Pathology of the Spinal Cord of C57BL/6J Mice Infected with Rabies Virus (CVS-11 Strain)

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ABSTRACT. Fixed rabies viruses (CVS-11 strain) were inoculated intramuscularly to C57BL/6J mice, and the pathomorphological changes of the spinal cord including dorsal root spinal ganglion cells were investigated. At 4 days postinoculation (PI), viral antigens were first detected in the spinal neurons and dorsal root spinal ganglion cells without producing morphological changes. At 5 days PI, mild infiltration of lymphocytes was observed around the central canal, small blood vessels and leptomeninges. Cells positive to anti-Iba1 and anti-GFAP antibodies increased significantly from 3 to 5 days PI, respectively. Microglia changed their morphological forms to be ramified or amoeboid, and astrogliosis extended their cytoplasm from the leptomeninges to the parenchyma. At 7 days PI, apoptotic cells were found in the spinal cord and dorsal root spinal ganglion using TUNEL. We confirmed that most of T lymphocytes and a minority of microglial cells underwent apoptosis, using a combination of TUNEL and immunostaining with antibodies to viral phosphoprotein, CD3, Iba1 and GFAP. On the other hand, astroglial cells and virus-infected nerve cells were negative against TUNEL and cleaved caspase-3 antibody. These findings indicate that T lymphocytes and microglial cells died by apoptosis, whereas virus-infected nerve cells died by necrosis. This was accompanied by increased numbers and morphological changes of glial cells associated with the pathogenesis of CVS-11 in the C57BL/6J mouse.

KEY WORDS: experimental study, mouse spinal cord, pathogenesis, rabies (CVS-11).

FULL PAPER

Pathology

Rabies is a neurotropic virus that causes fatal encephalomyelitis in humans and animals. It enters the nervous system via a motor neuron through the neuromuscular junction or via a sensory nerve through nerve spindles after replication at the infection site. It then travels along the spinal cord to the brain before spreading to the salivary glands [5, 12, 16]. Most neurons in the brain and spinal cord of patients infected with street viruses are intact and have few abnormalities in organelle structure or cell to cell relationships [18, 20]. On the other hand, fixed viruses cause widespread damage to neurons in the brain and spinal cord in laboratory rodents. This difference is probably related to the mechanism of neuronal dysfunction, the mode of viral spread in the brain and the nature of the stimulus for inflammatory infiltration. The challenge virus standard strain (CVS-11) is widely used for the study of rabies pathogenesis because it has a low infection risk for human compared with wild type rabies virus, and shows strong neurotropism for experimental animals [4, 14, 24]. This virus causes severe neuronal apoptosis in the hippocampus, cerebral cortex and cerebellum after intracerebral inoculation in the mouse [13, 15, 24–26]. On the other hand, spinal neurons infected with the virus are resistant to apoptosis when the virus is inoculated into the footpad [11] or into hind limb muscles, which are early targets of the rabies virus [2]. Previously, we reported that virally infected neurons and inflammatory cells increased as the infection progressed and that there are different pathways of cell death between neurons and inflammatory cells after infection [24]. To obtain further information about the pathogenesis of rabies, CVS-11 was injected into mice intramuscularly and the localizations of viral antigens in the spinal cord and dorsal root spinal ganglion cells were investigated. Cell types undergoing apoptosis were identified using double labeling immunohistochemistry. Finally, we will discuss the roles of increased glial cells and their morphological changes during pathogenesis in CVS-11 infected mice.

MATERIALS AND METHODS

Virus, mice and viral inoculation: The fixed rabies strain (CVS-11) was obtained from Dr C. E. Rupprecht (Rabies section, Virus and Rickettsia Zoonoses Branch, Center for Disease Control and Preservation, Atlanta, GA, U.S.A.) and was replicated in mouse A/J (H-2a) neuroblastoma cells. Twenty-one specific pathogen-free female C57BL/6J mice (SLC, Inc, Shizuoka, Japan), six weeks old, were purchased and injected in the right triceps surae muscle with 10^7 plaque-forming units of the CVS-11 virus strain suspended in phosphate-buffered saline (PBS, pH 7.4). Three mice were inoculated with PBS as negative controls. The mice were observed daily for their neurological condition and killed at 2, 3, 4, 5, 7, 9 and 11 days postinoculation (PI), (three mice per day). All experiments were performed in Level 2 Biosafety laboratories according to the Committees on Biosafety and Animal Handling and Ethical Regulation...
of the National Institute of Infectious Disease, Japan. Animal care, breeding, virus inoculation and observation were performed in accordance with the guidelines of institutional committees.

