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Biphasic disease of central nervous system induced in DBA/2 mice by the D variant of encephalomyocarditis virus (EMC-D)

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Summary. DBA/2 mice infected with the D variant of encephalomyocarditis virus (EMC-D) (10^1 PFU/head) developed biphasic hind limb paralysis. At 12 days post inoculation (12 DPI), 60% of the infected mice developed hind limb paralysis and two-thirds of them showed recovery by 33 DPI. Thereafter, about 30% of the mice which once showed paralysis developed hind limb paralysis again by 56 DPI. Histopathologically, the spinal cord lesion of paralysed mice was characterized by demyelination associated with infiltration of macrophages in the funiculus lateralis and by degeneration of neurons in the cornu ventrale. Virus antigens were detected in the cytoplasm of degenerated neurons and oligodendrocytes in the demyelinated lesions from 3 to 14 DPI. At 28 DPI, demyelinated lesions reduced in size due to prominent remyelination. At 56 DPI, infiltration of mononuclear cells mainly composed of anti-L3T4-positive (CD4+) T cells were observed in the cornu ventrale of the mice showing recurrence of hind limb paralysis. These results suggested that the early paralysis was mainly due to demyelination in funiculus lateralis caused by EMC-D and macrophages and that the late paralysis was due to degeneration of motor neurons, probably brought about by CD4+ T cells.

Keywords: biphasic paralysis, CD4+ T cell, DBA/2 mice, EMC-D, macrophage, spinal cord lesion

Encephalomyocarditis (EMC) virus was first isolated from non-human primates (Helwig & Schmidt 1945) and then from pigs (Murane *et al.* 1960), and it is now considered to be an important causative agent of fetal death (Kim *et al.* 1989) and acute necrotizing myocarditis in pigs (Acland & Littlejohn 1986). On the other hand, in the field of experimental medicine, since Yoon *et al.* (1980) established the highly diabetogenic D variant of EMC virus (EMC-D) by repeated plaque purification of the M variant of EMC virus (EMC-M) (Craighead & McLean 1968), many studies focused on diabetes have been done in mice using EMC-D (Yoon *et al.* 1982; Doi *et al.* 1989).

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Recently, Takeda *et al.* (1991) reported that almost all the DBA/2NCrj mice (DBA/2) inoculated with 10^3 PFU/head of EMC-D developed hind limb paralysis and that they had lesions in the central nervous sytem with a characteristic distribution (neuronal degeneration in the st. pyramidale hippocampi nuc. amygdaloideus corticalis and st. granulosum cerebelli of the brain and in the cornu ventrale of the thoracic to lumbar spinal cord, and demyelination in the funiculus ventralis and funiculus lateralis of the spinal cord). In the subsequent preliminary examination, we observed biphasic hind limb paraly-

sis with characteristic spinal cord lesions in DBA/2 mice inoculated with 10^1 PFU/head of EMC-D.

The purpose of this study is to elucidate the virological and pathological backgrounds of biphasic paralysis in DBA/2 mice inoculated with 10^1 PFU/head of EMC-D.

Materials and methods

Animals

One hundred 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 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 virus stock, determined by plaque assay on L-929 cell cultures, was 4×10^7 plaque forming units (PFU)/ml. The serial tenfold dilution of the virus was prepared in 0.01 M phosphate-buffered saline (PBS) to get the final dilution of 10^2 PFU/ml, and 0.1 ml of this dilution (10^1 PFU/head) was inoculated intraperitoneally (i.p.) into 94 mice. The remaining 6 mice were inoculated i.p. with 0.1 ml of PBS and served as controls.

The mice inoculated with EMC-D were randomly divided into two groups. Forty mice (Group 1) were kept mainly for checking the sequence of clinical signs until the end of this experiment (56 DPI), when surviving mice were all killed by exsanguination under anaesthesia for virological and pathological examinations. Six mice each of the remaining 54 (Group 2) were randomly chosen and killed in the same way at 3, 7, 14, 21, 28 and 42 DPI, respectively. At 28 and 56 DPI, 3 control mice were sacrificed in the same way, respectively.

Clinical signs and mortality

During the experimental period, particular changes in behaviour and appearance of all mice and mortality were recorded.

