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Advantages of using recombinant measles viruses expressing a fluorescent reporter gene with vibratome slice technology in experimental measles neuropathogenesis

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Aims: In this study of experimental measles neuropathogenesis, the utility of enhanced green fluorescent protein (EGFP) as a sensitive indicator of measles virus (MV) cell-to-cell spread in the central nervous system (CNS) has been assessed in vibratome-cut brain slices to demonstrate the degree and mechanism of viral spread in the rodent CNS. **Methods:** Recombinant MVs expressing EGFP were visualized at different levels in 200-μm vibratome-cut brain sections from infected animals by confocal scanning laser microscopy (CSLM). Comparison was made with 7-μm microtome sections, stained for the N protein of measles by immunocytochemistry (ICC). **Results:** The recombinant viruses were readily visualized in infected brain tissue, with no loss of neuropathogenicity. No difference was found in the sites of infection when MV infection was detected through EGFP fluorescence or by ICC. MV-infected cells were detected in the cerebral cortex, olfactory bulb and tract, hippocampus, thalamus, hypothalamus, ependyma and subventricular zone. However, the 200-μm vibratome-cut sections and confocal microscopy proved excellent for demonstrating virus distribution in neurites and for in-depth analysis of the extent of tract infection in the white matter of the cerebral hemispheres such as selective infection of the internal capsule and anterior commissure. **Conclusions:** The use of self-tracing recombinant MVs, viewed in thick vibratome-cut sections by CSLM, demonstrated that in experimental MV neuropathogenesis the infection is selective and spreads predominately by neurites using defined anatomical pathways.

Keywords: confocal scanning laser microscopy, eGFP, measles virus, vibratome, viral neuropathogenesis

Introduction

It is recognized that acute measles is occasionally complicated by three distinct neurological complications. Acute postinfectious measles encephalomyelitis is the most common of these and occurs in approximately 1:1000 cases with associated mortality rate from 10% to 20% [1]. Measles inclusion body encephalitis (MIBE) is a fatal complication of measles occurring in immunosuppressed or immunodeficient individuals. It is characterized by progressive neurological deterioration, seizures and coma [2]. Subacute sclerosing panencephalitis (SSPE) occurs in approximately 1:10 000 cases [3] after an incubation period of 5–10 years. Despite the presence of measles virus (MV) specific antibody titres [4], the condition is

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invariably fatal, although the illness may be prolonged. Pathologically, MIBE and SSPE are characterized by the demonstration of viral antigen in many neurones, predominantly in the cerebral cortex [2,5].

In spite of extensive research on both *post mortem* SSPE brain tissue and a number of rodent model systems of MV-induced central nervous system (CNS) disease, details of the specific mechanisms of MV cell-to-cell spread in the CNS are unknown. Extensive analysis of SSPE brain tissue has rarely resulted in the observation of cell-to-cell fusion between MV-infected cells [6]. The paucity of cell-to-cell fusion in SSPE brain tissue may be due to the absence of signalling lymphocyte activation molecule (SLAM), the primary MV cellular receptor, on the surface of neurones or oligodendrocytes [7]. These observations have given credence to the theory that MV may spread within the CNS through localized fusion events at synaptic contacts, thus negating the need for any specific virus receptor [5]. This theory is supported by the observation that MV can spread between differentiated human NT2 neurones and between primary murine hippocampal neurones in the absence of both CD46 and SLAM [8].

Measles virus has been demonstrated in axons and dendrites in SSPE and in various animal models [5,9,10], and transneuronal virus spread is likely to be transynaptic [11,12]. The pattern of viral infection should therefore reflect the host's neuroanatomical structures and involve nerve fibre tracts, commissures and so on. The technical challenge of demonstrating this by conventional microscopical methods in experimentally infected animals is considerable and labour-intensive.

The availability of a reverse genetics system for the Edmonston vaccine strain of MV further enhances the usefulness of this model system in the study of MV neuropathogenesis. One application of this technology has been the development of recombinant strains of MV which express enhanced green fluorescent protein (EGFP) from an additional transcription unit (ATU) located at the promoter proximal position of the genome. For instance, MVeGFP [13] has been used in a number of *in vitro* studies to monitor in real time the cell-to-cell spread of MV in human oligodendroglioma and neuronal cells [14,15]. This approach has also been used to track the spread of MVeGFP in infected *ex vivo* rat hippocampal cultures [16].

