Degeneration of Neuronal Processes after Infection with Pathogenic, but Not Attenuated, Rabies Viruses

Xia-Qing Li, Luciana Sarmento, and Zhen F. Fu

Department of Pathology and Department of Infectious Diseases, University of Georgia, Athens, Georgia

Received 9 December 2004/Accepted 18 April 2005

Despite extensive investigation, the mechanism by which rabies virus (RV) infection causes neurological disease and death is still not completely understood (3). RV enters the peripheral nervous system at the bite site by binding to one or more specific neural receptors (14, 22, 25) with or without local replication (19, 21). Once inside neurons, RV is spread by retrograde transport to the spinal cord and then to the brain (4, 12). Clinical signs include severe agitation, depression, hydrophobia, and paralysis followed by impaired consciousness and coma (9). Patients eventually die of circulatory insufficiency, cardiac arrest, and respiratory failure (9, 23). Despite the dramatic and severe clinical course, postmortem examination of rabies patients reveals only a few pathological lesions, such as cerebral edema (18). Inflammatory reactions and other histological lesions are mild with relatively little neuronal loss (15, 18). These observations led to the hypothesis that fatal rabies may result from neuronal dysfunction rather than neuronal damage (24).

Studies of neuronal dysfunction have revealed electroencephalographic abnormalities, including the disappearance of rapid eye movement sleep and the development of sleep-related myoclonus (6). Brain electrical activity terminated about 30 min before cardiac arrest, indicating that cerebral death in experimental rabies occurs prior to failure of vegetative functions (6, 7). RV infection of neurons also induces dysfunction of ion channels, for example, reduction in sodium channels and inward rectifier potassium channels (10, 11), which could prevent infected neurons from firing action potentials and generating synaptic potentials, resulting in functional impairment. Decreased binding of serotonin (particularly the subtype 5-HT1D) to its receptors has also been reported (2). Recent studies of the release of norepinephrine, dopamine, and serotonin in the hippocampi of rats infected with RV indicated that at the terminal stage of the disease, neurons are no longer capable of releasing neurotransmitters at the synaptic junctions (5). Hence, there is evidence of impaired release of neurotransmitters and binding of neurotransmitters to the receptors, which may result in neuronal dysfunction in patients infected with rabies. To investigate the basis of neuronal dysfunction, we studied the morphological alteration of neuronal processes after RV infection and found degeneration of neuronal processes after infection with pathogenic, but not attenuated, RVs.

In the present study, two viruses were used to determine whether RV infection induces degeneration in neuronal processes. One was the pathogenic N2C virus that is derived from CVS-24 by passaging in neuroblastoma cells (16) and the other was the attenuated SN-10 virus derived from the SAD B19 vaccine strain (20). Initially, we compared the pathogenicities of these two viruses by determining the intracerebral pathogenic indices as described previously (16). The pathogenic indices were found to be 0.38 and 0.00066 for N2C and SN-10, respectively, indicating that 500 times more viral particles are required for SN-10 than for N2C to kill infected animals. This suggests that N2C is more pathogenic than SN-10 in the mouse model. To investigate RV-induced changes in neuronal processes, 4- to 6-week-old ICR mice were infected by intracranial injection with each of the two viruses at 10% intracerebral lethal doses. Brain samples were harvested by transcardial perfusion with 10% formaldehyde when infected mice developed severe seizures and/or paralysis (26). Serial brain sections (40 μm) were obtained by cryostat, and these sections were used for immunohistochemistry to detect viral antigen by using anti-RV nucleoprotein antibody 802-2 (8) or for silver staining to examine the morphologies of neurons and neuronal processes. Paraffin sections were prepared and stained with hematoxylin-eosin (HE) for examination of histological changes.

RV antigen was detected in almost all brain areas, particularly in the hippocampus (data not shown). Thus, we selected adjacent hippocampal sections for morphological studies. HE staining showed pathological changes of pyramidal neurons, including necrosis, apoptosis, and neuronal loss, particularly in animals infected with SN-10 (Fig. 1C). Very few lesions were observed in N2C-infected mice, and neuronal bodies were normal, at least morphologically (Fig. 1B), compared to those of...
FIG. 1. Degeneration of neuronal process in the hippocampi of mice infected with pathogenic RV. Mice were infected with pathogenic virus N2C or attenuated virus SN-10 and were transcardially perfused with 10% formalin when moribund. Consecutive hippocampal sections were subjected to HE staining (A through C), silver staining (D through F), and immunohistochemistry for detection of viral antigen (G through I). Magnification, ×40. CON, sham-infected controls.
uninfected controls (Fig. 1A). However, obvious destruction of neuronal processes in N2C-infected animals was observed by silver staining (Fig. 1E). The processes (axons and dendrites) showed severe disorganization and various degrees of degradation. In contrast, neuronal processes in SN-10 infected (Fig. 1F) or sham-infected mice (Fig. 1D) did not show disorganization or degradation. When brain sections were stained with anti-RV antibodies, viral antigens were found in the neuronal bodies, as well as in the processes (Fig. 1H and I). The neuronal processes in mice infected with SN-10 were clearly labeled, whereas the normal arbor-like structure was not seen in mice infected with N2C. All these results indicate that pathogenic RV induced degeneration of neuronal processes despite very little pathology in neuronal bodies. On the other hand, more severe lesions were found in the neuronal bodies, but not in the processes, of animals infected with attenuated SN-10.