Virus titre

Virus titration by plaque assay on L-929 cell cultures was done on the spinal cord of 3 infected mice according to the method of Matsuzaki *et al.* (1989).

Histopathology

At autopsy, immediately after measuring the body weight, the spinal cord was fixed in 10% neutral-buffered formalin, and 4-μm paraffin sections were stained with haematoxylin and eosin (H&E).

In addition, in order to investigate the changes in myelination in the funiculus lateralis, small pieces of the lumbar spinal cord of infected mice killed at 7 and 28 DPI were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), post-fixed in 1.0% osmium tetroxide in the same buffer, and embedded in epoxy resin, Quetol 812 (Nissin EM Co. Ltd, Tokyo). Semithin sections were stained with toluidine blue (TB). Ultrathin sections were double-stained with uranyl acetate and lead citrate, and observed under a JEM-1200EX electron microscope (JEOL Co. Ltd, Tokyo).

Immunohistochemistry

To elucidate the distribution of virus antigens and to identify the infiltrated cells, small pieces of the fresh lumbar spinal cord from each mouse were embedded in OCT-compound (Miles Inc., Elkhart, USA), frozen quickly in dry-ice–ether and stored at −80°C until used. Then, 10-μm cryostat sections were made, fixed with acetone and stained with the antibodies shown in Table 1 by the avidin–biotin–peroxidase complex (ABC) method using Vectastatin Elite ABC kit (Vector Lab. Inc., USA).

Results

Clinical signs and mortality

Clinical signs and their sequences were common to

Table 1. Antibodies used for immunohistochemistry

Antibodies	Source	Dilution	Specificity
Polyclonal anti-EMC-D guinea-pig IgG	In-house	1:20000	Viral proteins
Polyclonal anti-galactocerebroside rabbit IgG	Chemicon International Inc., Temecula, CA	1:1000	Oligodendrocyte
Polyclonal anti-GFAP rabbit IgG	Dako Japan Ltd, Kyoto, Jpn	1:200	Astrocyte
Monoclonal anti-Mac-1 rabbit IgG	Boehringer Mannheim Yamanouchi, Tokyo, Jpn	1:1000	Macrophage/microglia
Monoclonal anti-L3T4 rabbit IgG	Biosys, Compiegne, FR	1:500	Helper/inducer T cell
Monoclonal anti-Lyt2 rabbit IgG	Biosys, Compiegne, FR	1:500	Suppressor/cytotoxic T cell

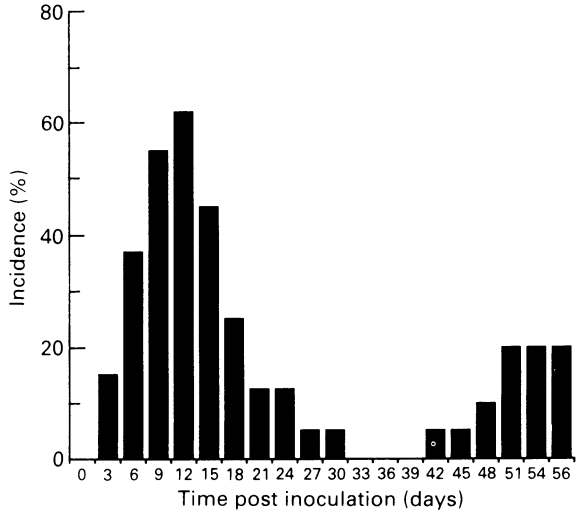

Figure 1. Changes in incidence of paralytic mice.

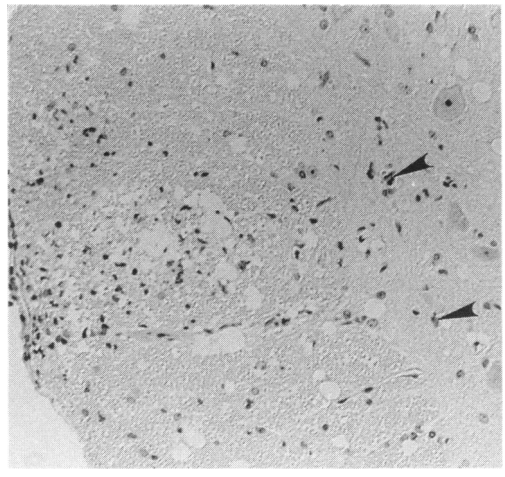

Figure 3. Lumbar spinal cord of an infected mouse showing paralysis at 7 DPI. Demyelination with mononuclear cell infiltration in the funiculus lateralis and pyknosis of neurons (arrowheads) in the cornu ventrale. H&E. × 200.