The detection by confocal scanning laser microscopy (CSLM) of recombinant MVs expressing EGFP in vibratome-cut sections from infected animals enables a novel approach to be taken to the study of MV neuropathogenesis, as the increased thickness of vibratome-cut brain sections (200 μm), in comparison with traditional immunohistochemical methods on microtome-cut brain sections (7 μm), allows MV-infected neurones to be tracked through multiple cell layers and neuroanatomical tracts. In this study, two recombinant MVs which express EGFP have been used to assess the extent of MV infection in the brains of infected animals.

Materials and methods

Cells and viruses

Vero cells were grown in Dulbecco's modified Eagle medium (Invitrogen) containing 8% (v/v) foetal-calf serum (PAA Laboratories, Pasching, Austria) and were used for the growth of recombinant MVs, which were propagated in Optimem (Invitrogen, Paisley, UK). The recombinant virus, Edtag, was rescued from the full-length infectious antigenomic clone p(+)MV [17] as previously described [18]. The recombinant virus, MVeGFP, was rescued from the full-length infectious vaccine antigenomic clone pMeGFPNV as previously described [13]. EGFP is expressed from an ATU inserted between the 3′ end of the genome and the gene encoding the nucleocapsid protein. The recombinant virus, MVeGFP^{CAMH} was rescued from p(+)MVeGFPCAMH, a modified full-length infectious antigenomic clone which contains an equivalent ATU to pMeGFPNV [13]. Plasmid p(+)MVeGFPCAMH was produced by removing the 10 624 bp *Cla*I fragment from p(+)MVCAMH [19], and introducing it into *Cla*I restricted pMeGFPNV. The recombinant virus was recovered using an analogous procedure to that described previously for the generation of EdtagCAMH [19]. UV microscopy was used to verify that all syncytia contained high levels of EGFP. The rodent brain-adapted MV (CAM/RB) was obtained from U.G. Liebert, Würzburg, Germany. The virus was passaged on Vero cells, and retention of the neurovirulent phenotype was verified as previously described [19]. Virus titres were obtained by 50% end-point dilution assays and are expressed in 50% tissue culture infectious doses [20].

Infection of nontransgenic and transgenic mice

Suckling C57/BL/6 and Ifnar^{ko}CD46Ge mice, which contain the human CD46 gene and lack the interferon type 1 receptor gene [21], were obtained from in-house

breeding colonies in the Laboratory Service Unit, The Queen's University of Belfast and were used in animal experimentation under approved University regulations and United Kingdom Home Office license. The mice were kept in a barrier system with slight negative pressure and a 12-h artificial light day.

Two litters of 4-day-old suckling Ifnar^{ko}-CD46Ge mice (no. 8) were infected into the right cerebral hemisphere (RCH) under mild isofluorane anaesthesia with 2000 $TCID_{50}$/ml of Edtag or MVeGFP in a total volume of 20 µl or with an equivalent volume of tissue culture maintenance medium. One litter of 4-day-old suckling C57/BL/6 mice (no. 4) were infected in an identical manner with 2000 $TCID_{50}$/ml of CAM/RB or MVeGFP^{CAMH}. Mice were checked daily for clinical symptoms. If clinical symptoms such as hindlimb paralysis and seizures were observed, mice were sacrificed by overexposure to the anaesthetic isofluorane. Brains were removed immediately and immersed in either 10% (v/v) buffered formalin or freshly prepared 4% (w/v) paraformaldehyde (PFA).

Preparation of tissue samples for vibratome sectioning

Brains were fixed in 4% (w/v) PFA for 3 days at room temperature (RT). Brains were then transferred to a solution of 4% (w/v) PFA in 0.1 M sodium cacodylate buffer (pH 7.2), and the samples were incubated overnight at 4°C. Samples were immersed in 0.2 M sodium cacodylate buffer (pH 7.2) for 20 min and embedded in 5% (w/v) agarose (Type VII low gelling temperature, Sigma, Gillingham, Dorset, UK) in phosphate buffered saline (PBS). A vibratome (Leica Microsystems, Milton Keynes, UK) was used to cut serial 50-µm or 200-µm brain slices into 0.2 M TRIS buffered saline. Sections were stored at 4°C. The nuclei in a number of brain sections were counterstained by incubation for 60 s in 1 µg/ml propidium iodide (Sigma). Sections were mounted in Eukitt mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA) on glass slides and coverslips.

