Lesions of the Central Nervous System Induced by Intracerebral Inoculation of BALB/c Mice with Rabies Virus (CVS-11)

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ABSTRACT. BALB/c mice were inoculated intracerebrally with fixed rabies virus (CVS-11) and pathomorphological changes in the central nervous system were studied. Infected mice showed ruffled hair, hunchback, anorexia, emaciation and ataxia at 5 days postinoculation (DPI), but paralysis did not occur. Viral antigens were first detected in the pyramidal cells of the cerebral cortex and hippocampus at 3 DPI, and these cells exhibited apoptosis at 5 DPI. Microglial cells and astroglial cells significantly increased in the areas of the nerve cells which showed apoptosis. However, spinal neurons and spinal dorsal root ganglion cells did not exhibit apoptosis despite virus infection. These observations indicate that different mechanism which causes apoptosis exists among the neurons of the brain and spinal cord, and glial cells play an important role in pathogenesis of the experimental rabies.

KEY WORDS: BALB/c mice, intracerebral inoculation, pathogenesis, rabies virus (CVS-11).

Rabies is an ancient disease that is still endemic in many parts of the world and is a serious public health problem in developing countries, especially in Asia. Approximately 55,000 human deaths are caused by rabies each year [9, 10]. An understanding of the pathogenesis of rabies is important for developing novel therapies and preventive measures for rabies, which may be fatal for humans and animals.

Previously, we demonstrated that C57BL/6J mice inoculated intramuscularly with CVS-11 showed paralysis with severe spinal lesions [15]. The spinal lesions were composed of the infiltration of T lymphocytes, the increase of microglial cells and astroglial cells and the majority of T lymphocytes exhibited apoptosis. In contrast, marked neuronal apoptosis was detected in the cerebral cortex, hippocampus and cerebellum of mice that were inoculated intracerebrally with CVS-11, but paralysis was absent [21]. Induction of apoptosis by fixed strain of rabies virus has been reported in vitro [19] and experimental animal models [11, 13] to be associated with the expression level of the virus protein. On the other hand, the street strain of human rabies virus, which may be fatal for humans and animals.

To obtain the more information about pathogenesis of rabies in mice, CVS-11 was inoculated into the cerebrum of BALB/c mice and the target cells of apoptosis and the kinetics of glial cells in the CNS were studied during the infection.

MATERIALS AND METHODS

Virus, mice and viral inoculation: The CVS-11 strain of fixed rabies virus, which was obtained from Dr. C. E. Rupprecht (Rabies Section, Virus and Rickettsia Zoonooses Branch, Centers for Disease Control and Preservation, Atlanta, GA, U.S.A.), was grown in mouse A/J (H-2a) neuroblastoma cells as previously described [24]. Twenty-four 6-week-old female BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Twenty-one mice were inoculated intracerebrally (right frontal lobe of the cerebrum) with viral doses of $10^5$ plaque-forming units of the CVS-11 strain suspended in phosphate-buffered saline (PBS, pH 7.4) and three uninfected control mice were inoculated with PBS alone. The inoculated mice were observed daily for neurological symptoms and were killed at 2, 3, 4, 5, 7, 10 and 11 DPI (three mice per day). All experiments were performed in level-2 biosafety laboratories according to the Committee on Biosafety and Animal Handling and Ethical Regulation of the National Institute of Infectious Diseases, Japan. Animal care, breeding, virus inoculation and observation were performed in accordance with the guidelines of the committee.

Necropsy and preparation of tissue sections: Each mouse was anesthetized with chloroform and perfused transcar-
dially 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 (RT) for less than 24 hr and were decalcified in K-CX solution (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan). Transverse sections of spinal cords at the cervical (C3–4), thoracic (T1–3), lumbar (L1–2) and sacral (S1–3) vertebrae were prepared. A complete series of paraffin sections about 3 μm thick was cut and mounted on glass slides. Serial sections were subjected to hematoxylin and eosin (HE) staining, immunohistochemistry and in situ terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL).

