Int. J. Exp. Path. (1995), **76**, 441–447

Distribution of viral RNA in the spinal cord of DBA/2 mice developing biphasic paralysis following infection with the D variant of encephalomyocarditis virus (EMC-D)

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Received for publication 3 April 1995
Accepted for publication 11 September 1995

Summary. DBA/2 mice infected with the D variant of encephalomyocarditis virus (EMC-D) (10^1PFU/head) developed biphasic hind limb paralysis. As a first step in clarifying its pathogenesis, we examined the distribution of viral RNA in the spinal cord using *in situ* hybridization. At 3 days post inoculation (DPI), in the spinal cord of mice showing slight paralysis, viral RNA was observed in capillary endothelial cells and a few adjacent glia cells in the funiculus lateralis from thoracic to lumbar enlargement. At 7 DPI, in the spinal cord of mice showing apparent paralysis, viral RNA was observed in a larger number of glia cells in the demyelinated lesion associated with infiltration of macrophages in the funiculus lateralis and in a small number of degenerated neurons in the cornu ventrale. In the funiculus lateralis, viral RNA could not be observed after 28 DPI. On the other hand, viral RNA was observed in degenerated neurons in the cornu ventrale of mice showing the second phase paralysis at 42 DPI. Many $CD4^+$T cells infiltrated around these degenerated neurons. These results suggest that: (1) the viral entry zone was the capillary endothelial cells in the funiculus lateralis; (2) first phase paralysis was due to demyelination caused by EMC-D and associated with macrophage infiltration; (3) second phase paralysis was due to degeneration of motor neurons bearing viral RNA associated with infiltration by $CD4^+$T cells.

Keywords: biphasic paralysis, viral RNA, *in situ* hybridization, EMC-D

Since Yoon *et al.* (1980) established the highly diabetogenic D variant of encephalomyocarditis virus (EMC-D) by repeated plaque purification of the M variant (EMC-M; Craighead & McLean 1968), many studies of diabetes in mice have made use of this variant (Yoon *et al.* 1982; Doi *et al.* 1988). Recently, Takeda and colleagues have reported that DBA/2NCrj mice (DBA/2) inoculated with 10^3 plaque forming units (PFU)/head of EMC-D developed monophasic hind limb paralysis (Takeda *et al.* 1991), and that DBA/2 mice inoculated with 10^1 PFU/head of EMC-D developed biphasic hind limb paralysis (Takeda *et al.* 1993). In the latter case, about 60% of the infected mice developed hind limb paralysis by 12 days post inoculation

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(DPI), two-thirds of them showed recovery by 33 DPI and, thereafter, 30% of the mice which had shown recovery again developed paralysis by 56 DPI. The spinal cord lesion in the early phase was characterized mainly by demyelination associated with infiltration of macrophages in the funiculus lateralis from thoracic to lumbar enlargement, whilst that in the late phase was characterized mainly by degeneration of neurons associated with infiltration of $CD4^+$T cells in the cornu ventrale from thoracic to lumbar enlargement.

As a first step in clarifying the pathogenesis of this biphasic syndrome, we have studied the distribution of viral RNA in the spinal cord using *in situ* hybridization, and we have compared our findings with immunohistochemistry using an anti virus antibody, to try to clarify the kinetics of the virus in the tissues.

Materials and methods

Animals

Eighty 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 $23 \pm 2°C$ with a relative humidity of $55 \pm 5\%$ and fed MF pellets (Oriental Yeast Co. Ltd., Tokyo) and water *ad libitum*.

Virus infection

Plaque-isolated D variant of EMC virus (EMC-D: a gift from Dr J.W. Yoon, the University of Calgary, Alberta, Canada) was cultured on mouse L-929 cells and stored at $-80°C$ until used. The titre of this stock virus, determined by plaque assay on L-929 cells, was 4×10^7 PFU/ml. Serial tenfold dilutions of virus were prepared in 0.01M phosphate buffered saline (PBS) to a final dilution of 10^2 PFU/ml, and 0.1 ml of this dilution (10^1PFU/head) was inoculated intraperitoneally (i.p.) into 74 mice. The remaining 6 mice were inoculated i.p. with 0.1 ml of PBS and served as controls.

Six to 10 mice inoculated with EMC-D were randomly chosen and sacrificed by exsanguination under ether anaesthesia at 3, 7, 14, 28, 42 and 56 days post inoculation (DPI). At 28 and 56 DPI, 3 control mice were sacrificed in the same way.

