Title: Viral neuronotropism is important in the pathogenesis of Murray Valley encephalitis

Tzeh Long Fu, Kien Chai Ong¹, Yang David Tran², Catriona A. McLean^{2,3}, Kum Thong Wong*

Department of Pathology and ¹Department of Biomedical Science, Faculty of Medicine,
University of Malaya, Kuala Lumpur, Malaysia

²Department of Medicine, Monash University, Melbourne, Victoria, Australia

³Department of Anatomical Pathology, Alfred Hospital, Melbourne, Victoria, Australia

*Wong Kum Thong (Corresponding author)

Address: Department of Pathology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.

Telephone Number: +603-79492064; Fax Number: +603-79556845

E-mail: wongkt@ummc.edu.my

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Murray Valley Encephalitis (MVE) virus, an RNA flavivirus, was first isolated in 1951 during an outbreak of encephalitis in the Murray Valley, Australia [1]. Subsequent outbreaks have been reported in 1956, 1974 and 2011 [2, 3]. Similar to other flaviviral encephalitides such as Japanese Encephalitis (JE), West Nile (WN) encephalitis and Tick Borne Encephalitis (TBE) [1], MVE is often severe with significant morbidity and mortality [2]. Since MVE viral tropism in the central nervous system (CNS) is still unknown, we have examined 3 suspected (not culture-

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proven) cases of MVE, and report in 1 case, neuronal localisation of viral antigens and viral RNA using immunohistochemistry (IHC) and *in situ* hybridization (ISH), respectively. These findings strongly suggest that MVE virus is primarily neuronotropic, i.e. has a predilection for neurons, and that neuronal viral cytolysis plays an important role in neuropathogenesis.

During the 1974 MVE outbreak in New South Wales (NSW) [3], Australia, a 21 month old boy was admitted to the Royal Children's Hospital (RCH), Melbourne, Australia, with a 7-day history of drowsiness, malaise, convulsions and coma. He was transferred from another hospital in NSW, where he had a temperature of 39.5°C, generalized hypertonia and neck stiffness. His pupils were small, equal and reactive to light and he maintained response to painful stimuli. The lumbar puncture showed cerebrospinal fluid pressure of 150 mmH₂O (normal, 70-180 mmH₂O); 18×10^6 lymphocytes/L with no polymorphs; chloride of 106 mmol/L (normal, 118-132 mmol/L), glucose 3.36 mmol/L (normal, 2.2-3.9 mmol/L) and protein <0.1 g/L (normal, <0.4 g/L). On admission to RCH, he was comatose and had opisthotonus and muscle spasms. A repeat lumbar puncture showed 3.7×10^6 polymorphs/L, 3.6×10^6 lymphocytes/L, 0.3×10^6 red cells/L, 0.5×10^6 "mononuclear" cells/L, protein 0.5g/L and glucose 3.08 mmol/L. Organisms and cultures were negative. He died after 10 days of illness with a clinical diagnosis of viral encephalitis.

At autopsy, the congested brain weighed 1040g (normal 1050g) and showed no asymmetry, herniation or abnormalities in the meninges or other brain areas. However, on later examination, a few small necrolytic areas were noted in the left thalamus (Figure 1A). These relatively circumscribed lesions of 500-900µm consisted of neuropil vacuolation/oedema, neuronal loss and a few degenerate/necrotic neurons (Figure 1B). There was mild meningitis (Figure 1C), perivascular cuffing and focal parenchymal infiltration by inflammatory cells mainly in grey matter, including cerebral cortex (temporal lobe and cingulate gyrus) (Figure 1D), thalamus, subthalamic nucleus, corpus striatum, hippocampus, hypothalamus, cerebellum (Figure 1C), midbrain, pons and spinal cord. Viral inclusions were not detected. A pathological diagnosis of MVE was made because of the on-going MVE outbreak [4], but this was never proven by viral culture.