Necropsy and preparation of tissue sections: Each mouse was anesthetized with chloroform and perfused transcardially with 10-15 ml of PBS followed by freshly prepared 4% paraformaldehyde in 0.1 M PBS. Spinal samples were removed and fixed in 4% paraformaldehyde at room temperature for less than 24 hr and were decalcified in K-CX (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) solution. Transverse sections of spinal cords at C3-4, T1-3, L1-2 and S1-3 were prepared. Complete series of paraffin sections about 3 μm thick were cut and mounted on glass slides. Serial sections were subjected to hematoxylin and eosin (HE) staining, to immunohistochemistry and to TUNEL assay.

Immunohistochemistry: For detection of the rabies virus antigens in tissues, all sections were stained using the streptavidin-biotin-peroxidase complex (LSAB) method (Nichirei Biosciences, Tokyo, Japan) using rabbit anti-phosphoprotein (P) [28] and anti-nucleoprotein (N) antibodies [10, 24]. For the detection of each cell type, the following primary antibodies were used: anti-glial fibrillary acidic protein (GFAP) for astroglial cells (Nichirei Biosciences); anti-ionized calcium binding adaptor molecule 1 (Iba1) for microglial cells (Wako, Osaka, Japan); anti-CD3 for T lymphocytes (DAKO, Kyoto, Japan); anti-CD20 for B lymphocytes (Spring Bioscience, Fremont, CA, U.S.A.) and anti-activated caspase-3 for apoptotic cells (Cell Signaling Technology, Inc., Beverley, MA, U.S.A.). Immunohistochemistry was performed essentially as described [24]. Briefly, tissue sections were treated for the activation of antigens with 0.25% trypsin at room temperature (RT) for 30 min for anti-N and anti-P, by microwaving at 750W for 5 min for anti-CD3 and anti-CD20, or in a water bath at 95°C for 15 min for anti-Iba1 and anti-cleaved caspase-3.

To remove endogenous peroxidase activity, sections were treated with 0.3% H2O2 in methanol for 30 min at RT. Then, sections were treated with 20 mg/ml proteinase-K in 0.1 M PBS for 15 min at RT to activate antigens. After washing in PBS, sections were covered with 50 μl of the TUNEL reaction mixture (Chemicon, Temecula, CA, U.S.A.), which containing terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP, and incubated under a coverslip in a humidified chamber for 1 hr at 37°C. The reaction was stopped by washing slides in PBS. Slides were then incubated with anti-digoxigenin peroxidase for 30 min at RT. After a 15 min wash in PBS, sections were stained with hematoxylin.

Double or triple labeling by combination of TUNEL and immunostaining: Double labeling of a single tissue section was used for analysis of apoptosis and the coexpression of antigens. Firstly, TUNEL assays were performed as above, and slides were incubated with DAB until color developed. The reaction was stopped by washing the slides in distilled water, and then antibodies to P, CD3, Iba1 or GFAP were added. Histofine® Simple Stain AP (Nichirei Biosciences) was used as a secondary antibody. A color reaction was developed with Histofine® New Fuchsin (Nichirei Biosciences). For triple labeling, anti-P, Iba1 and GFAP antibodies were visualized with New Fuchsin, BCIP/NBT substrate solution (DAKO) and DAB, respectively. The slides were counterstained with hematoxylin and mounted for light microscopy.

Electron microscopy: After fixation in 4% paraformaldehyde (pH 7.4), the spinal cord and dorsal root spinal ganglia were removed under a dissection microscope (SMZ-10, Nikon, Tokyo, Japan) and postfixed in 0.5% glutaraldehyde. Samples were cut into ~1 mm³ blocks, fixed in 1% buffered osmium tetroxide and embedded in epoxy resin. Sections (~70 nm) were stained with uranyl acetate and lead citrate and were examined using a transmission electron microscope (H-700, Hitachi, Tokyo, Japan).

Counts of TUNEL and immunostained cells in the cervical spinal cord and spinal ganglion: All of the TUNEL-positive cells and those immunostained for cleaved caspase-3, P, Iba1, GFAP and CD3 were counted in the cervical spinal cord and spinal ganglion at 2, 5, 9 days PI (three mice per day).

RESULTS

Clinical signs and macroscopic findings: Mice showed hind limb paralysis at 5 days PI, quadriplegia at 8 days PI and died at 11 days PI. No macroscopic findings were found at necropsy throughout the experimental period.