At necropsy, the spinal cord was fixed by the AMeX method (Sato *et al.* 1992), and serial 4-μm paraffin sections were made for histopathology and *in situ* hybridization.

Histopathology

Paraffin sections were stained with haematoxylin and eosin (HE) for histopathological observation. In addition, in order to elucidate the nature of the infiltrating cells, serial sections were stained with anti-Mac1 and anti-CD4 monoclonal antibodies (Boehringer Mannheim, JPN) by the avidin–biotin–peroxidase complex (ABC) method using Vectastain Elite ABC kit (Vector Lab. Inc., USA).

Preparation of EMC-D-probe

L-929 cells were infected for 24 hours with 0.1 multiplicity of infection (MOI) of EMC-D. After washing with PBS, EMC-D was ultrafiltered and purified by density gradient centrifugation. Viral RNA was isolated by the guanidine isothiocyanate (GTC) method from purified EMC-D.

To amplify the most specific region of EMC-D which was complementary to structural viral proteins, VP3-VP1 (Eun *et al.* 1988; Bae *et al.* 1989; Kang & Yoon 1993), the following primers were synthesized using DNA Cyclone apparatus (Milligen Bioresearch, USA).

Forward primer: 5′ TGAATAGTCCAATGCGAAAGT-3′
Reverse primer: 5′ AGCACCACGGCATGTTAAGAG-3′

Viral RNA was reverse transcribed into cDNA using First-strand cDNA synthesis kit (Pharmacia, USA), and then the 1000-bp specific region of EMC-D was amplified using these primers by the polymerase chain reaction (PCR) method.

Agarose gel purified PCR product (1000bp) was subcloned into the Sma I site of plasmid pBluescript (SK-) (Stratagene, CA), which was transformed in *E. coli* XLI-blue. DNA sequencing of the clone was performed by the dideoxy chain termination method using Auto-Read sequencing kit and ALF DNA sequencer (Pharmacia LKB, Sweden). The sequence corresponded to the above mentioned most specific region.

After large-scale plasmid extraction, circular plasmid DNA was cut with Hind III or Bam HI (Boehringer Mannheim, JPN). Digoxygenin labelled antisense probe was synthesized from linearized plasmid DNA cut with Hind III by T3 RNA polymerase (Stratagene, CA) using DIG RNA labelling kit (Boehringer Mannheim, JPN). This probe was partially digested to 500 bases by alkaline hydrolysis in carbonate buffer.

Northern blot analysis

To determine the specificity of our EMC-D-probe, Northern blot analysis was performed using RNA obtained from spinal cords of 3 EMC-D-infected mice at 7 DPI and an uninfected mouse.

Ten 10 μg of each RNA sample were electrophoresed on formaldehyde gel, blotted to nitrocellulose paper,

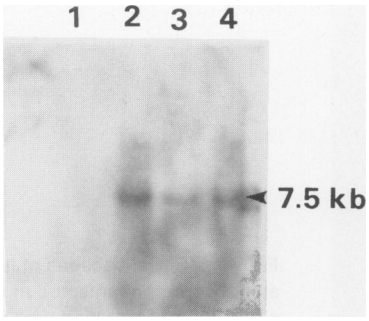

Figure 1. Northern blot analysis with digoxygenin-labelled RNA probe for EMC-D-RNA. Lane 1 : 10 μg RNA from spinal cord of mouse uninfected with EMC-D. Lanes 2 to 4 : 10 μg RNA from spinal cords of 3 mice infected with EMC-D. The probe hybridizes a single band at about 7.5 kb in lanes 2, 3 and 4 but not in lane 1, indicating specificity of this probe for EMC-D.

hybridized with digoxygenin-labelled RNA probe, and luminesced by ECL (Amersham, USA) according to the method of Nomura and Inazawa (1994).