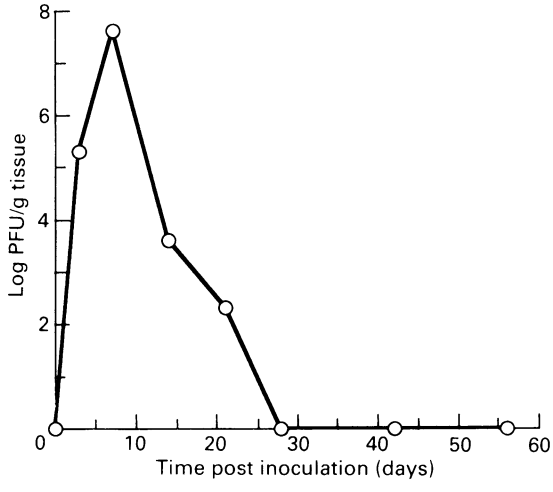

Figure 2. Changes in virus titre of spinal cord.

Groups 1 and 2. Hind limb paralysis was first seen in some mice at 3 DPI and its incidence increased markedly to about 60% at 12 DPI (Figure 1). Thereafter, recovery from such a neurologic syndrome was observed in some mice, and two-thirds of paralysed mice appeared normal at 33 DPI. Another third of the paralysed mice showed no recovery, and died by 21 DPI. At 42 DPI, hind limb paralysis recurred in some mice, and about 30% of the mice which once showed paralysis developed the sign again by 56 DPI (Figure 1). A total of 30 mice (Group 1, 12; Group 2, 18) died during the experimental period, and 80% of the deaths occurred in the first 3 weeks. Histopathological examinations revealed that 20 paralysed mice died by marked encephalitis, and 10 non-paralysed mice died by severe myocarditis.

Virus titre

As shown in Figure 2, virus proliferation in the spinal cord reached a maximal level at 7 DPI. In the early phase, the virus titre of paralysed mice was higher than that of non-paralysed mice. The virus titres decreased thereafter and were no longer detected even in paralysed mice at 28 DPI. After that, viral replication was never detected, even in mice showing a recurrent paralysis.

Histopathological and immunohistochemical findings

In the spinal cord of mice showing hind limb paralysis, the lesion was observed in the funiculus lateralis of the white matter and in the cornu ventrale of the gray matter from thoracic to lumbar enlargement at 7 DPI. The lesion in the cornu ventrale was minimal and characterized by degeneration of neurons exhibiting pyknosis (Figure 3), and that in the funiculus lateralis was characterized by focal demyelination (Figures 3 and 6A). The lesions of paralysed mice were more severe than those of non-paralysed mice. The lesions were associated with infiltration of mononuclear cells, almost all of which were positive for anti-Mac-1 antibody. A few mononuclear cells were positive for anti-L3T4 or anti-Lyt2 antibodies (Figure 4). Virus antigens were detected in the cytoplasm