Immunocytochemical staining of 50-µm vibratome-cut brain slices

Vibratome-cut brain sections (50 µm) were permeabilized in PBS with 0.2% (v/v) Triton X-100 (TX-100, Sigma) for 30 min at RT to facilitate the dissemination of primary and secondary antibodies through the brain slice. Sections were incubated overnight at 4°C in an appropriate primary antibody diluted in PBS with 0.1% (v/v) TX-100 and 1% (w/v) bovine serum albumin (BSA). Monoclonal antibodies to neurofilament protein (NFP) (1:100, DAKO, Glostrup, Denmark) and synaptophysin (1:100, DAKO) were used to detect neurones. A monoclonal antibody to glial fibrillary acidic protein (GFAP) (1:200, DAKO) was used to detect astrocytes. After incubation in primary antibodies, the brain slices were rinsed three times in PBS with 0.1% (v/v) TX-100 for 10 min to remove unbound antibodies. Sections were incubated for 2 h at RT in rabbit anti-mouse Cy3 (1:50, Sigma) diluted in PBS with 0.1% TX-100 (v/v) and 1% (w/v) BSA. Sections were rinsed several times in PBS with 0.1% (v/v) TX-100 to remove unbound secondary antibody and were mounted as previously described.

Imaging of vibratome-cut brain slices

Photomicrographs of vibratome-cut brain slices were collected by CSLM. Brain slices were viewed using an upright DM-IRBE fluorescence microscope (Leica) and appropriate fields selected. A Leica TCS/NT confocal microscope equipped with a krypton–argon laser as the source for the ion beam was used to detect EGFP and Cy3 autofluorescence as described previously [13]. Image stacks were collected through an optical plane of 50–200 µm. Composite images of whole or regions of vibratome-cut brain slices from infected animals were constructed by aligning overlapping individual images in Microsoft PowerPoint (Microsoft Corporation, USA).

Histology and immunohistochemical detection of N protein in mouse brain tissue

Formalin-fixed brains were processed to paraffin and embedded for coronal sectioning. Microtome sections (7 µm) were cut coronally at 40-µm intervals from the frontal lobes to the brainstem. Sections from control and MV-infected brain tissue were stained with haematoxylin and eosin (H&E), and MV antigen was detected as described previously [22], using a monoclonal antibody which recognizes the nucleocapsid (N) protein (MAS182, Harlan Seralabs, Loughborough, UK 1:2500). Specific antibody-antigen binding sites were detected using a streptavidin – biotin–peroxidase system. Liquid 3, 3′-diaminobenzidine substrate-chromogen system (DAKO) was used to visualize bound antibody complexes. MV antigen was also visualized

in mouse brain sections using tyramide-enhanced signal amplification as described previously [23]. Immunohistochemically stained brain sections were examined with a DMLB light microscope fitted with N Plan objectives (Leica) and were photographed using a Nikon digital coolpix 950 camera. H&E-stained brain sections were digitized with a Kodak professional RFS 2035 Plus Film scanner directly from the slide.

Results

Detection of EGFP fluorescence is a sensitive indicator of recombinant MV infection