In order to confirm MVE, we retrospectively performed IHC to detect flaviviral antigen and specific ISH to detect MVE viral RNA on formalin-fixed, paraffin-embedded CNS tissues archived in the Department of Anatomical Pathology, Alfred Hospital, Melbourne, Australia. The study was approved by the ethics committee of the Alfred Hospital. In addition, standard IHC was also performed to characterise inflammatory cells.

Flaviviral IHC was performed using a standard immunoperoxidase technique [5]. Briefly, dewaxed, rehydrated 4µm tissue sections were antigen-retrieved and blocked with H₂O₂/methanol and serum. A primary mouse monoclonal anti-flavivirus NS1 antibody (4G4, 1:500 dilution; courtesy of Roy Hall, University of Queensland, Australia), was added and incubated overnight at 4°C followed by HRP-conjugated secondary antibody, 3,3'-diaminobenzidine (DAB) substrate and haematoxylin counterstaining steps. Similarly, inflammatory cells were detected using specific primary antibodies to human CD3 (1:200 dilution), CD4 (1:100 dilution), CD8 (1:150 dilution), CD20 (1:1000 dilution) and CD68 (1:250 dilution) (Novocastra, UK). Negative tissue controls included normal human brain, mouse brain infected with Enterovirus 71 (EV71), WN, TBE, dengue (courtesy of Noriyo Nagata, National Institute of Infectious Diseases (NIID), Japan) and JE viruses. MVE virus-infected culture cells (courtesy of Tomohiko Takasaki, NIID, Japan) and mouse brain (courtesy of John Bingham, CSIRO, Australia) were used as positive controls. Duplicate negative control IHC assays were also done by omitting the primary antibody.

As IHC to detect viral antigens was only flavivirus-specific, to confirm MVE, we developed an MVE virus specific oligo-riboprobe for ISH. Its sequence was determined by aligning MVE (MVE-151, AF161266), WN, JE, TBE and dengue viral sequences from the GenBank with CLUSTALW and MEGA 5.2 softwares. A conserved, 35-base sequence (5'-AGACGGTTCTGGGAGGCTTTCCAACCGTGGAGAAT-3'; position 10582-10616) in the MVE virus NS5 gene, confirmed by BLAST to be specific, was custom-synthesised and prelabeled with digoxigenin (DIG) at the two ends (Integrated DNA technologies, USA). The GC% content (54.3%) and T_m (69.1°C) were determined using MEGA 5.2 and AnnHyb v4.946 programs.

The ISH procedure with slight modifications, was previously described [5]. Briefly, 0.2N hydrochloric acid and proteinase-K pretreated tissue sections were hybridised overnight with riboprobes in a standard hybridisation solution at 60°C, followed by washing twice in 2X saline-sodium citrate (SSC) (60°C), once in 0.1X SSC (RT) and incubation with 0.5% blocking reagent, alkaline phosphatase-conjugated, anti-DIG antibody, nitroblue tetrazolium/5-bromo-4-chloro-indol-3-indolyl phosphate (Roche diagnostics, Germany), and Mayer haematoxylin counterstaining. The tissue controls for ISH were the same as IHC. A “minus probe” ISH assay was included as a negative control.

MVE viral antigens/RNA, were mainly localized to degenerate neuronal bodies and processes of single or small groups of neurons, in some but not all inflamed areas. Surviving neurons in necrolytic lesions in the thalamus showed strong IHC and ISH positivity (Figure 1E, F). In areas without necrolytic lesions, e.g. hippocampus, neurons also showed strong positive staining for viral antigens/RNA (Figure 1G). Positive neurons could also be found focally in the temporal lobe and hypothalamus. Antigens/RNA were not detected in glial cells, blood vessels or choroid plexus. The positive MVE controls, and WN, JE and dengue controls were IHC positive, whereas normal human brain, TBE and EV71 tissue controls were negative with no significant background staining. The ISH showed specific positive staining only in MVE controls; all other controls were negative with no significant background staining. The absence of cross reactivity with other viruses suggests that the riboprobe designed for the ISH was useful to confirm the diagnosis of MVE even in tissue blocks > 4 decades old. In contrast to routine ISH, in which the hybridisation temperature is usually 42°C, we found that greater specificity could be obtained when hybridisation was performed at 60°C. The reasons are uncertain but may be related to the use of an oligo-riboprobe for ISH.