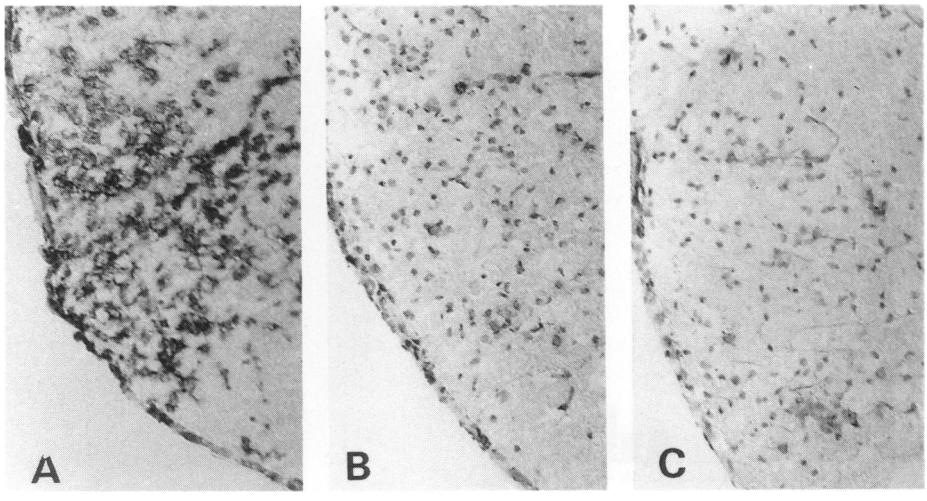

Figure 4. Serial cryosections of lumbar spinal cord of an infected mouse showing paralysis at 7 DPI stained with A, anti-Mac-1; B, anti-L3T4; and C, anti-Lyt2 antibody. Anti-Mac-1-positive cells are predominant. ABC method × 150.

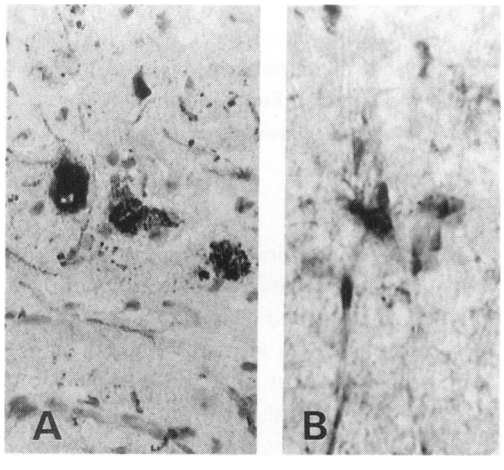

Figure 5. Lumbar spinal cord of an infected mouse showing paralysis at 7 DPI. Virus antigens are observed in the cytoplasm of A, neurons and B, oligodendrocytes. ABC method. A, × 300; B, × 600.

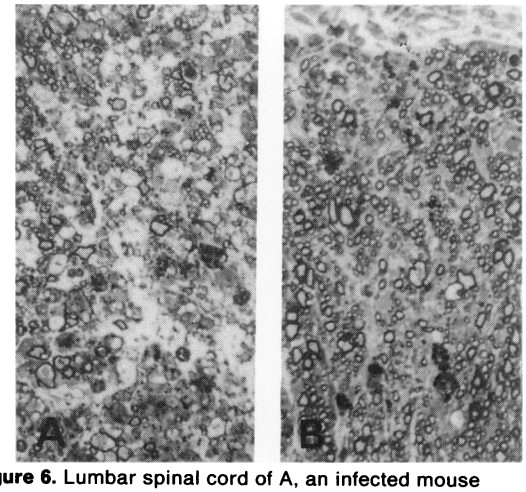

Figure 6. Lumbar spinal cord of A, an infected mouse showing paralysis at 7 DPI and B, that of an infected mouse showing recovery at 28 DPI. In comparison with A, B shows a prominent reduction in size of demyelinated lesions. Toluidine blue. × 150.

of degenerated and/or adjacent intact neurons in cornu ventrale (Figure 5A) and of oligodendrocytes in the demyelinated lesion in funiculus lateralis from 3 to 14 DPI (Figure 5B). These glial cells were also positively stained with anti-galactocerebrocide antibody. On and after 21 DPI, virus antigens were not detected in any of the cells.

At 28 DPI, in the mice showing recovery from paralysis, the size of demyelinated lesion apparently reduced with a decrease in number of infiltrated cells (Figure 6B).

At 56 DPI, in the mice showing recurrent paralysis, prominent infiltration of mononuclear cells was characteristic in the cornu ventrale showing degeneration and necrosis of motor neurons (Figure 7). These mononuclear cells were mainly composed of anti-L3T4-positive cells (CD4+ T cells) and anti-Mac-1-positive cells, and anti-Lyt2-positive cells (CD8+ T cells) were scarce (Figure 8). On the other hand, in the mice without recurrent paralysis, there was no apparent lesion in the spinal cord at 56 DPI.

