Lesions in the Central Nervous System of DBA/2 Mice Infected with the D Variant of Encephalomyocarditis Virus (EMC-D)

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(Received 15 July 1991/Accepted 10 August 1991)

ABSTRACT. The central nervous system (CNS) of DBA/2 mice inoculated i.p. with the D variant of encephalomyocarditis virus (EMC-D) (10^5 PFU/head) was examined up to 28 days postinoculation (28 DPI). The virus titer of CNS reached a maximum level at 4 DPI, and infectious viruses became undetectable by 28 DPI. Histopathologically, degeneration of neurons with virus antigens was observed in St. pyramidal hippocampi, Nuc. amigdaloides corticalis and St. granulosum cerebelli of the brain and in Cornu ventrale of the thoracic to lumbar spinal cord at 6 DPI. In addition, in the spinal cord, demyelination was found in Funiciulus ventrais and Funiciulus lateralis at 6 DPI and it progressed to form spongiosis at 10 DPI. In correspondence with these virological and histopathological findings, hind limb paralysis developed in some mice at 6 DPI, and its incidence increased markedly to about 60% at 10 DPI. —KEY WORDS: CNS lesion, DBA/2 mouse, EMC-D, paralysis.

Encephalomyocarditis (EMC) virus was first isolated from non-human primates [7] and then from pigs [13], and it is now considered to be an important causative agent of fetal death [9] and acute necrotizing myocarditis in pigs [1]. Since Yoon et al. [18] established the highly diabetogenic variant of EMC virus (EMC-D) by repeated plaque-purification of the M variant (EMC-M) [4], many studies focused on diabetes have been done in mice using EMC-D [5, 6, 19]. Recently, during the process of investigating diabetic nephropathy in EMC-D-induced diabetic DBA/2NCrj (DBA/2) mice, a highly susceptible strain to EMC virus-induced diabetes, Doi et al. [5] observed hind limb paralysis at a high frequency. In the subsequent preliminary examinations at 3 months postinoculation, we observed uni-phasic or bi-phasic paralysis in DBA/2 mice according to the dose of EMC-D inoculated.

As the first step of investigating EMC-D-induced CNS lesions in mice, this paper describes the virological and histopathological changes in the central nervous system (CNS) of DBA/2 mice. During the subacute stage of infection mice showed uni-phasic hind limb paralysis. The data of mice showing bi-phasic paralysis will be published elsewhere.

MATERIALS AND METHODS

Animals: Seventy seven 8-week-old male DBA/2 mice were obtained from Charles River Japan Inc. (Kanagawa). The mice were housed in an animal room at a temperature of 23±2°C with a relative humidity of 55±5% and fed MF pellets (Oriental Yeast Co., Ltd., Tokyo) and water ad libitum.

Virus infection: Plaque-isolated D variant of EMC virus (EMC-D: gifts from Dr. Ji-Won Yoon, the University of Calgary, Calgary, Alberta, Canada) was cultured on mouse L-929 cells and stored at −80°C until used. The titer of this virus stock determined by plaque assay on L-929 cell cultures was 4×10^7 plaque forming units (PFU)/ml. The ten-fold dilution of virus was prepared in 0.01 M phosphate buffered saline (PBS) (10^4 PFU/ml), and 0.1 ml of the dilution (10^3 PFU/head) was inoculated intraperitoneally (i.p.) into 74 mice. The remaining 3 mice were inoculated i.p. with 0.1 ml/head of PBS and served as histological controls.

Six EMC-D-inoculated mice were randomly chosen and killed by exanguination under ether anesthesia at 2, 4, 6, 10, 14, 21 and 28 days postinoculation (DPI), respectively. A half was subjected to virus titration and the remainings to histopathological examination. Control mice were sacrificed at 28 DPI in the same way.

Clinical signs and mortality: During the experimental period, particular changes in behaviour and appearance and mortality of mice were observed and recorded.

Virus titer: Virus titration by plaque assay on L-929 cell cultures was done on the brain and spinal
cord according to the method of Matsuzaki et al. [11].

**Histopathology:** At autopsy, immediately after measuring the body weight, the brain and spinal cord were fixed in 4% paraformaldehyde. Quasi-serial sections (4 μm) were made and stained with hematoxylin and eosin (HE) or luxol-fast blue counterstained with HE (LFB). Orientation of coronal sections was performed according to atlas of the mouse brain and spinal cord drawn by Sidman et al. [14]. For detecting virus antigens, some sections were also stained by avidin-biotin-peroxidase complex (ABC) method using Vector ABC kit (Vector Lab. Inc., U.S.A.). Anti-EMC-D guinea pig serum maintained in our laboratory [11] was used as the first antibody.

**RESULTS**

**Clinical signs and mortality:** Uni- or bi-lateral hind limb paralysis was first seen in some mice at 6 DPI, and its incidence increased markedly to about 60% at 10 DPI (Fig. 1). A total of 31 mice died from 6 to 14 DPI (Fig. 1).

**Virus titer:** As shown in Fig. 2, the kinetics of virus proliferation in the brain was similar to that in the spinal cord, and the titers reached a maximum level at 4 DPI. Thereafter, the virus titers decreased, and infectious viruses were no longer detected at 21 DPI in the spinal cord and at 28 DPI in the brain, respectively (Fig. 2).

**Histopathological findings:** The distribution of brain lesions is shown in Fig. 3. The lesions were first found in St. pyramidal hippocampi, Nuc. amigdaloideus corticalis and St. granulosum cerebelli at 6 DPI. At 10 DPI, the brain lesions were also seen in T. olfactorium, Lamina pyramidal areae pyriformis and St. granulosum dentatae.

