Pathologic Characterization of a Murine Model of Human Enterovirus 71 Encephalomyelitis

Kien Chai Ong, BSc, Munisamy Badmanathan, MMedSc, Shamala Devi, PhD, Kum Loong Leong, DipMLT, Mary Jane Cardosa, PhD, and Kum Thong Wong, MBBS, Mpath, FRCPath

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MATERIALS AND METHODS

Preparation of Mouse-Adapted Virus Strain

Vero cells grown in Dulbecco’s modified Eagle's medium supplemented with 5% fetal bovine serum were used for virus preparation and titration. The unadapted parental EV71 strain (genotype B3; GenBank accession number AY207648) (18) was previously isolated in the Department of Medical Microbiology, University of Malaya, from a child who died of EV71 encephalomyelitis (10).

To prepare EV71 mouse-adapted virus strain (MAVS), serial passages were initiated by intracerebral (i.c.) inoculation of 10^5 cell culture infective dose 50% (CCID50) of the parental strain into day-old ICR mice (n = 6). Brains from a litter of 6 infected mice were harvested and pooled after 3 to 4 days postinoculation (PI). Homogenate suspension (10% wt/vol) was prepared with PBS containing calcium chloride, magnesium chloride, and antibiotics (19). Approximately 30 μL of the supernatant was i.c.-inoculated into each mouse of a new mouse litter for the second and subsequent passages. Mouse-adapted virus strain derived from the fifth passage was used for experiments because it showed the highest virus titer in the mouse brains (data not shown). To increase virus stock, the MAVS was further propagated in Vero cells once a multiplicity of infection of 0.01 CCID50 per cell and incubated at 36°C until complete cytopathic effect was achieved. The cells and supernatant were frozen at −80°C, thawed 3 times, and centrifuged to remove cell debris. The final virus titer was determined by a standard microtitration assay in Vero cells as previously described (20).

Animal Infection Experiments

Three sets of animal experiments were performed. These were approved by, and in accordance with, institutional guidelines on animal welfare and use of the University of Malaya. Because preliminary testing of the MAVS on 2- and 4-week-old ICR mice showed that only the former were susceptible and showed evidence of CNS infection (results not shown), a 50% lethal dose study (LD50) was performed on groups of four 2-week-old mice by i.c., intraperitoneal (i.p.), intramuscular (i.m.), subcutaneous (s.c.), and oral (o.l.) routes. The LD50 was calculated by the method of Reed and Muench (21).

Based on the LD50 study, a second study was performed on groups of 2-week-old mice using i.c., i.m., i.p., s.c., and o.l. routes to confirm virus infection in the CNS and other tissues. Thirteen animals per group were each inoculated with 10^5 CCID50 MAVS. Mock-infected animals (n = 5) for each route received diluents with no virus. Whenever possible, mice were observed up to 14 days for signs of infection, for example, paralysis and death. All animals that showed paralysis were killed under ether anesthesia. At the time of death, cardiac puncture was performed to obtain serum samples for virus titration. Other tissues were also harvested for histology, IHC, ISH, EM, IEM, and virus titration. Tissues from animals found dead in the cages were not studied.

To investigate viral spread into and within the CNS, a third (kinetic) study was done. The design of this experiment was partly suggested by the results of the first and second studies that showed that IM-inoculated animals had the lowest LD50 and shortest incubation periods (see Results section). Two-week-old mice (n = 32) were inoculated i.m. with 10 μL PBS containing 3 × 10^5 CCID50 MAVS into the caput medialis of the right hindlimb gastrocnemius muscle under ether anesthesia. The virus suspension was administered slowly and carefully via a 27-G needle attached to a micropipette. At 24, 36, 48, and 72 hours postinfection (HPI), 5 mice at each point were killed, and tissues were collected. Mock-infected mice (n = 10) were given PBS and kept either in the same cage with the infected mice or in a separate cage. Control animals were always handled prior to infected animals.