Electron microscopic findings

In coincidence with the histopathological findings,

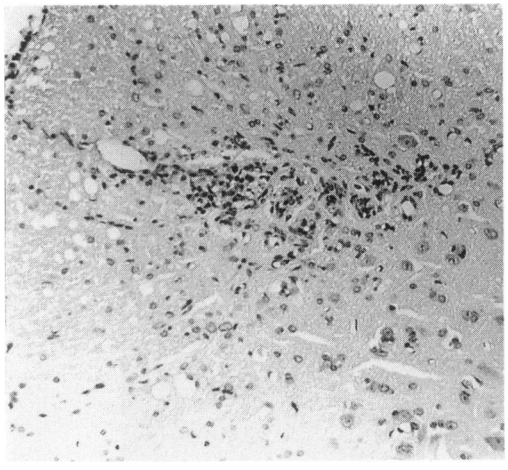

Figure 7. Lumbar spinal cord of an infected mouse showing recurrent paralysis at 56 DPI. Infiltration of mononuclear cells in the cornu ventrale. H&E. × 150.

marked demyelination with infiltration of macrophages was found in the funiculus lateralis at 7 DPI (Figure 9A). At 28 DPI, remyelination occurred in the previous lesion (Figure 9B).

Discussion

As mentioned above, Takeda *et al.* (1991) reported that the high dose (10^3 PFU/head) of EMC-D induced unphasic paralysis in DBA/2 mice. In that case, the mice which developed paralysis did not recover; almost all of them died by 28 DPI. On the other hand, in the case of this paper, it was confirmed that biphasic paralysis could be induced in mice given a low dose of EMC-D. Thus, about 60% of the infected mice developed paralysis by 12 DPI and two-thirds of them showed recovery by 33 DPI. Thereafter, about 30% of the mice which once showed paralysis developed paralysis again by 56 DPI.

The virus titre of the spinal cord reached a maximal level at 7 DPI and infectious virus became undetectable by 28 DPI. Virus antigens were detected in the cytoplasm of degenerated and/or adjacent intact neurons in the cornu ventrale and of oligodendrocytes in the funiculus lateralis showing focal demyelination until 14 DPI. Therefore, the initial lesions are considered to be brought about mainly by direct attack of EMC-D on neurons and oligodendrocytes, resulting in hind limb paralysis. However, we must take account of participation by macrophages which infiltrated in the early lesions. In this regard, Baek and Yoon (1990) reported that macrophages participated in the damage of pancreatic β cells of mice infected with EMC-D.

During the following stage, the spinal cord lesion appeared to be reduced. In particular, demyelinated areas in the funiculus lateralis were reduced by remyelination as demonstrated by electron microscopic examinations.

At 56 DPI, CD4+ T cells were seen to predominate in the cornu ventrale including degenerative or necrotic motor neurons in the mice showing recurrent hind limb paralysis. The infiltration of these cells was not observed in the mice without recurrent paralysis. This suggests that the late paralysis is probably caused by degenera-