In situ *hybridization*

Prior to hybridization, sections deparaffinized with xylene and dexylenized with acetone were treated with 4% paraformaldehyde (PFA)/PBS for 30 min, 0.3% Triton X-100/PBS for 10 min, 0.2M HCl for 20 min, 2 μg/ml proteinase-K/PBS for 30 min at 37°C, 4% PFA/PBS for 5 min, 0.2% glycine/PBS for 20 min and 0.25% acetic anhydrate/0.1M triethanolamine-HCl (pH 8.0) for 10 min. These sections were then dehydrated in graded ethanol and air dried. After treatment, sections were hybridized

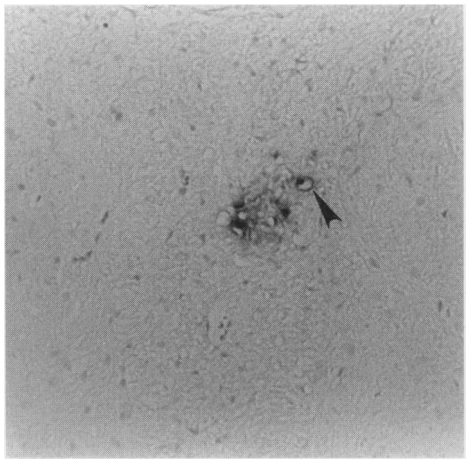

Figure 2. Lumbar spinal cord exhibiting normal structure of an infected mouse showing slight paralysis at 3 DPI. Virus RNA is observed in capillary endothelial cells (arrowhead) and a few adjacent glial cells in the funiculus lateralis. *In situ* hybridization. ×150.

for 18 hours at 50°C in the following solutions (probe concentration : 2 μg/ml): 50% deionized formamide, 100 μg/ml yeast t-RNA, 10% dextran sulphate, 1×Denhardt's solution, 0.05M Tris-HCl (pH 7.5), 5mM EDTA and 0.6M NaCl.

The next day, the sections were rinsed with 5×SSC at 50°C, and then treated with 50% formamide/2×SSC for 30 min at 50°C and 20 μg/ml RNase A/RNase buffer (10mM Tris-HCl (pH 7.5), 0.5M NaCl for 30 min at 37°C. The sections were then treated with 2×SSC for 1 hour at 50°C, following by 0.2×SSC for 1 hour at 50°C. These sections were blocked (1% Blocking Reagent (Boehringer

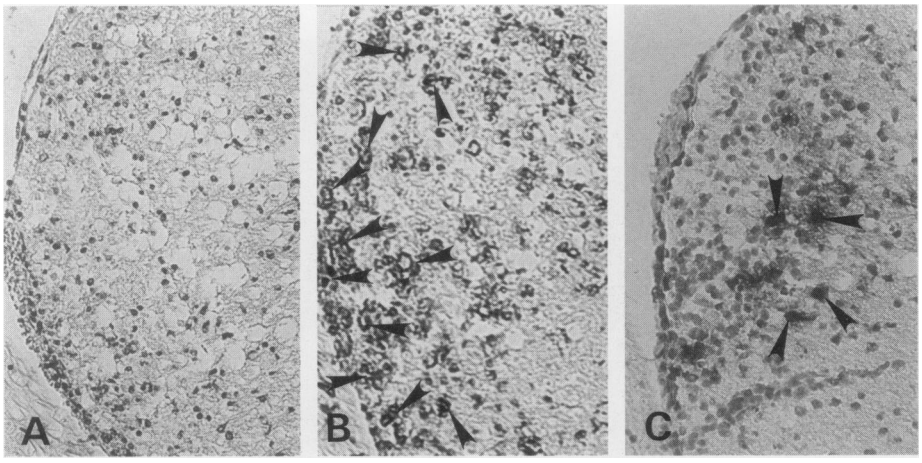

Figure 3. Serial sections of lumbar spinal cord of an infected mouse showing apparent hind limb paralysis at 7 DPI. A, Demyelination associated with infiltration of mononuclear cells in the funiculus lateralis. HE.×150. B, The infiltrating cells are positive for anti-Mac1 antibody (arrowheads). ABC method.×150. C, Viral RNA is observed in glial cells (arrowheads) around the demyelinated lesion. *In situ* hybridization.× 150.