Histopathology

In the second study, for each route of inoculation, tissue samples from killed animals (n = 10) were fixed in 10% neutral-buffered formalin. Seven standard tissue blocks were taken from each animal. The first block contained coronal sections of the whole brain; the subsequent blocks contained whole axial sections of the head, neck, thorax, abdomen, pelvis, and thighs. This provided a wide range of organs, multiple levels of spinal cord, and dorsal root and autonomic ganglia for study. The blocks were fixed for 2 more days, decalcified in formic acid, and routinely processed.

Paraffin-embedded tissue sections 4-μm thick were placed onto 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO)-coated slides, dried overnight, and stained with hematoxylin and eosin. Additional unstained sections were used for IHC and ISH. Tissues from the third study were prepared in the same way. For the CNS tissues, left-right orientation was carefully maintained throughout processing. Brainstem and spinal cord nuclei and tracts were identified using an atlas (22).

Immunohistochemistry

Immunohistochemistry was performed using a standard avidin-biotin immunoperoxidase technique as described previously (14). Briefly, tissue sections were dehydrated, dehydrated, and microwaved for 20 minutes at 99°C in a citrate buffer. Polyclonal rabbit anti-EV71 (a gift from Dr Shimizu, Japan) was applied for 2 hours at room temperature. A secondary biotinylated swine anti-rabbit immunoglobulin G (Dako, Glostrup, Denmark) was added, followed by avidin-biotin-peroxidase complex and 3, 3'-diaminobenzidinetetrahydrochloride (Sigma-Aldrich) chromogen. Tissues were counterstained with hematoxylin and eosin. Additional unstained sections were used for ISH and ISH. Tissues from the third study were prepared in the same way. For the CNS tissues, left-right orientation was carefully maintained throughout processing. Brainstem and spinal cord nuclei and tracts were identified using an atlas (22).

In Situ Hybridization

Digoxigenin-labeled DNA probes were generated from a 500-bp PCR product derived from the 5'-nontranslated region of the EV71 genome using published primers (23) and used for ISH as previously described (14). Briefly, dewaxed tissue sections were pretreated with HCl and proteinase K.
IHC specificity testing.

omitted probes were performed. In situ hybridization assays that either used RNAase-treated tissue sections or phosphate substrate (Roche). For negative controls, duplicate nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Roche). For negative controls, duplicate

Mannheim, Germany) conjugated to alkaline phosphatase was detected using an anti-digoxigenin antibody (Roche, Organs were weighed and homogenized in PBS to 10%

K (100 g/ml; 20 minutes; 37°C). Approximately 1 ng of probe in a hybridization solution (6 × saline sodium citrate, 5 × Denhardt solution, 100 μg/ml of denatured salmon sperm DNA, 5% dextran sulphate) was added onto tissue sections and heated to 95°C for 10 minutes, followed by incubation for 16 to 20 hours at 42°C in a moist chamber. Hybridization was detected using an anti-digoxigenin antibody (Roche, Mannheim, Germany) conjugated to alkaline phosphatase and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Roche).

For IEM, similar pieces of tissue were fixed in 4% paraformaldehyde/0.1% glutaraldehyde solution for at least 4 hours in 4°C and washed 2 to 3 times in cacodylate buffer before postfixation in 1% osmium tetroxide for 2 hours at 4°C. The tissues were then washed in double-distilled water and dehydrated through graded ethanol and propylene oxide, before Epon embedding at 60°C overnight. Selected 60- to 80-nm ultrathin sections were collected on copper grids and stained with uranyl acetate and lead citrate before viewing by transmission EM (Zeiss Libra 120, Zeiss, Germany).

For IEM, similar pieces of tissue were fixed in 4% paraformaldehyde/0.1% glutaraldehyde solution for 4 hours at 4°C. Tissues were dehydrated and embedded in LR White resin (ProSciTech, Queensland, Australia). Ultrathin sections on 200-mesh nickel grids were incubated in 0.01 mol/L PBS, pH 7.2, containing 5% normal goat serum, then in 0.01 mol/L PBS-1% bovine serum albumin before incubation with anti-EV71 primary antibody (dilution, 1:50). Goat anti-rabbit secondary antibody coupled with 10-nm colloidal gold (BB International, Cardiff, UK) were then added. Finally, the sections were stained with uranyl acetate and lead citrate and examined as previously discussed.