**Immunohistochemistry:** For detection of rabies virus antigens in tissues, all sections were stained using the streptavidin-biotin-peroxidase complex method using anti-rabbit phosphoprotein (P) [15]. For detection of cell type, the following primary antibodies were used: anti-glial fibrillary acidic protein (GFAP) for astroglial cells (Nichirei Biosciences, Tokyo, Japan); anti-ionized calcium binding adaptor molecule 1 (Iba1) for microglial cells (Wako, Osaka, Japan); anti-CD3 for T lymphocytes (DAKO, Kyoto, Japan); and anti-CD20 for B lymphocytes (Spring Bioscience, Fremont, CA, U.S.A.). Brieﬂy, tissue sections were treated with 0.25% trypsin (for P) at RT for 30 min, with 750 W microwaves (for CD3 and CD20) for 5 min or in a water bath (for Iba1) at 95°C for 15 min.

To remove endogenous peroxidase, immunostained sections were treated with 0.3% H2O2 in methanol (for P) or 3% H2O2 in methanol (for GFAP, Iba1, CD3 and CD20). To block nonspecific reactions, each immunostained section was treated with 5% normal goat serum (for P) or 10% normal goat serum (for GFAP, Iba1, CD3 and CD20). Primary antibodies were diluted in PBS (1:1000 for P and 1:500 for Iba1) and incubated at 4°C overnight. Antibodies against GFAP, CD3 and CD20 were incubated at RT for 1 hr. Anti-rabbit IgG (Nichirei) was used as a secondary antibody for immunostaining for P and Iba1. The Envision + System Labeled Polymer-HRP anti-rabbit antibody (DAKO) was used for immunostaining for CD3 and CD20. Histofine® Simple Stain MAX-PO (Nichirei) was used for immunostaining for GFAP. Finally, each antibody was visualized using 3-3’-diaminobenzidine (DAB, DAKO). Slides were counterstained with hematoxylin.

**TUNEL assays:** The presence of fragmented DNA was evaluated using TUNEL (Chemicon, Temecula, CA, U.S.A.). After deparaffinizing the sections, endogenous peroxidase activity was removed by exposure to 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 (DAKO) for 15 min at RT to retrieve antigens. After washing in PBS, sections were prepared according to the manufacturer’s instructions and were counterstained with hematoxylin.

**Double labeling with 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 and slides were incubated with DAB until color developed. The reaction was stopped by washing the slides in distilled water, and then antibodies against P, CD3, Iba1 or GFAP were added. Histofine® Simple Stain AP (Rabbit) (Nichirei) was used as a secondary antibody. The color reaction was developed using Histofine® New Fuchsin (Nichirei). The slides were counterstained with hematoxylin.

**Counting of TUNEL-positive and immunostained cells in the cerebrum and cervical spinal cord:** All TUNEL-positive cells and cells immunostained for P, Iba1, GFAP and CD3 in the cerebrum (cerebral cortex, thalamus and hippocampus) and cervical spinal cord, including the dorsal root spinal ganglion, were counted using a light microscope with a magnification of ×100–200 on 3, 5 and 10 DPI (three mice per day).

**RESULTS**

**Clinical signs and macroscopic findings:** Mice showed ruffled hair, hunched backs, anorexia, emaciation and ataxia at 5 DPI and became moribund at 8 DPI. All mice died by 11 DPI, and no mice showed paralysis before death. No macroscopic findings were observed at necropsy.

**Histology:** Nuclear pyknosis and fragmentation and cytoplasmic shrinkage were first observed in the pyramidal cells (CA3) of the hippocampus at 4 DPI, and most of the pyramidal cells had been destroyed by 7 DPI (Fig. 1). Lesser changes were observed in the neurons of the cerebral cortex pyramidal cells, thalamus neurons, cerebellum Purkinje cells and cerebellum granule cells. Nuclear pyknosis and cytoplasmic shrinkage were detected in the dorsal root spinal ganglion cells at 5 DPI (Fig. 2). No histological changes were observed in the spinal neurons.

On the other hand, mild inflammatory cells composed mainly of lymphocytes were observed around the small vessels and cerebral ventricles and under the leptomeninges, and large activated microglial cells were observed throughout the CNS at 5 DPI. More advanced lesions appeared at 7 DPI, and the majority of lymphocytes showed nuclear fragmentation and pyknosis.