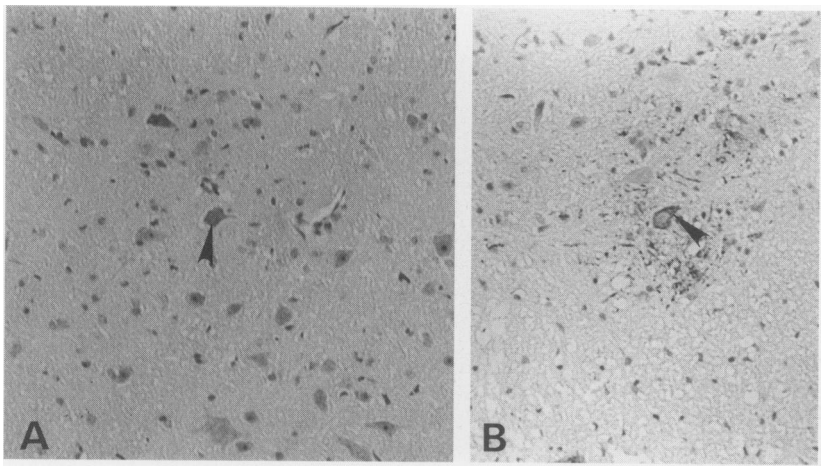

Figure 4. Serial sections of lumbar spinal cord of an infected mouse showing apparent hind limb paralysis at 7 DPI. A, Degeneration of neuron (arrowhead) in the cornu ventrale. HE.×150. B, Viral RNA (arrowhead) is observed in the degenerated neuron. *In situ* hybridization method.×150.

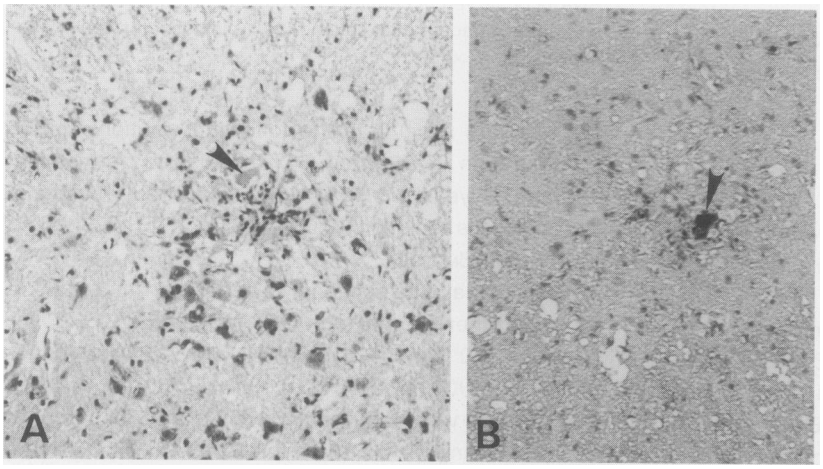

Figure 5. Serial sections of lumbar spinal cord of an infected mouse showing recovery at 28 DPI. A, Degenerated neuron (arrowhead) with a few mononuclear cells in the cornu ventrale. HE.×150. B, Viral RNA is observed in this degenerated neuron (arrowhead). *In situ* hybridization.×150.

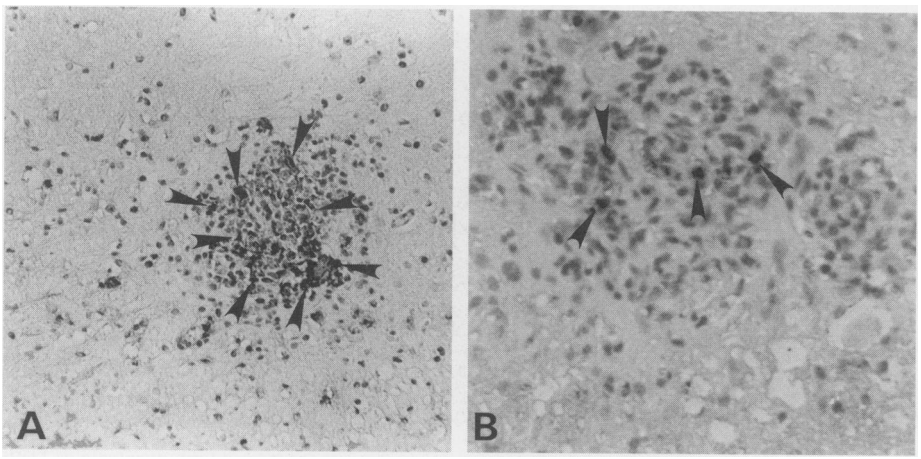

Figure 6. Serial sections of lumbar spinal cord of an infected mouse showing the second paralysis at 42 DPI. A, The infiltrating cells around degenerated neurons in the cornu ventrale are positive for anti-CD4 antibody (arrowheads). ABC method.×150. B, Viral RNA in these degenerated neurons (arrowheads). *In situ* hybridization.×300.