Electron Microscopy and Immuno-electron Microscopy

Small pieces (~ 2 mm³) of the spinal cords from i.c. and i.m.-infected mice (second animal study) were fixed in 4% glutaraldehyde for at least 4 hours in 4°C and washed 2 to 3 times in cacodylate buffer before postfixation in 1% osmium tetroxide for 2 hours at 4°C. The tissues were then washed in double-distilled water and dehydrated through graded ethanol and propylene oxide, before Epon embedding at 60°C overnight. Selected 60- to 80-nm ultrathin sections were collected on copper grids and stained with uranyl acetate and lead citrate before viewing by transmission EM (Zeiss Libra 120, Zeiss, Germany).

Virus Titration

In the second study, 3 mice in each inoculation group were killed to obtain fresh tissues (serum, brain, spinal cord, and other major organs) for virus titration. Utmost care was taken to minimize cross-contamination between animals and between tissues in the same animal. Samples were frozen at –80°C immediately for later titration. Organs were weighed and homogenized in PBS to 10% (wt/vol). Virus titer was determined using CCID<sub>50</sub>, and the virus infectivity was calculated using the method of Karber (24).

In the third study, 3 mice each were killed at 24, 36, 48, and 72 HPI to obtain tissues for virus titration by the same methods. In this study, however, only serum and tissues from the brainstem; “upper spinal cord” (cervical and upper half of the thoracic cord); “lower spinal cord” (lower half of the thoracic and lumbosacral cord); and bilateral, front, and hindlimb skeletal muscles were analyzed.

Statistics

The Student t-test was used to determine the significance of differences in mean viral titers using the software SigmaStat version 3 (SPSS, Inc, Chicago, IL). The results are expressed as mean ± SEM. p values less than 0.05 were considered significant.

RESULTS

LD<sub>50</sub> Study (First Study)

Mice could be infected by the MAVS via all of the inoculation routes examined. Signs of infection included progressive paralysis, ruffled fur, weight loss, and a humped posture. In ascending order, the LD<sub>50</sub> values were i.m. group (1 × 10<sup>4</sup> CCID<sub>50</sub>), i.c. group (3 × 10<sup>4</sup> CCID<sub>50</sub>), i.p. group (6 × 10<sup>4</sup> CCID<sub>50</sub>), and s.c. group (28 × 10<sup>4</sup> CCID<sub>50</sub>). Because only 2 of 4 o.l.-inoculated mice developed paralysis and none died, the LD<sub>50</sub> was not calculated.

Animals Infected by Different Routes (Second Study)

Clinical Observations

All animals inoculated by parenteral routes developed infection. On average, i.m.-inoculated animals developed paralysis or died at 2 to 3 days PI; for other routes of infection (i.c., i.p., s.c.), paralysis or death occurred at 3 to 4 days PI. Only 1 mouse in the o.l.-inoculated group (10%) developed paralysis and none died.

Macroscopic Pathology

At death, none of the infected mice showed any cutaneous or mucosal lesions. Hardened whitish foci in the interscapular brown adipose tissues were observed in some mice, and there was pallor of hindlimb and spinal muscles. There were no apparent macroscopic CNS lesions in infected mice.

CNS Histopathology

The spinal cord and brainstem seemed to be the most severely affected portions of the CNS in all infected groups. The major features were neuropil vacuolation and neuronal damage or loss with little or no evidence of inflammatory response (Fig. 1A). These findings were most severe in the anterior horn cells, brainstem reticular formation, and motor trigeminal nucleus. No obvious neuronal abnormalities were identified in the cerebral cortex, thalamus, hypothalamus, cerebellum, or dorsal root sensory and autonomic ganglia. There were no cytoplasmic or nuclear viral inclusions. Tissues from control mice showed no abnormalities.