The lesions were characterized by degeneration of neurons exhibiting pyknosis with rounding and amphophilic cytoplasm, among which vacuolar spaces with irregular contours were present (Fig. 4). Perivascular infiltration of mononuclear cells and meningitis were sometimes associated. Virus antigens were detected in the cytoplasm of degenerated neurons and/or adjacent intact neurons from 6 to 10 DPI (Fig. 4). On and after 14 DPI, focal loss of neurons and gliosis were noticed in the previous lesions (Fig. 5).

Brain lesions were more prominent in mice with hind limb paralysis than in mice without clinical signs.

The distribution of spinal cord lesions is shown in Fig. 6. At 6 DPI, minimal to small focal lesions were first observed in Funiculus ventralis and Funiculus lateralis and in Cornu ventrale from the thoracic to lumbar spinal cord. The lumbar spinal cord was most frequently involved. Although varied in degree, lesions in the white matter were characterized by demyelination (Fig. 7), while changes in the gray matter were similar to those in the brain (Fig. 8). Virus antigens were mainly detected in the cytoplasm of degenerated neurons and/or adjacent intact neurons from 6 to 10 DPI (Fig. 9). Spinal cord lesions became predominantly severer in the white matter at 10 DPI, resulting in formation of spongiosis (Fig. 10). On and after 14 DPI, mononuclear
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Fig. 3. Distribution of degenerative changes in the brain at 10 DPI (■: frequently affected, □: less frequently affected).
1, T. olfactorium; 2, Lamina pyramidal area piriformis; 3, St. pyramidal hippocampi; 4, St. granulosum dentatae; 5, Nuc. amigdaloideus corticalis; 6, St. granulosum cerebelli.

Fig. 4. Cerebrum of an infected mouse at 6 DPI. Degeneration and pyknosis of neurons (arrowhead) in St. pyramidal hippocampi. HE stain. ×150. Inset. Virus antigen (arrowhead) in the cytoplasm of a nerve cell. ABC method. ×600.

Fig. 5. Cerebrum of an infected mouse at 21 DPI. Loss of neurons and gliosis in St. pyramidal hippocampi. HE stain. ×150.

Fig. 6. Distribution of degenerative changes in the spinal cord at 10 DPI (■: frequently affected, □: less frequently affected). 1, Funiculus lateralis; 2, Cornu vevtrale; 3, Funiculus ventralis.
Fig. 7. Thoracic spinal cord of an infected mouse at 6 DPI. Small focal demyelination (arrowhead) in Funiculä lateralis. HE stain. ×200.

Fig. 8. Thoracic spinal cord of an infected mouse at 6 DPI. Small focal degeneration and pyknosis of neurons (arrowhead) in Cornu ventrale. HE stain. ×300.

Fig. 9. Lumbar spinal cord of an infected mouse at 6 DPI. Virus antigen (arrowhead) in the cytoplasm of a nerve cell in Cornu ventrale. ABC method. ×400.

Fig. 10. Lumbar spinal cord of an infected mouse at 10 DPI. Spongiosis in Funiculä lateralis. LFB. ×150.

Fig. 11. Lumbar spinal cord of an infected mouse at 21 DPI. Gliosis in Cornu ventrale. HE stain. ×150.

cell infiltration and gliosis were observed in the previous lesions in the gray matter (Fig. 11). The spinal cord lesions were noticed only in mice with paralysis.

DISCUSSION

In addition to the brain lesions similar to those previously described in EMC-D-infected BALB/c mice [6], DBA/2 mice infected with EMC-D developed spinal cord lesions with characteristic localization. Such characteristic localization of CNS lesions has not been reported in animals infected with other variants of EMC virus [2, 3, 12, 16, 17]. In the present experiment, St. pyramidale hippocampi and Nuc. amigaloideus corticalis, a part of
cerebral limbic system, were most frequently affected in the cerebrum, and *St. granulosum cerebelli* was exclusively attacked in the cerebellum. In the thoracic and lumbar spinal cord, motor neuron in *Corpus ventrale*, and *Funiculus ventralis* and *Funiculus lateralis*, which constitute the descending nerve tract dominating mobility and tension of the skeletal muscle, were selectively affected. Distribution of the virus antigens in CNS corresponded well with that of histopathological lesions and they were detected from 6 to 10 DPI. The spinal cord lesions were observed only in mice showing hind limb paralysis while the brain lesions were observed even in mice without paralysis. Moreover, the incidence of the hind limb paralysis increased markedly at 10 DPI in coincidence with the prominent progression of the spinal cord lesions in the white matter. From the above-mentioned mode of occurrence and distribution of lesions, development of the hind limb paralysis is thought to be more closely related to spinal cord lesions than to brain lesions.

Histopathologically, CNS lesions were characterized by degeneration of neurons containing the viral antigens in the particular portions of the brain and spinal cord and by demyelination in the descending nerve tract of the spinal cord. Based on these histopathological findings and virological data, it is reasonable to consider that CNS lesions were caused by direct attack of EMC-D, resulting in hind limb paralysis.

Paralytic syndrome was also reported in BALB/c mice infected with a low dose (60 PFU/head) of EMC-M and CNS lesions in these mice were observed in the white matter and characterized by immune-mediated demyelination [15]. Moreover, Theiler's mouse encephalomyelitis virus, which is a member of the Picornaviridae as EMC virus is, can induce an early poliomyelitic phase followed by chronic demyelination, resulting in posterior flaccid paralysis in mice [8, 10]. We are now carrying out detailed investigations of CNS lesions in DBA/2 mice infected with a low dose of EMC-D to clarify the mechanism of bi-phasic paralysis. In addition, we will compare the mechanisms of CNS lesions in EMC-D-infected mice with those in the above-mentioned reports [8, 10, 15].

REFERENCES