**Immunohistochemistry:** Virus antigens were first detected as small spots in the pyramidal cells of the cerebral cortex and hippocampus at 3 DPI and in the cerebellum Purkinje cells, neurons of the thalamus, brain stem, spinal cord (C, T, L, S) and dorsal root spinal ganglion at 5 DPI. As infection progressed, virus antigens were also detected in the dendrites and axons of neurons.

At 2 DPI, rod-like small microglial cells were scattered throughout the brain and spinal cord. From 3 to 5 DPI, the number of microglial cells increased and their morphology changed from rod to ramified or amoeboid (Fig. 3). These morphological changes were more evident in the cerebral cortex, hippocampus and brain stem than in the spinal cord. Astroglial cells were scattered throughout the parenchyma, under the leptomeninges, around the small vessels and the central canal of the brain and spinal cord at 3 DPI. From 5 DPI, astroglial cells were activated in the brain and their
morbidity changed. The processes of fibrous astroglial cells elongated from the leptomeninges to the deep parenchyma and many large protoplasmic astroglial cells were detected simultaneously in the parenchyma of the brain (Fig. 4), particularly around apoptotic neurons. There were no significant changes of astroglial cells in the spinal cord throughout the experimental period.

At 5 DPI, CD3-positive T lymphocytes appeared under the leptomeninges and around the blood vessels and ventricles of the entire CNS, but the extent of infiltration of T lymphocytes became greater in the brain than in the spinal cord as the infection progressed. On the other hand, CD20-positive B lymphocytes were not detected in the CNS throughout the experimental period. The mean of the number of glial cells around apoptotic neurons and lymphocytes in multiple areas (cerebral cortex, thalamus, hippocampus) of brain and cervical spinal cords at time course was summarized in Table 1.

**TUNEL assays and double staining:** At 5 DPI, TUNEL positive nerve cells appeared in the cerebral cortex pyramidal cells, thalamus neurons, hippocampus pyramidal cells, cerebellum Purkinje cells and cerebellum granule layer cells, and those of TUNEL positive neurons were significantly increased at 10 DPI, particularly in the pyramidal cells of the hippocampus (Table 2 and Fig. 5). On the other hand, TUNEL positive signal was not detected in the spinal neurons, including the dorsal root spinal ganglion cells (Table 2). The majority of microglial cells and astroglial cells were TUNEL negative. Double staining confirmed that all apoptotic neurons, except for the cerebellum granule layer cells, were infected with CVS-11 (Fig. 6). T lymphocytes that exhibited nuclear fragmentation and pyknosis by HE staining were TUNEL positive.

**DISCUSSION**

In this study, BALB/c mice that were inoculated intracebrally with CVS-11 did not exhibit paralytic signs and the pathological changes were mainly located in the cerebral cortex, hippocampus, thalamus neurons, cerebellum Purkinje cells and cerebellum granule layer cells and were accompanied by inflammation. In addition, the majority of virus-infected neurons of the brain and T lymphocytes underwent apoptosis, but spinal lesions were mild and apoptotic features were not observed in the virus-infected spinal neurons or dorsal root spinal ganglion cells. These findings differ substantially from those of mice that were inoculated intramuscularly [15]. Guigoni and Coulon [6] reported that in primary cultures of CVS-infected pyramidal cells of the hippocampus, more than 90% of cells showed apoptosis within 3 DPI, whereas the rat spinal motor neurons did not show major evidence of apoptosis over a period of 7 DPI. In addition, primary dorsal root spinal ganglion cells show long term survival up to 4 weeks when they are chronically infected in cell culture with rabies virus [16]. Our previous report and those of others suggest that various types of neurons are susceptible to CVS-11 [6, 15], and different mechanism which causes apoptosis exists among the neurons of the brain and spinal cord.