Viral RNA and antigens were mainly found in neuronal cytoplasm and processes (Figs. 1B, C). In addition, efferent motor axons adjacent to positive anterior horn cells occasionally demonstrated viral RNA and antigens (Figs. 1D, E). No nuclear staining was detected. Infected neurons were either single or in small groups; some seemed morphologically normal. In situ hybridization and IHC in positive controls gave clear, crisp signals with little background
FIGURE 1. Pathologic findings in mice infected with Enterovirus 71 by various inoculation routes at 3 days postinfection. Neutrophil vacuolation and degeneration surrounding cervical cord anterior horn cells (A; arrows). These cells demonstrated viral RNA (B; arrow) and antigens (C; arrow). In the lumbar cord, from a right hindlimb intramuscular-inoculated mouse, viral antigens are detected in the ipsilateral anterior horn cells and axons (D, E; arrows). Motor trigeminal nucleus positive for viral RNA (F; arrows) and antigens (G; arrows). Viral RNA in the reticular formation (H) and lateral cerebellar nucleus (I; arrows). A single neuron in the motor cortex contains viral antigen (J). Myositis and extensive necrosis in skeletal muscle (K) with numerous viral RNA-positive fibers (L; arrows). (A, K) Hematoxylin and eosin stain. (B, F, H, I, L) In situ hybridization with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate and hematoxylin counterstain. (C–E, G, J) Immunohistochemistry with 3, 3'-diaminobenzidinetetrahydrochloride chromogen and hematoxylin counterstain. Original magnification: 4× (D); 10× (F, G, I); 40× (A–C, E, H, J–L).
staining, and controls were negative. In general, ISH seemed to be slightly more sensitive than IHC.

By electron microscopy, viral particles (~30 nm) were observed scattered throughout the cytoplasm of infected neurons. Abnormal membrane-bound cytoplasmic vesicles were also frequently observed in damaged neurons. Viral particles were occasionally demonstrated within these vesicles (Fig. 2A). Immunoelectron microscopy confirmed the presence of virus (Fig. 2B).

The distribution of viral RNA and antigens in CNS tissues is summarized in the Table. Consistent with the histology findings, anterior horn neurons in the cervical, thoracic, and lumbar cord were very frequently (50%–100%) positive, independent of the route of infection. The sacral cord and posterior horn cells were consistently negative. In the brainstem, viral RNA and antigens were always found in the reticular formation (Fig. 1H) and frequently (78%–100%) in the motor trigeminal nucleus (Figs. 1F, G). The numbers of positive neurons were also the highest in these areas.

Positive neurons in the red, facial, hypoglossal, and lateral cerebellar (equivalent to the human dentate nucleus) nuclei (Fig. 1I) and the locus coeruleus were less frequently observed; except for the red nucleus, the density was also generally low (Table). Rare positive neurons were also found in the motor cortex (Fig. 1J), thalamus, and hypothalamus. The pontine nuclei, cerebral sensory, and cerebellar cortex showed no evidence of virus. Meningeal, ependymal and other glial cells, blood vessels, dorsal root, and autonomic ganglia were all negative.

Non-CNS Organ Pathology

Skeletal muscle fibers showed striking degeneration, necrosis, and varying degrees of inflammation (Fig. 1K) in

![Figure 2](http://jnen.oxfordjournals.org/) Virus particles in the cytoplasm of a neuron shown by electron microscopy (A; arrows; inset) and immunogold labeling (B). Scale bar = (A) 1 μm; (inset and B) 100 nm.

