neurons. A smaller number of CNS neurons became infected, although their topographic distributions in the CNS were similar. It is unclear whether the less efficient spread of RV194-2 virus between neurons occurs at the level of interaction of the

virus with receptors, penetration into neurons, replication, or maturation. Further studies comparing the infections caused by these viruses at the cellular level are needed.

I thank William H. Wunner (Wistar Institute) for variant virus RV194-2 and K. M. Charlton (Animal Diseases Research Institute) for anti-rabies virus serum. The technical assistance of Dorothy Reimer and secretarial assistance of Martha Steacy are gratefully acknowledged.

This work was supported by grant MA-10068 from the Medical Research Council of Canada and the Violet E. Powell Fund (Queen's University).

REFERENCES

- Coulon, P., C. Derbin, P. Kučera, F. Lafay, C. Prehaud, and A. Flamand. 1989. Invasion of the peripheral nervous systems of adult mice by the CVS strain of rabies virus and its avirulent derivative AvO1. *J. Virol.* **63**:3550-3554.
- Dietzschold, B., T. J. Wiktor, J. Q. Trojanowski, R. I. MacFarlan, W. H. Wunner, M. J. Torres-Anjel, and H. Koprowski. 1985. Differences in cell-to-cell spread of pathogenic and apathogenic rabies virus *in vivo* and *in vitro*. *J. Virol.* **56**:12-18.
- Dietzschold, B., W. H. Wunner, T. J. Wiktor, A. D. Lopes, M. Lafon, C. L. Smith, and H. Koprowski. 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. *Proc. Natl. Acad. Sci. USA* **80**:70-74.
- Gillet, J. P., P. Derer, and H. Tsiang. 1986. Axonal transport of rabies virus in the central nervous system of the rat. *J. Neuropathol. Exp. Neurol.* **45**:619-634.
- Goodpasture, E. W. 1925. A study of rabies, with reference to a neural transmission of the virus in rabbits, and the structure and significance of Negri bodies. *Am. J. Pathol.* **1**:547-584.
- Jackson, A. C. Analysis of viral neurovirulence. *In* J. Brosius and R. T. Freneau (ed.), *Molecular genetic approaches to neuropsychiatric diseases*, in press. Academic Press, Inc., Orlando, Fla.
- Jackson, A. C., and D. L. Reimer. 1989. Pathogenesis of experimental rabies in mice: an immunohistochemical study. *Acta Neuropathol.* **78**:159-165.
- Kucera, P., M. Dolivo, P. Coulon, and A. Flamand. 1985. Pathways of the early propagation of virulent and avirulent rabies strains from the eye to the brain. *J. Virol.* **55**:158-162.
- Lycke, E., and H. Tsiang. 1987. Rabies virus infection of cultured rat sensory neurons. *J. Virol.* **61**:2733-2741.
- Martin, X., and M. Dolivo. 1983. Neuronal and transneuronal tracing in the trigeminal system of the rat using the herpes virus suis. *Brain Res.* **273**:253-276.
- Murphy, F. A. 1985. The pathogenesis of rabies virus infection, p. 153-169. *In* H. Koprowski and S. A. Plotkin (ed.), *World's debt to Pasteur, Proceedings of a Centennial Symposium Commemorating the First Rabies Vaccination Held at the Children's Hospital of Philadelphia, January 17-18, 1985*, sponsored by The Wistar Institute, Philadelphia, Pa. Alan R. Liss, Inc., New York.
- Seif, I., P. Coulon, P. E. Rollin, and A. Flamand. 1985. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. *J. Virol.* **53**:926-935.
- Torres-Anjel, M. J., J. Montano-Hirose, E. P. I. Cazabon, J. K. Oakman, and T. J. Wiktor. 1984. A new approach to the pathobiology of rabies virus as aided by immunoperoxidase staining, p. 1-26. *American Association of Veterinary Laboratory Diagnosticians, 27th Annual Proceedings. American Association of Veterinary Laboratory Diagnosticians, Madison, Wis.*
- Tsiang, H. 1979. Evidence for an intraaxonal transport of fixed and street rabies virus. *J. Neuropathol. Exp. Neurol.* **38**:286-296.
- Tsiang, H., E. Lycke, P.-E. Ceccaldi, A. Ermine, and X. Hirardot. 1989. The anterograde transport of rabies virus in rat sensory dorsal root ganglia neurons. *J. Gen. Virol.* **70**:2075-2085.