All MVeGFP- and Edtag-infected Ifnar^{ko}CD46Ge mice, which contain the human CD46 gene and lack the interferon type 1 receptor gene, showed signs of neurological illness at 4 or 5 days post infection, respectively, and were sacrificed under terminal anaesthesia. Expression of EGFP from an ATU inserted at the 3′ end of the genome did not affect either the duration or the severity of clinical symptoms in comparison to Edtag-infected mice. Clinical signs of neurological illness included initial hyperactivity and an awkward gait, which quickly progressed to a loss of balance, ataxia, hindlimb paralysis and seizures. Significant levels of EGFP fluorescence were observed in the hippocampus, olfactory bulb, basal ganglia, thalamus, ependymal and subependymal zone (SVZ) in 200-μm vibratome-cut coronal brain sections from MVeGFP-infected animals. Application of a semiquantitative scoring system to assess the numbers of foci of infection in approximately 60 immunohistochemically stained microtome-cut sections, cut at 40-μm intervals from the frontal lobes to the brainstem of an MVeGFP- and an Edtag-infected mouse brain, revealed no differences in either the levels or the distribution of MV-infected cells (data not shown). A detailed examination of the hippocampus showed that numerous pyramidal neurones in the CA1 and CA2 fields of the hippocampus and neurones in the oriens layer and stratum radiatum of the hippocampus contained high levels of EGFP (Figure 1**A**). In contrast, only baseline levels of background autofluorescence were observed in vibratome-cut brain sections from mock-infected Ifnar^{ko}-CD46Ge mice (data not shown). The efficacy of EGFP as a sensitive indicator of MV infection was indicated by a comparison with immunohistochemically stained microtome-cut sections (Figure 1**B**). The increased thickness of vibratome-cut sections in comparison with microtome-cut sections enabled the easier detection of extended cellular processes connecting MV-infected neurones in the hippocampus and cerebral cortex. While both methods are sensitive, the use of EGFP/vibratome/CSLM technology permits analysis throughout up to 200 μm of tissue, permitting analysis of viral distribution throughout multiple cell layers and axonal tracts.

Demonstration of cortical columnar, axonal tract, bundle and commissural infection indicates intrahemispheric and transhemispheric virus spread

The potential role of cortical connections, axonal tracts, bundles and commissures in mediating long-range virus spread in Ifnar^{ko}-CD46Ge transgenic mice was investigated in 200-μm vibratome-cut coronal brain sections and in 7-μm immunohistochemically stained sections. Examination of serial vibratome-cut coronal brain sections using UV microscopy revealed that while cortical infection was focal, these foci involved whole columns of the cortex. In addition, a number of axonal tracts in different areas of the brain contained significant amounts of EGFP, frequently adjacent to anatomically related areas of the cortex. In contrast to microtome-cut brain sections, MV-infected axonal tracts could be visualized over much longer distances in vibratome-cut brain slices. For instance, MV infection of the anterior commissure was observed in consecutive serial brain slices (Figure 2**A**) to extend from a large focus of infection in the piriform cortex towards the midline of the brain (levels 210–255, Harvard Mouse Brain Atlas). MVeGFP-infected axonal tracts were also observed in the vicinity of extensive ependymal and SVZ cell infection and in the subependymal region of the fourth ventricle of different brain sections (data not shown). A number of infected cell bodies were visible adjacent to infected axonal tracts (Figure 2**B**, arrows). In one section, the involvement of axonal tracts in mediating transhemispheric spread of MVeGFP was indicated by the detection of MV antigen in a number of axons in the corpus callosum by immunohistochemistry (Figure 2**C**).

Utility of EGFP expression in the identification of MV-infected cells

Having observed that recombinant strains of MV which express EGFP are powerful tools to assess rapidly the dis-