**TABLE.** CNS Distribution of Enterovirus 71 RNA and Antigens in Severely and Terminally Infected Mice

<table>
<thead>
<tr>
<th>CNS Region</th>
<th>Density* of RNA/Antigens and Percentage† of Positive Areas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intracerebral Route, n = 10</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>+ 20% (2/10)</td>
</tr>
<tr>
<td>Sensory cortex</td>
<td>– 0% (0/10)</td>
</tr>
<tr>
<td>Thalamus</td>
<td>+ 30% (3/10)</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>+ 30% (3/10)</td>
</tr>
<tr>
<td>Brainstem</td>
<td>++ 43% (3/7)</td>
</tr>
<tr>
<td>Red nucleus</td>
<td>+ 20% (1/5)</td>
</tr>
<tr>
<td>Locus coeruleus</td>
<td>++ 78% (7/9)</td>
</tr>
<tr>
<td>Motor trigeminal nucleus</td>
<td>+++ 100% (10/10)</td>
</tr>
<tr>
<td>Facial nucleus</td>
<td>+ 50% (4/8)</td>
</tr>
<tr>
<td>Reticular formation</td>
<td>+++ 100% (10/10)</td>
</tr>
<tr>
<td>Hypoglossal nucleus</td>
<td>+ 40% (4/10)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>– 0% (0/10)</td>
</tr>
<tr>
<td>Cervical anterior horn</td>
<td>– 0% (0/10)</td>
</tr>
<tr>
<td>Thoracic anterior horn</td>
<td>+ 50% (5/10)</td>
</tr>
<tr>
<td>Lumbar anterior horn</td>
<td>++ 50% (5/10)</td>
</tr>
<tr>
<td>Sacral anterior horn</td>
<td>– 0% (0/10)</td>
</tr>
</tbody>
</table>

* Density of positive neurons per microscopic field (10x objective): +, 1 positive neuron; ++, 2–5 positive neurons; ++++, 6–10 positive neurons.
† Percentages were derived from dividing total number of mice with positive area by the total number of mice in which these specific areas were found in the tissue sections. Raw data are in parentheses.
‡ Because only 1 sick animal was studied, percentages were not displayed.
infected mice. The limb muscles were the most severely affected, with massive and diffuse necrotizing myositis. Tongue involvement was also common, but myositis was far less severe than in other muscle groups. Other major organs, including the heart, lung, liver, kidney, pancreas, and gastrointestinal tissues, were all normal. In particular, there was no evidence of myocarditis. Tissues of all mock-infected animals were also normal.

Viral RNA and antigens were demonstrated in the entire skeletal muscle system (Fig. 1L), but the head, neck, masster, spinal, limbs, and pelvic muscles most often showed the strongest ISH/IHC staining. Many foci of RNA and antigen-positive cells were observed in interscapular brown adipose tissues and adipose tissues attached to the heart, thymus, kidney, and spleen (data not shown). The thymus, lymph nodes, spleen, heart, lung, liver, kidney, pancreas, oral cavity epithelium, and gastrointestinal tissues were all negative for viral RNA and antigens.

**Viral Titers**

The results of virus titration by CCID$_{50}$ are shown in Figure 3. In general, all infected mice showed evidence of virus in the serum, CNS, and other tissues. The highest titers were in the skeletal muscle, spleen, and spinal cord.

**Clinical Observations**

In all hindlimb i.m.-inoculated mice, paralysis started at 48 HPI in the inoculated right limb. Paralysis was initially mild with decreased limb movements, but severe paralysis was observed at 72 HPI. Paralysis involving all other limbs became evident at approximately this time.

**Pathologic Findings**

Figure 4 summarizes the distribution of viral RNA and antigens in the CNS tissues of each mouse at 24, 36, 48, and 72 HPI points.

**24 and 36 Hours Postinfection**

At 24 HPI, viral RNA and antigens were found only in Mouse 1 (Fig. 4), on the right side of the lumbar cord, ipsilateral to the inoculation site, and exclusively in the anterior horn cells and adjacent axons (Fig. 1D). There was no evidence of virus on the contralateral side at the same or other cord levels or in the brain. At 36 HPI, 4 animals (Fig. 4; Mice 6–9) showed these same features. Additionally, Mice 8, 9, and 10 showed rare positive neurons in the thalamus, cervical cord, and motor cortex and hypothalamus, respectively.