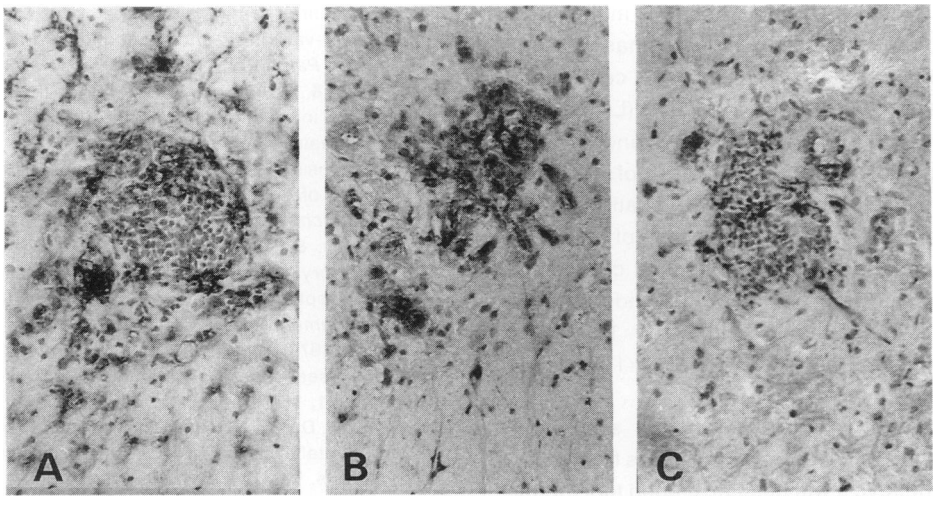

Figure 8. Serial cryosections of the lumbar spinal cord of an infected mouse showing recurrent paralysis at 56 DPI stained with A, anti-Mac-1; B, anti-L3T4; and C, anti-Lyt2 antibody. Anti-L3T4-positive cells are predominant. ABC method. × 150.

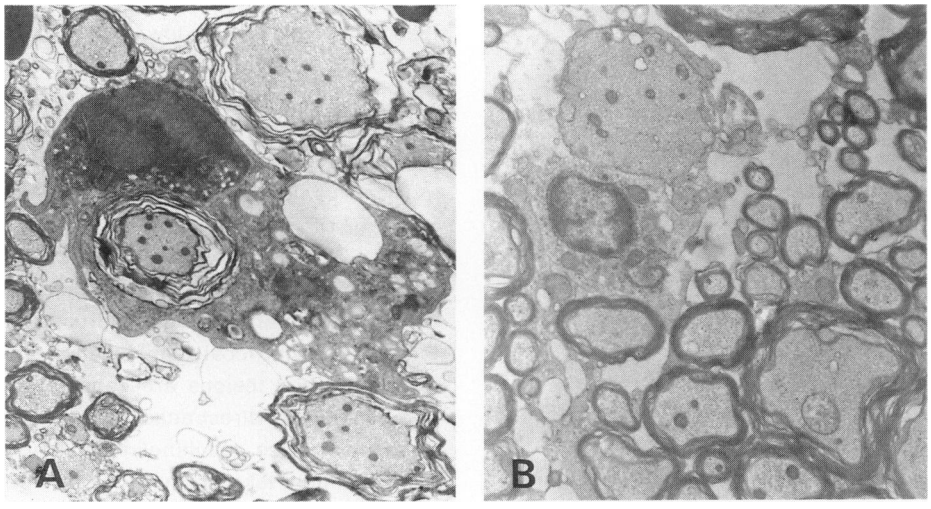

Figure 9. Electron microscopic pictures of Figure 8. Marked demyelination and infiltration of macrophage are seen in A, and prominent remyelination is noticed in B. A, × 6000; B, × 10500.

tion of motor neurons and that this neuronal damage may be brought about by infiltration of CD4+ T cells. However, the exact relationship between infiltration of CD4+ T cells and degeneration of motor neurons is still obscure.

Paralysis was also reported in BALB/cCum mice infected with EMC-M (Sriram *et al.* 1989; Craighead *et al.* 1990; Topham *et al.* 1991). In that case, demyelination and perivascular cuffing were detected in the white matter of the spinal cord and participation of T cells in demyelination was suggested. The DA strain of Theiler's murine encephalomyelitis virus (DAV) is well known to cause biphasic nervous symptoms in mice (Lipton 1975). In mice infected with DAV, virus antigens were detected in nerve cells, astrocytes and macrophages in the early phase and in oligodendrocytes in the late phase (Rodriguez *et al.* 1983). Furthermore, CD8+ T cells were suggested to cause demyelinated lesions (Lindsley & Rodriguez 1989; Rodriguez & Sriram 1988), and Yamada *et al.* (1990) suggested the synergetic effect of viral RNA and inflammatory cells using in-situ hybridization. In this connection, we are now trying to detect RNA of EMC-D in neurons and oligodendrocytes in the spinal cord using in-situ hybridization. Furthermore, we are now investigating the role of macrophages and CD4+ T cells by treating EMC-D-infected mice with anti-Mac-1 and anti-CD4 antibodies.

As mentioned above, our experimental system of biphasic hind limb paralysis somewhat differs from other systems and we believe it will become a good tool for the investigation of the relevance of the immune system and viral infection to central nervous disorders.

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