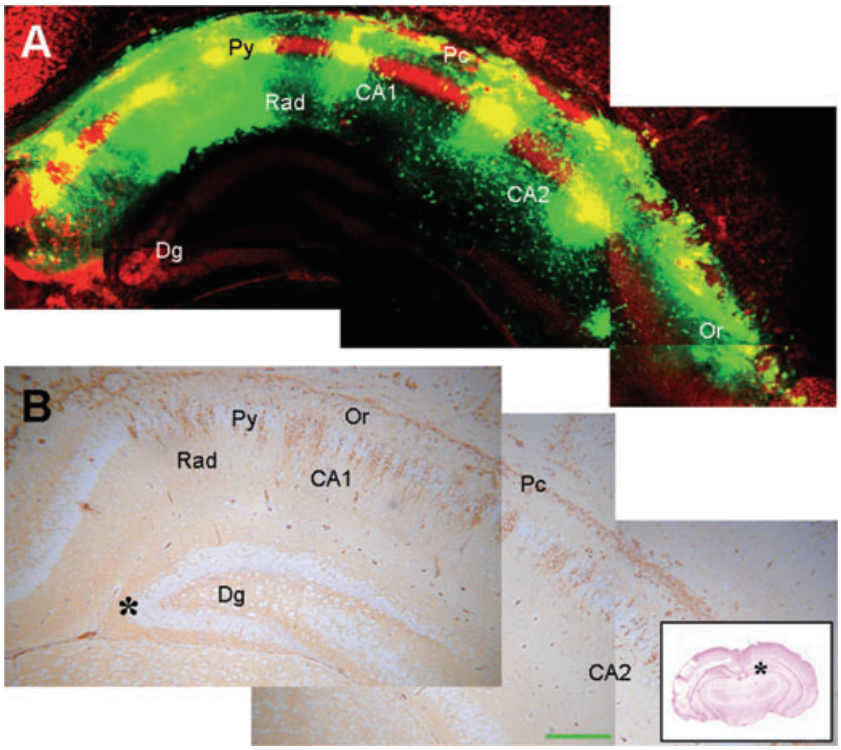

Figure 1. Enhanced sensitivity of virus detection by EGFP fluorescence in vibratome-cut coronal sections (200 μm) from an MVeGFP-infected Ifnar^{ko}-CD46Ge transgenic mouse brain. (**A**) Infected pyramidal neurones (Py) are visible in the CA1 and CA2 fields of the hippocampus. Infected neurones are also visible in the oriens layer of the hippocampus (Or) and in the stratum radiatum of the hippocampus (Rad) adjacent to the dentate gryrus. Infected progenitor cells (Pc) are visible above the hippocampus. (**B**) Distribution of MV antigen in the hippocampus of a microtome-cut coronal brain section (7 μm) from an Edtag-infected Ifnar^{ko}-CD46Ge transgenic mouse brain. Infected pyramidal neurones are visible in the CA1 and CA2 fields of the hippocampus. The anatomical location of the immunostained sections are highlighted in the adjacent haematoxylin and eosin (asterisks on B and inset). Bar: A–B = 45 μm. EGFP, enhanced green fluorescent protein; Dg, dentate gyrus; MV, measles virus.

tribution of MV-infected cells in infected mice, the potential use of MVeGFP in the examination of individual infected cells was investigated using vibratome-cut brain sections. A multitude of infected neurones with extended neuronal processes were visible in the cortex of such sections (Figure 3**A**) and individual neurones with multiple branched processes were readily visualized (Figure 3**B**) without the necessity for tissue processing, microtomy and immunohistochemical staining (Figure 3**C**). Only the EGFP/vibratome/CSLM technique permitted the identification of very fine neuronal processes connecting MVeGFP-infected neurones in the outer cortex of infected animals (Figure 3**D**, arrows).

Enhanced green fluorescent protein was also visible in many ependymal cells in the SVZ surrounding the lateral ventricle of MVeGFP-infected animals (Figure 3**E**). Infection of ependymal cells surrounding the lateral ventricle of an MVeGFP-infected animal was associated with severe disruption of the integrity of the ependymal cell layer (Figure 3**F**, arrows). MV antigen was also observed in a distinct and extended submeningial cell layer adjacent to the midline of the right cerebral hemisphere (Figure 3**G**). Infected cells with the morphological appearance of neurones were visible adjacent to this cell layer. Although numerous infected subependymal progenitor cells were visible in the RCH, no MV antigen was observed in similar cells in the left cerebral hemisphere.

The identity of fluorescent cells in the cortex of MVeGFP-infected mice could be confirmed by immunocytochemical staining of vibratome-cut brain sections (50 μm). No GFAP-positive astrocytes were observed to contain EGFP despite the proximity of these cells to EGFP-positive cells (Figure 3**H**), but a number of EGFP-positive cells were observed to contain the neuronal cell marker synaptophysin (Figure 3**I**). In addition, bundles of