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**FIGURE 3.** Virus titers in tissues of mice infected with same doses of Enterovirus 71 by various routes. Virus titer is expressed as the mean log$_{10}$ CCID$_{50}$ ± standard error of mean per 10% pooled tissue homogenates from groups of 3 mice at Day 3 postinfection. No virus was isolated from heart tissue of intracerebral- and subcutaneous-inoculated mice. Virus titers in pooled sera are expressed as the log$_{10}$ CCID$_{50}$ per milliliter. BF, interscapular brown adipose tissue; LSC, lower spinal cord (lumbosacral and lower half thoracic cord segments); SM, skeletal muscle (hindlimb); USC, upper spinal cord (cervical and upper half thoracic cord segments).
At 24 to 36 HPI, there were large numbers of virus-positive skeletal muscle fibers in the inoculated limb. In contrast, there were only a few positive muscle fibers in the left hindlimb, front limbs, tongue, head, and facial muscles detected.

48 Hours Postinfection
At 48 HPI, lumbar, thoracic, and cervical cord segments began to show infected anterior horn cells, but the highest densities were still in the right lumbar anterior horn area (Fig. 4; Mice 11–15). One or 2 infected neurons (unilateral and bilateral) were also detected in medulla, pons, thalamus, hypothalamus, and motor cortex. Virus-positive muscle fibers in the inoculated limb were as extensive as at the earlier time point, but the numbers of positive fibers in other limbs and elsewhere were still small, and viral antigen or RNA was localized.

72 Hours Postinfection
Viral RNA/antigens were detected mainly in bilateral anterior horn areas from lumbar to cervical cord segments. There were, however, a few positive neurons detected in the intermediate and posterior horns (Mice 16, 19, and 20). In the brainstem, viral RNA and antigens were found in many more neurons in the motor trigeminal nucleus and reticular formation than at earlier points. Usually, the involvement was bilateral. The distribution of the viral RNA and antigens in skeletal muscle fibers at this time point was extensive in all muscle groups and was more or less of uniform density throughout.

Motor System Involvement
Immunohistochemistry-positive efferent motor axons adjacent to positive anterior horn cells were found in 14 of 20 mice (70%) (Fig. 4). Mice 1, 6, 8, 9, and 11 to 20 had ipsilateral (right side), and Mice 14 to 18 and 20 had contralateral, IHC-positive efferent motor axons at the lumbar cord level. Mice 11 to 20 also had either unilateral or bilateral efferent motor axon involvement in cervical and thoracic levels.

Motor cortex involvement was detected in 3 mice (Fig. 4; Mice 10, 13, and 18) and was always on the left side, contralateral to the site of inoculation. Dorsal root and autonomic ganglia (sympathetic and parasympathetic ganglia) showed no evidence of virus in any of the animals examined.

Viral Titers
Virus titers in the brain, upper and lower spinal cords, bilateral front and hindlimb muscles, and sera are shown in Figure 5. At 24 HPI, virus was isolated from the spinal cord but not from the brain (Fig. 5A). The lower spinal cord showed the highest titers; they were significantly higher than titers from the upper spinal cord (p < 0.05). The lower spinal cord titers up until 48 HPI were higher than those of the upper spinal cord and brain, whereas at 72 HPI, the titers were more similar.

Viremia was detected as early as 24 HPI and persisted at high levels throughout the period of observation. In the inoculated right hindlimb muscles, the titers were the highest compared with all other limb muscles especially at 24 HPI (Fig. 5B).

DISCUSSION
We have demonstrated susceptibility of 2-week-old mice to EV71 MAVS infection by parenteral and oral routes. Similar to human EV71 encephalomyelitis (14), our mouse model demonstrated strong neuronotropism in the CNS. Viral RNA, antigens, and particles were detected in neuronal somata and processes but not in glial or other cells. In a previously reported mouse model, viral antigens were shown...
FIGURE 5. Viral titers in pooled (n = 3 each) CNS samples and sera (A), and limb muscles (B) after right hindlimb intramuscular inoculation with Enterovirus 71. Virus was not isolated from brain tissues at 24 hours postinfection. Titers are expressed as the mean log CCID$_{50}$ ± standard error of mean of 10% (wt/vol). LSC, lower spinal cord (lumbosacral and lower half thoracic cord segments); USC, upper spinal cord (cervical and upper half thoracic cord segments).

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in neurons (17), but our study demonstrates the presence of viral RNA and virions for the first time.