In this study, the inflammatory cells in the brain consisted mainly of T lymphocytes and microglial cells; their numbers increased as infection progressed and most T lymphocytes in the brain and spinal cord underwent apoptosis. These results correspond with those for mice that were inoculated with CVS-11 intramuscularly [15]. Infection of experimental animals with an attenuated strain of the rabies virus induces a strong specific immune response that results in a nonlethal infection, whereas mice that were infected using salivary glands from a naturally infected dog showed severe suppression of immunity caused by lymphocyte apoptosis [14, 25]. In addition, Iwasaki *et al.* [8] reported that immunocompetent mice show severe paralytic disease and
marked inflammation and degeneration of the CNS. In contrast, immunosuppressed mice developed encephalitic symptoms with only minor paralysis. Individual neuronal degeneration and microglial reaction were mild even though the level of antirabies antibody was similar to that of immunocompetent mice. These findings suggest that T lymphocyte immune reactions play a role in the pathogenesis of rabies. In the present study, the reasons of apoptosis of lymphocytes were not investigated, but currently it is suggested that T lymphocyte mediated Fas/Fas ligand pathways

Table 1. The mean of the number of glial cells and lymphocytes in three mice infected with CVS-11

<table>
<thead>
<tr>
<th>Cerebral cortex, thalamus and hippocampus</th>
<th>3 DPI (n=3)</th>
<th>5 DPI (n=3)</th>
<th>10 DPI (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>67</td>
<td>272</td>
<td>454</td>
</tr>
<tr>
<td>Iba1</td>
<td>974</td>
<td>1,732</td>
<td>2,045</td>
</tr>
<tr>
<td>CD3</td>
<td>0</td>
<td>76</td>
<td>294</td>
</tr>
<tr>
<td>CD20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

| Cervical spinal cord/ spinal ganglion    |             |             |             |
| GFAP                                     | 16/0        | 15/0        | 21/0        |
| Iba1                                     | 37/0        | 124/0       | 148/0       |
| CD3                                      | 0/0         | 17/15       | 45/32       |
| CD20                                     | 0/0         | 0/0         | 0/0         |

GFAP: astroglial cell (protoplasmic astroglial cells type only counted), Iba1: microglial cell (ramified or amoeboid microglial cells type only counted), CD3: T lymphocyte, CD20: B lymphocyte.

Fig. 3. Hippocampus. There are no morphological changes (a) and small rod-like microglial cells are observed 2 DPI (a, inset). In contrast, an increase in the number of microglial cells with a ramified or amoeboid shape (b, inset) was observed 5 DPI. Immunohistochemistry (anti-Iba1). Bars=250 μm and 50 μm (inset).

Fig. 4. Hippocampus. There are no morphological changes (a) and few astroglial cells 3 DPI (a, inset). In contrast, an increased number of astroglial cells (b) of protoplasmic shape are observed 5 DPI (b, inset). Immunohistochemistry (anti-GFAP). Bars=250 μm and 25 μm (inset).
expressed within infected neurons and lymphocytes apoptosis [2].

In the present study, the number of microglial cells positive to anti-Iba1 antibody increased significantly at 3 DPI and their morphological features changed to a ramified or amoeboid form in areas of apoptosis. Those findings were more significant in the brains than spinal cords throughout the experimental period. Activated microglial cells release proinflammatory cytokines such as interleukin (IL)-1 [5], tumor necrosis factor (TNF)-α [22, 26] and nitric oxide (NO) [18], which cause neuronal cell death both directly and indirectly via the induction of NO and free radicals [7, 23]. IL-1 and TNF-α are released by microglial cells and macrophages, and play an important role in coordinating the inflammatory response associated with rabies encephalopathy [3, 17].

Astroglial cells provide structural, metabolic and trophic support for neurons. As might be expected from their wide range of activities, both beneficial and detrimental effects are attributed to activated astroglial cells [1, 4, 20]. In a previous study in which mice were inoculated with CVS-11 intramuscularly, we observed that numerous spinal neurons underwent necrosis and that the number of astroglial cells increased as the infection progressed [15]. In the present study, the reaction of astroglial cells was mild and there were no morphological changes in the spinal cord despite infection of many spinal and spinal ganglion neurons. Therefore, it was suggested that the number of astroglial cells increased in response to neuronal cell death by apoptosis.

In the present study, most pathomorphological changes after intracerebral inoculation are located in the brain, and spinal lesions are slight compared with those of intramuscularly infected mice. These findings suggest that expression of a paralytic symptom in rabies infection in mice is determined by differences in the route of inoculation. In addition, increasing of glial cells is essential features of CVS-11 infection in mice, this process may also play an important role in pathogenesis of experimental rabies.

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