Histology and immunohistochemistry: Mild infiltrations of lymphocytes under the leptomeninges and perivascular cuffing of mononuclear cells in the gray matter of the spinal cord was observed from 5 days PI. Dorsal root spinal gan-
glion cells with nuclear pyknosis and eosinophilic cytoplasm appeared, and occasional mononuclear cells surrounded these degenerated gangliocytes from 5 days PI (Fig. 1). From 7 days PI, more advanced lesions appeared, including increased microglial cells and numerous apoptotic-like features, necrosis of nerve cells and neuronophagia. Histological lesions were always associated with the presence of viral antigens in the cytoplasm of neurons. The numbers of virus antigens, glial cells, inflammatory cells and apoptotic cells in spinal cord and spinal ganglion was summarized in Table 1. Viral antigen positive cells were first detected in the gray matter of the lumbar cord and dorsal root spinal ganglion cells at 4 days PI and then spread throughout the spinal cord at 5 days PI. Viral antigens were first detected as small spots in the cytoplasm of nerve cells, and as infection progressed, they were detected in the dendrites and axons (Fig. 2). Iba1 positive cells increased significantly in the gray matter of spinal cords from 3 to 5 days PI, and they increased to uncountable levels at 11 days PI. In addition, these microglia changed their morphology from small rod cells to ramified or amoeboid cells at 5 days PI (Fig. 3). At 2 days PI, a few GFAP positive cells were mainly located under the leptomeninges and around the central canal of the spinal cord. At 5 days PI, astroglial cells elongated to reach the deep white matter of the spinal cord, and simultaneously many protoplasmic astroglial cells were observed in the gray matter (Fig. 4). As infection progressed, the numbers of GFAP positive cells increased, and they changed to a ramified shape at 9 days PI. The localization of nerve cells, microglial cells and astroglial cells in the spinal cord was more clearly confirmed by triple labeling for P, Iba1 and GFAP antibodies (Fig. 5). At 5 days PI, CD3 positive T lymphocytes appeared under the leptomeninges, around blood vessels and dorsal root spinal ganglion cells and increased as infection progressed. CD20 positive cells were not detected.

**Detection of apoptotic cells by TUNEL assays and cleaved caspase-3 antibody:** In the spinal cord, TUNEL and cleaved caspase-3 antigen positive microglial cells and lymphocytes were found under the leptomeninges and white matter (Fig. 6) and around the small blood vessels. On the other hand, TUNEL positive lymphocytes were only observed in dorsal root spinal ganglions at 5 days PI. The numbers of apoptotic cells increased as infection progressed. However, the nerve cells of the spinal cord and dorsal root spinal ganglion cells were negative for TUNEL and cleaved caspase-3 antibody until 9 days PI.

**Identification of apoptotic cells by double staining:** For coexpression analysis of TUNEL and each of the primary antibodies, most T lymphocytes and a minority of microglial cells showed apoptosis in the spinal cord (Fig. 7). T lymphocytes also showed apoptosis in the dorsal root spinal ganglia, whereas virus-infected spinal nerve cells and dorsal root spinal ganglion cells were negative. Positive reactions for Iba1 and GFAP were not observed in the dorsal root spinal ganglion cells throughout the experimental period.

**Electron microscopy:** Until 5 days PI, most nerve cells were intact. Virus particles and various sized matrices were observed in the perikarya of spinal gray matter neurons and dorsal root spinal ganglion cells (Fig. 8). These matrices were round to oval in shape and consisted of granular material and tubular nucleocapsid structures. Most matrices were located in the cell margins and interspersed among organelles of the perikaryon, especially the rough endoplasmic reticulum (Nissl bodies). Occasionally, virus particles and matrix were observed in the plasma membranes of myelinated axons. No virus particles or nucleocapsid matrices were observed in the nonneuronal satellite cells or glial cells.

At 7 days PI, a few viral infected spinal nerve cells and dorsal root spinal ganglion gangliocytes displayed cytopathological changes such as the accumulation of Nissl bodies, a dark appearance, shrunken nuclei, irregular bundles of fine fibers, and cytoplasmatic vacuolization containing swollen mitochondria. By contrast, mononuclear cells surrounding the infected spinal nerve cells and dorsal root spinal ganglion cells showed characteristic morphological features of apoptosis, including well-demarcated chromatin masses and nuclear fragmentation (Fig. 9).

**DISCUSSION**

Rabies virus infection result in 2 syndromes: encephalitic disease and paralytic disease. However, little explanation has been provided that would account for these diverse manifestations of infection with a common agent. In experimen-
In transgenic models, clear hind limb paralysis is only observed in an immunocompetent mouse, whereas immunosuppressed and athymic mice never show paralysis [11]. Other reports described that limb paralysis results from a peripheral immunopathological process in which both the CD4 and CD8 subsets of T lymphocytes are implicated, and which causes degeneration of the motor neurons of the local sciatic nerve [31]. In this study, viral antigen was first detected in the grey matter of lumbar cord and dorsal root spinal ganglion cells at 4 days PI, and limb paralysis appeared at the same time as the numbers of virus infected nerve cells and inflammatory cells increased at 5 days PI. These findings indicate that the virus reached the spinal cord and dorsal root ganglion cells in a retrograde direction via a motor neuron or sensory nerve following replication in the muscles [21, 24], and activation of immune responses in the spinal cord and
the dysfunction of virus infected spinal neurons were prece-
dence of paralytic manifestations.