Despite the presence of only sparse inflammation, the distribution of CNS viral RNA and antigens seems to parallel the distribution of inflammation and virus in human EV71 encephalomyelitis. In the mouse model, viral RNA and antigens were mostly found in the anterior horn cells of the spinal cord, medulla, pons (sparing the pontine nuclei), midbrain, and lateral cerebellar nucleus. These are also the areas in which inflammation was found to be severe in human cases (14, 25). In addition, the rarity and absence of virus in the murine motor cortex and cerebellar hemisphere, respectively, also seem to resemble the human infection. In contrast, however, the mouse hypothalamus, unlike the human counterpart (14), did not seem to be severely involved. To our knowledge, a detailed description of neuronal infection in the mouse brain with comparisons to human EV71 encephalomyelitis has not been previously reported.

Our data show that after inoculation, viremia occurs early, and that there is spread of the infection to the CNS and other organs. The mechanism for viral entry into the murine CNS was previously suggested to be via retrograde peripheral nerve transmission (16). There was, however, no indication as to which portion of the peripheral nervous system (motor, sensory, or autonomic) might be implicated. The present study provides evidence for viral transmission into the murine CNS via the peripheral motor nerves, similar to what seems to occur in human encephalomyelitis (14).

Independent of the route of inoculation, all sick mice exhibited more or less the same stereotyped viral distribution, suggesting that the virus was likely to have used the same pathway to enter and spread within the CNS. Moreover, the kinetic study suggests that the virus enters the CNS via peripheral somatic motor nerves and first infects ipsilateral spinal cord anterior horn motor neurons from 24 to 36 HPI. This was supported by the high density of viral RNA and antigens localized only to the anterior horn at these early points (Fig. 4). Moreover, viral RNA and antigens were also found in the efferent somatic motor axons adjacent to these positive anterior horn neurons.

Our findings show that unilateral or bilateral involvement of anterior horn cells above the lumbar cord and brainstem motor neurons also occurs, but generally only after 36 HPI. We think that retrograde transmission up peripheral motor nerves that innervate skeletal muscles in the un inoculated limbs and head and neck regions led to the infection of these neurons. Viremia could have spread the infection to skeletal muscle first in these areas; the short but significant delay in finding positive neurons in these areas is consistent with this scenario. Moreover, viral titers were highest in the lower spinal cord at 24 and 36 HPI. Our data did not support viral entry into the CNS by sensory and autonomic pathways because the dorsal root and autonomic ganglia were not found to be involved. Figure 6 summarizes the major findings and our hypothesis for viral spread in this model.

Once inside the CNS, one of the pathways for rostral spread of virus could be the corticospinal/corticobulbar tracts. The paucity of positive neurons in the contralateral motor cortex in 3 mice (Fig. 3; Mice 10, 13, and 18) compared with numerous positive neurons in the anterior horn/brainstem seems to suggest a delay of viral transmission (i.e. additional time) would be needed to travel up the motor tracts. In addition, rare positive motor cortical neuron might have been missed as a result of sampling error. Nevertheless, similar findings have been observed in human EV71 encephalomyelitis (14). The severely involved reticular formation is likely to facilitate viral spread within the CNS to the hypothalamus and other areas, and lateral cerebellar nucleus involvement may be via pathways that connect it to the red nucleus and

FIGURE 6. Hypothesis for Enterovirus 71 infection and spread into and within the murine CNS after hindlimb intramuscular inoculation.
the medulla. The rare positive neurons in the thalamus and principal sensory and spinal V nucleus (data not shown) may have been infected via the complex interlink of other neural pathways in the CNS. There was no evidence of anterograde viral spread to the dorsal root ganglion from anterior horn cells similar to that reported in rabbits.