Mannheim, JPN), 0.3% Tween 20/0.1M Tris-HCl (pH 7.6) 0.15M NaCl) for 30 min, and then incubated with 1 : 500 AP-conjugated antidigoxygenin antibody (Boehringer Mannheim, JPN) blocking solution overnight at 4°C. The next day, the sections were coloured by NBT/BCIP and counterstained with haematoxylin.

Results

Northern blot analysis

The probe did not hybridize with RNA from spinal cord of an uninfected mouse (Figure 1). However, the probe did hybridize with RNA samples from spinal cords of EMC-D-infected mice at a single band of about 7.5 kb (Figure 1). This indicated that the probe was specific for EMC-D-RNA.

Histopathological findings

At 3 DPI, the spinal cord of infected mice showing slight hind limb paralysis appeared normal (Figure 2). At 7 DPI, in the spinal cord of mice showing apparent hind limb paralysis, the lesion was observed in the funiculus lateralis and in the cornu ventrale from thoracic to lumbar enlargement. The lesion in the funiculus lateralis was characterized by demyelination associated with infiltration of macrophages (Figure 3A and B) and that in the cornu ventrale was characterized by degeneration of neurons (Figure 4A). At 28 DPI, in the mice showing recovery from paralysis, a few infiltrating cells were observed around degenerated neurons in the cornu ventrale (Figure 5A). On the other hand, the size of demyelinated lesion apparently reduced with a decrease in number of infiltrating cells. At 42 DPI, the second phase hind limb paralysis developed in 2 mice which appeared to recover. In these mice, infiltration of mononuclear cells around the degenerated neurons become more prominent, and these cells were positive for anti-CD4 antibody ($CD4^+$T cells) (Figure 6A). At 56 DPI, infiltration of mononuclear cells was more prominent than at 42 DPI (Figure 7A).

In situ *hybridization*

At 3 DPI, in mice showing slight hind limb paralysis, viral RNA was observed in capillary endothelial cells and a few adjacent glial cells in the funiculus lateralis (Figure 2B).

At 7 DPI, in the spinal cord of mice showing apparent hind limb paralysis, viral RNA was observed in a larger number of glial cells in demyelinated lesions in the funiculus lateralis (Figure 3C) and in a small number of degenerated neurons in the cornu ventrale (Figure 4B).

At 28 DPI, in the spinal cord of mice showing recovery from paralysis, viral RNA was observed in degenerated neurons associated with infiltration of a few mononuclear cells in the cornu ventrale (Figure 5B). On the other hand, in the funiculus lateralis, viral RNA was not observed.

At 42 DPI, in mice showing the second phase hind limb paralysis, viral RNA was observed in degenerated neurons in the cornu ventrale associated with infiltration of many $CD4^+$T cells (Figure 6A and B).

At 56 DPI, in mice showing the second phase hind limb paralysis, viral RNA was no longer observed in this area (Figure 7B).

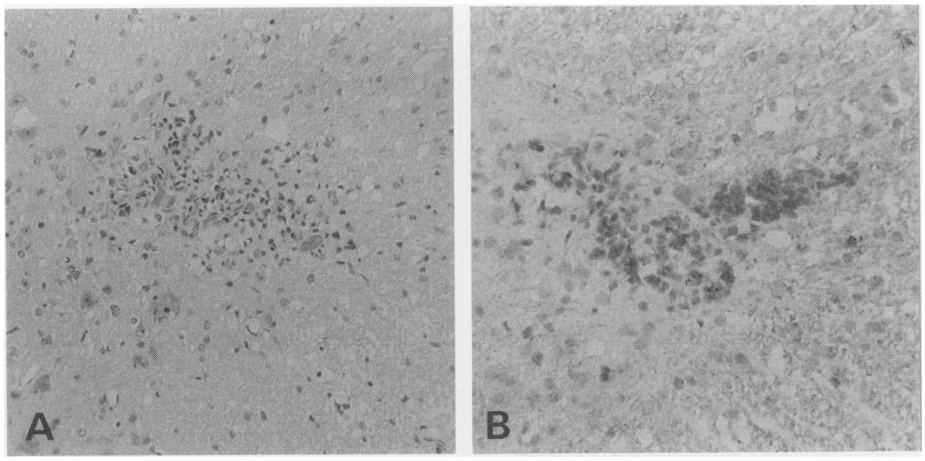

Figure 7. Serial sections of lumbar spinal cord of an infected mouse showing the second hind limb paralysis at 56 DPI. A, Infiltration of mononuclear cells in the cornu ventrale. HE. ×150. B, Viral RNA is not detected in this area. *In situ* hybridization. ×150.