In the spinal cord, virus infected nerve cells showed
necrotic features [23] by light and electron microscopy, and
they were negative for cleaved caspase-3 antibody and
TUNEL assays. On the other hand, the numbers of lympho-
cytes and microglial cells increased significantly as the
infection progressed, and most of T lymphocytes and a
minority of microglial cells showed apoptosis using double-
labeling immunohistochemistry. Baloul et al. [2] reported
that a limited number of infected neurons die by apoptosis in
mice infected with CVS-11, whereas 75% of CD3 T lym-
phocytes are apoptotic compared with only 5% in attenuated
strain (PV) infected spinal sections. These differences in
pathogenicity are related to the neurotoxic and neuroprotec-
tive function of TNF-α, interleukin(IL)-6 and CD3 T lym-
phocyte-mediated Fas/Fas ligand apoptotic pathways
expressed within infected neurons and lymphocytes [1, 2].

In the dorsal root spinal ganglia, migration of microglial
and astroglial cells from spinal cords was not observed until
a late stage of infection. T lymphocytes surrounding the
dorsal root spinal ganglion gangliocytes showed apoptosis
by electron microscopy and TUNEL assays but were nega-
tively stained for anti-caspase antibody. Therefore, it was
suggested that apoptosis of T lymphocytes between the spi-
nal cords and dorsal root spinal ganglia are induced by dif-
f erent pathways.

Within the central nerve system (CNS), microglial cells are
tissue macrophages with dual neuroprotective and detri-
tmental functions and acts as scavenger cells by phagocy-
tosing dead cells [3, 19, 29, 30]. Microglial cells changes
their morphological forms such as ramified and amoeboid
types after CNS injury [3, 29, 30], and they induce neuro-
protection by releasing trophic factors and neuroprotective
substances such as plasminogen and IL-6 [9]. On the other
hand, the release of cytokines and inflammatory molecules
such as IL-1 [8], TNF-α [27] and nitric oxide [17] causes
neuronal cell death. In the present study, microglial cells
were significantly increased and changed their morphologi-
ical forms in the spinal cord from 3 days PI. These findings
suggest that inflammatory cytokine released by activated
microglial cells and related to the necrosis of spinal neurons.
In the present study, fibrous and protoplasmic astroglial

Fig. 1. Lumbar spinal ganglion. Nuclear pyknosis and eosinophilic cytoplasm were observed in the dorsal root spinal ganglion cells (arrowhead) at 9 days PI. Hematoxylin and eosin staining. Bar=20 μm.

Fig. 2. Lumbar spinal ganglion. Viral antigens were detected in the cytoplasm (arrowhead) and axon (arrows) of dorsal root spinal ganglion cells at 5 days PI. Immunohistochemistry (anti-N antibody). Bar=20 μm.

Fig. 3. Cervical spinal cord. Many microglial cells were detected in the gray matter of spinal cord at 9 days PI, and they changed their morphology to be ramified or amoeboid shapes. Immunohistochemistry (anti-Iba1 antibody). Bar=30 μm.

Fig. 4. Thoracic spinal cord. Protoplasmic astroglial cells showed ramified shapes in the gray matter of the spinal cord at 9 days PI. Immunohistochemistry (anti-GFAP antibody). Bar=30 μm.

Fig. 5. Thoracic spinal cord. Many microglial cells (dark blue), astroglial cells (brown) and viral antigens (red) were observed in the gray matter at 7 days PI. Triple stain. Bar=30 μm.

Fig. 6. Cervical spinal cord. Cleaved caspase-3 immunopositive cells were observed in the white matter of spinal cord (arrows) at 9 days PI. Immunohistochemistry. Bar=30 μm.

Fig. 7. Cervical spinal cord. Microglial cells (arrow) in the spinal cord were positively stained both with TUNEL (brown) and with anti-Iba1 antibody (red) at 7 days PI. Double staining. Bar=20 μm.

cells increased in the spinal cord from 5 days PI. Subse-
quently, the numbers of necrotic neurons increased as infec-
tion progressed. Astroglial cells perform essential roles for
preservation of neuronal tissues and restriction of inflamma-
tion by produce cytokines and neurotrophic factors after CNS injury [6, 7, 22], however in the present study, the direct roles of glial cells in the pathogenesis of CVS-11 infected mice remains unresolved, therefore, further experimental studies are required.

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