To determine if degeneration of neuronal processes is due to direct virus infection or inflammatory reactions, we detected and quantified CD3-positive cells by using anti-CD3 antibodies (Abcam, England) in the hippocampal sections. Three serial sections of each mouse were selected for quantification, and the average numbers of CD3-positive cells were obtained for statistical analysis by one-way analysis of variance. The average numbers of CD3-positive cells \( \pm \) standard deviations are 0.83 \( \pm \) 0.4 (Con), 39.5 \( \pm \) 13 (N2C), and 167.7 \( \pm \) 6.8 (SN-10).

![FIG. 2. Numbers of CD3-positive neurons in brains infected with RV. Hipocampal sections were made for immunohistology to quantify CD3-positive cells by using anti-CD3 antibodies. Three serial sections of each mouse were selected for quantification, and the average numbers of CD3-positive cells obtained for statistical analysis by one-way analysis of variance. The average numbers of CD3-positive cells \( \pm \) standard deviations are 0.83 \( \pm \) 0.4 (Con), 39.5 \( \pm \) 13 (N2C), and 167.7 \( \pm \) 6.8 (SN-10).](http://jvi.asm.org/)

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cesses. In those neuronal processes in which viral antigens are detected, MAP-2 staining disappeared. Likewise, viral antigens were not detected in cells showing MAP-2 staining. However, viral antigen staining did overlap with MAP-2 staining in neurons infected with SN-10 (Fig. 4B, panel i). Similar patterns were observed when the cells were stained with antiviral and anti-NF-200 antibodies. Positive staining of NF-200 was detected in control neurons including neuronal bodies as well as in the processes (Fig. 4C, panel a). In cells infected with N2C, a dramatic reduction in NF-200 staining was observed (Fig. 4C, panel d). In cells infected with SN-10, no reduction of NF-200 staining was seen although an abnormal pattern was apparent (Fig. 4C, panel g). When costaining for NF-200 and viral antigen, no overlapping in cells infected with N2C was found with NF-200 (Fig. 4C, panel f). In cells in which viral antigens were detected, NF-200 staining disappeared. Likewise, viral antigens were not detected in cells showing NF-200 staining. Neurons infected with SN-10 showed overlapping staining of viral antigen and NF-200 (Fig. 4C, panel i).

Despite the dramatic clinical expression in rabies patients,
pathological changes are usually mild with little neuronal destruction (15, 18), which has led to the hypothesis that neuronal dysfunction rather than structural damage may be responsible for the development of rabies (24). Neuronal dysfunction with regard to ion channels (10, 11), reduction of neurotransmitter release and uptake for gamma aminobutyric acid, noradrenaline, serotonin, and dopamine, and reduced binding of serotonin to its receptor has been reported (5, 13). In the present study, we examined the morphological alteration of neuronal processes after RV infection and found degeneration of neuronal processes and disruption of synaptic formation after infection with pathogenic, but not attenuated, RV. These findings may form the basis for neuronal dysfunction to occur in RV-infected individuals.

The structural integrity of neuronal processes (axons and dendrites) is important for neurotransmission, thus maintaining optimal neuronal function (17). Our study shows that infection with pathogenic, but not attenuated, RV induces degeneration and destruction of neuronal processes under both in vivo and in vitro conditions. Neuronal processes in mice infected with the pathogenic N2C virus showed obvious disorganization and various degrees of degradation. In the EM, both the transverse and longitudinal sections for the axonal and dendrite processes appeared severely disorganized or disappeared altogether. In addition, intracellular organelles such as RER and free ribosome disappeared almost completely, and only a few mitochondria were seen. The destruction and degeneration of neuronal processes can also be observed in pri-
mary neuronal cultures infected with the pathogenic RV. However, degeneration of neuronal processes in mice or neurons infected with the attenuated SN-10 virus was not detected. Instead, apoptotic and necrotic neuronal bodies were found in mice infected with attenuated SN-10. Furthermore, significantly more inflammatory cells are detected in mice infected with SN-10. It is thus possible that the pathogenic mechanisms by which the pathogenic and attenuated RV induces neurological diseases are different. Pathogenic RV induces degeneration of neuronal processes, while attenuated RV induces inflammations and, consequently, neuronal apoptosis and necrosis (15).

It is interesting to observe that MAP-2 and neurofilament immunoreactivity disappeared completely in neurons infected with pathogenic N2C virus and no overlapping staining of viral antigen and MAP-2 or NF-200 was observed. In contrast, neurons infected with vaccine strain SN-10 showed overlapping staining of viral antigen and MAP-2 or NF-200. These data, together with the morphological studies, suggest that pathogenic RV infects neurons and induces degenerative changes in the neuronal processes by disrupting cytoskeletal integrity. Without the integrity of the processes, neuronal transmission cannot be accomplished. Thus, our results may form the basis for the neuronal dysfunction found in RV-infected individuals (6, 7, 24). It is not known why only the pathogenic RV, but not the attenuated RV, induced degeneration of neuronal processes. Furthermore, there are more CD3-positive cells found in the hippocampi of mice infected with the attenuated virus than in mice infected with pathogenic virus, thus excluding the possibility that inflammatory cell infiltration is the cause of degeneration of neuronal processes. Thus, further studies to determine the mechanisms by which pathogenic RV induces degeneration of neuronal processes while keeping neuronal bodies intact are warranted.

This work is supported partially by Public Health Service grant AI-051560 from the National Institute of Allergy and Infectious Diseases (Z.F.F.).

We thank Charles E. Rupprecht at the Centers for Disease Control and Prevention for supplying anti-N monoclonal antibody 802-2 and Mary Ard in the Department of Pathology for help with the EM preparation.

REFERENCES