Discussion

The region of 1000 bases coding structural proteins of EMC-D, VP3-VP1, was reported to be most specific for EMC-D (Eun *et al.* 1988; Bae *et al.* 1989; Kang & Yoon 1993). The RNA probe for EMC-D-RNA used in this experiment was complementary to this region and, from the result of Northern blot analysis, was specific for EMC-D-RNA. In this study, judging from the view point of sensitivity, preservation of morphological structure and handling, we performed *in situ* hybridization using AMeX-fixed paraffin sections and digoxygenin-labelled RNA probe instead of using frozen sections and radioisotope-labelled RNA probe.

The histopathological changes were similar to those in the previous report (Takeda *et al.* 1993). In addition, in the early phase of infection, viral RNA was observed in capillary endothelial cells and a few adjacent glial cells in the funiculus lateralis from thoracic to lumbar enlargements at 3 DPI. Thereafter, viral RNA was observed in a larger number of glial cells in the demyelinated lesion in the funiculus lateralis and in a small number of degenerated neurons in the cornu ventrale at 7 DPI. These results indicate that the viral entry zone to the spinal cord is capillary endothelial cells in the funiculus lateralis from thoracic to lumbar enlargement, and that virus spread from this zone to adjacent glial cells and further to neurons in the cornu ventrale. The reason why EMC-D first appears in this area is not clear, but Juhler *et al.* (1985) reported that the blood–brain barrier in this area was leaky.

As to the role of macrophages infiltrated in the early phase of EMC-D-infection, Baek and Yoon (1990; 1991) reported that macrophages participated in the damage of pancreatic β-cells of mice. Macrophages infiltrated in the demyelinated lesion in the present study may also be involved in developing demyelination, in addition to the direct effect of the virus on oligodendrocytes (Takeda *et al.* 1993).

In the late phase, viral RNA was no longer observed in the funiculus lateralis after 28 DPI. On the other hand, in the spinal cord of mice showing second phase paralysis, viral RNA was observed in degenerated neurons in the cornu ventrale, and many $CD4^+$T cells infiltrated around these neurons.

With respect to persistence of EMC virus in the tissues, Cronin *et al.* (1988) used *in situ* hybridization to show that EMC-A211-RNA persisted in pyramidal cells of hippocampus by 28 DPI, and Kyu *et al.* (1992) used RT-PCR to show that EMC-M-RNA could be detected in cardiac cells by 90 DPI. However, this is the first report that EMC-D-RNA persisted in the spinal cord by 42 DPI.

The important role of $CD4^+$T cells was reported in the early phase of the demyelination disease induced by EMC-M (Sriram *et al.* 1989; Craighead *et al.* 1990; Topham *et al.* 1991). In contrast, in our experiment, $CD4^+$T cells were infiltrated around degenerated motor neurons bearing viral RNA only in mice showing second phase paralysis. Therefore, we are now examining whether treatment with an anti-CD4 antibody prevents the second phase paralysis. This should clarify the precise role of $CD4^+$T cells in the lesions.

The reason why $CD4^+$T cells infiltrate around the degenerated neurons is still obscure. Huber (1992) reported that 70 kDa heat-shock protein (hsp 70) was expressed on cardiocyte infected with EMC-M and coxsackie virus B3 (CVB3) *in vitro* and cytolytic T lymphocytes belonging to the $CD4^+$ population were detected in the mice infected with CVB3. Taking this report into account, it seems possible that $CD4^+$T cells recognized some signals from degenerated neurons exhibiting viral RNA and reacted in a cytotoxic fashion towards these neurons. Therefore we are investigating the relationship between $CD4^+$T cells and those degenerated neurons bearing viral RNA.

The DA strain of Theiler's murine encephalomyelitis virus (DAV) is well known to cause biphasic central nervous symptoms in mice (Lipton 1975). Different from EMC-D-induced biphasic symptoms, in the mice infected with DAV, the lesion in the early phase was characterized by degeneration of motor neuron, and in the late phase by demyelination caused by $CD8^+$T cells (Rodriguez & Sriram 1988; Lindsley & Rodriguez 1989). Viral antigen (Rodriguez *et al.* 1983) and viral RNA (Yamada *et al* 1990) persisted in oligodendrocytes even in the late phase. Our findings suggest that like DAV, EMC-D is a useful model of viral autoimmune neuritis.

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