In the CNS, macrophages were the most numerous inflammatory cells (Figure 1H) with varying number of CD8-positive, T lymphocytes (Figure 1I) and some CD4-positive cells; neutrophils were very rare. Inflammatory cells in thalamic necrolytic lesions comprised mainly CD68-positive macrophages/microglia and CD8-positive lymphocytes. Rare CD20-positive lymphocytes were observed especially in the perivascular spaces and meninges. The types of inflammatory cells found in MVE are apparently not significantly different from other

encephalitides in which varying degrees of infiltration by CD68-positive macrophages/microglia, CD4 and CD8-positive lymphocytes have also been found [6]. Similar to other flaviviral encephalitis, CD8-positive lymphocytes were found in higher densities than CD4 cells, and CD20-positive B cells were scarce and restricted to perivascular spaces and meninges [7].

Macroscopically-evident thalamic “degeneration” similar to the necrolytic lesions in our case, has been reported previously in chronic stages of MVE [8]. Although non-specific, these necrolytic lesions may be more common or prominent in JE virus infection [9, 10]. Brain MRI had also shown striking inflammation-associated, thalamic hyperintensity in MVE, JE, TBE and WN encephalitis [11]. Similar to other flaviviral encephalitides [9], MVE is a meningoencephalitis characterised by parenchymal inflammation, neuronal degeneration, neuronophagia and necrosis in the thalami and other CNS areas [8]. Viral neuronotropism has also been demonstrated in other flaviviral encephalitides [9, 12], and viral inclusions were also undetectable. The highest density of neurons with antigens/RNA were found in the characteristic thalamic necrolytic lesions, which were most likely the result of direct infection of groups of adjacent neurons. Although CNS inflammation is relatively widespread, viral antigens/RNA may be focal.

The clinical manifestations of MVE in this and other cases described in the literature were generally non-specific, and include fever, headache, nausea, vomiting followed by convulsions, neurological consequences of spinal cord and brainstem involvement, culminating in coma and death in about 20% of patients [2]. Long-term sequelae in survivors or complete recovery have been reported [1]. The broad clinical spectrum of the disease is consistent with widespread neuronal injury and inflammation.

In conclusion, MVE virus is neuronotropic, and neuronal viral cytolysis plays an important role in the pathogenesis. We believe this is the first time this has been demonstrated; more cases should be studied to confirm the findings.

Competing interests

None

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Figure 1. Pathology of Murray Valley encephalitis

A coronal brain slice showing macroscopically visible necrolytic lesions in the thalamus (A, arrow), corresponding to microscopic lesions (B, arrows). (C) Mild meningitis (arrow) and inflammation in the molecular layer of the cerebellum (★). (D) Perivascular (arrow) and parenchymal inflammation in the cerebral cortex (★). Viral antigens (E, arrows) and viral RNA (F, arrows) in infected neurons in and around necrolytic lesions. (G) Viral antigens in pyramidal cells of the hippocampus. Inflammatory cells included numerous CD68-positive macrophages/microglia (H, arrows) in microglial nodules, CD8-positive lymphocytes (I, arrows), and other inflammatory cells. H&E stains (B, C, D), Immunoperoxidase with haematoxylin counterstain (E, G, H, I) and *in situ* hybridisation with haematoxylin counterstain (F). Magnification: X10 objective (C); X20 objective (D-I); scale bar = 400µm (B), 50µm (C-I).

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Author/s:

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