It is possible that the generally stereotyped distribution of infected neurons in the murine CNS can be related to varying susceptibility of different types of neurons to EV71. If that was the case, then the virus may have entered the CNS by a hematogenous route and selectively infected only the susceptible neurons in the anterior horn, brainstem, and other areas. This possibility needs to be further investigated. To date, however, the viral receptor for EV71 has not been identified (26, 27), and it is not possible to speculate on relative susceptibility based on the presence or absence of receptors on neurons. In the case of poliovirus, some murine-adapted strains have been known to develop a preference for neural transmission over hematogenous spread (28). Although this might have happened to the wild EV71 strain after murine adaptation, we think that the similarity of the pathology in our model to human EV71 encephalomyelitis makes this possibility less likely. Interestingly, transgenic mouse models that express the human poliovirus receptor have suggested an important role for neural transmission for poliovirus infection (29, 30).

The data suggest that skeletal muscle infection plays a key role in the pathogenesis in this EV71 encephalomyelitis model (Fig. 6). Viral replication in the muscle was followed rapidly by viral spread into the CNS via peripheral motor nerves, presumably by crossing myoneural junctions. Direct ultrastructural demonstration of virions in the motor neuron junction can provide supportive evidence for this, but to date, this has not been reported. Muscle infection and retrograde nerve transmission have, however, been reported in poliovirus infection of murine models (31). Studies on small limited samples of human muscle have failed to demonstrated evidence of EV71 virus infection or myositis (14), but further investigations are needed to explore this possibility. Because severe myositis itself can cause paralysis, a more useful end point for encephalomyelitis in this model would be the direct demonstration of neuronal infection. In this regard, we think that the combination of ISH and IHC is more sensitive for detecting virus than using either alone.

Although a smaller proportion developed CNS disease, mice can be infected orally in our model in a manner consistent with natural human fecal-oral transmission. This resembles the human infection in that most human EV71 infections are not complicated by encephalomyelitis. Not surprisingly, however, the parenteral routes seem to be much more consistent and predictable for causing disease. It is assumed that orally inoculated viruses have to get past the effects of digestive juices and the mucosal barrier to cause systemic or CNS infection. Nevertheless, the pattern of disease in the mice that developed disease in this study by the oral route was the same as the mice inoculated by other routes.

Absence of significant inflammation in the mouse CNS might be a consequence of the comparatively short period of infection because most animals were paralyzed or died from 3 to 5 days PI. Another possibility may be related to lymphoid tissue depletion as previously reported (17) or to apoptosis/necrosis found in the thymus, spleen, and lymph nodes in some of our mice (data not shown). These observations need to be further investigated. This mouse model may not be suited to studying inflammation in EV71 encephalomyelitis.

Extensive involvement of brown adipose tissues has not been previously reported in EV71 infection. Similar findings were found in enterovirus, rabies, and cytomegalovirus infection animal models (32–35). So far, there is no information on viral replication in human brown fat. If human brown fat can be readily infected like in the model, it is conceivable that the relatively large deposits of brown fat in infants might predispose them to a higher EV71 replication rate and a more severe infection. The pathophysiologic significance of this adipotropism and how it may play a role in human encephalomyelitis remains speculative.

Viral RNA and antigens were not demonstrated in the heart, lung, spleen, or other organs. In particular, the absence of demonstrable viral RNA and antigens in the small intestines and lymph nodes was surprising because it is assumed that, like poliovirus, viral replication can occur in those sites. Whether murine EV71 replicates in these tissues needs further investigation. The apparent disparity between demonstrable viral presence and positive virus culture results may have arisen from contamination of tissues by attached infected brown fat or viremia. It is possible, however, that low concentrations of virus within cells may not be detected by ISH or IHC but can still be cultured.

Because the viral receptor for EV71 is unknown, there are currently no transgenic mouse models of EV71 encephalomyelitis as had been developed for poliovirus (29, 30). The present results suggest that this model can be used to study viral transmission pathways, mechanisms of neuronal damage, and vaccine studies.

ACKNOWLEDGMENT

The authors thank Hirofumi Shimizu, PhD, for providing the EV71 antibodies.

REFERENCES

15. Wong KT, Chua KB, Lam SK. Immunohistochemical detection of infected neurons as a rapid diagnosis of enterovirus 71 encephalomyelitis. Ann Neurol 1999;45:271–72