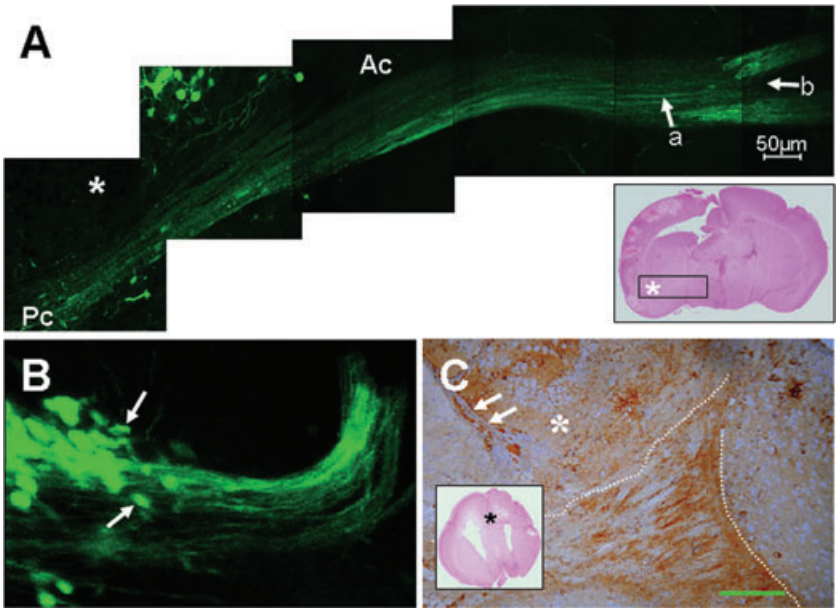

Figure 2. 200-µm thick vibratome sections obtained from MVeGFP-infected Ifnar^{ko}-CD46Ge transgenic mouse brains permit in-depth analysis of viral distribution in axonal tracts. (**A**) The anterior commissure (Ac) extends deep into the brain parenchyma from a focus of infection in the piriform cortex (Pc). Individual fluorescent axons are visible within the main body of the axonal tract (arrow a), which splits into two separate infected axonal tracts deep in the brain parenchyma (arrow b). Haematoxylin and eosin (H&E) of brain section (inset) were used to identify the fluorescent axonal tract as the anterior commissure. The open box indicates the location of the anterior commissure within the brain. The asterisk in the inset indicates the location of the asterisk shown in panel A. (**B**) Individual axons and associated cell bodies (arrows) are clearly visible within an axon bundle which extends from a focus of infection surrounding the lateral ventricle. (**C**) Immunohistochemical detection of MV antigen in axons in the corpus callosum (dashed line) transversing the midline (arrows) of a microtome-cut brain slice from an MVeGFP-infected Ifnar^{ko}-CD46Ge transgenic mouse brain at 6 days post infection. The neuroanatomical location of the asterisk is indicated by an asterisk in an H&E of an adjacent brain section (inset). Bar: A = 50 µm; B = 140 µm; C = 280 µm. MVeGFP, measles virus–enhanced green fluorescent protein.

EGFP-positive cellular processes stained positive for the neuronal cell marker NFP (Figure 3**J**).

Cloning, rescue and assessment of the neuropathogenicity of MVeGFP^{CAMH}

In order to investigate the utility of recombinant strains of MVs which express EGFP in the study of MV neuropathogenesis, a recombinant virus (MVeGFP^{CAMH}) based on MVeGFP which contains the H glycoprotein of the rodent brain-adapted strain CAM/RB has been generated (Figure 4**A**). Both MVeGFP and MVeGFP^{CAMH} grew to equivalent titres (1×10^6 plaque forming units/ml). To ensure that MVeGFP^{CAMH} retained its capacity to induce acute encephalitis in infected nontransgenic mice, rescued virus was amplified by successive passages in the brains of 4-day-old C57/BL/6 mice. The expression of EGFP was stably retained upon multiple viral passages in the murine CNS, as all syncytia (>100) which were observed in passage six (P6) MVeGFP^{CAMH}-infected Vero cells contained high levels of EGFP when viewed by UV microscopy (Figure 4**B**).

Intrinsic fluorescence was readily detectable using CSLM in PFA-fixed, agarose-embedded, vibratome-cut 200-µm coronal brain sections from MVeGFP^{CAMH}-infected C57/BL/6 mice (Figure 4**C**). Single cells and foci of infected neurones, many with extended neuronal processes, were observed throughout the cerebral cortex and hypothalamus. Fluorescence was also detected in axonal bundles which were observed in the striatum and internal capsule. The inability to detect virus in these axonal tracts in analogous immunohistochemically stained microtome-cut sections is indicative of the increased sensitivity of EGFP as a marker of recombinant MV infection.

Discussion

The use of a novel technical approach in this study of MV neuropathogenesis, in which recombinant MVs express-

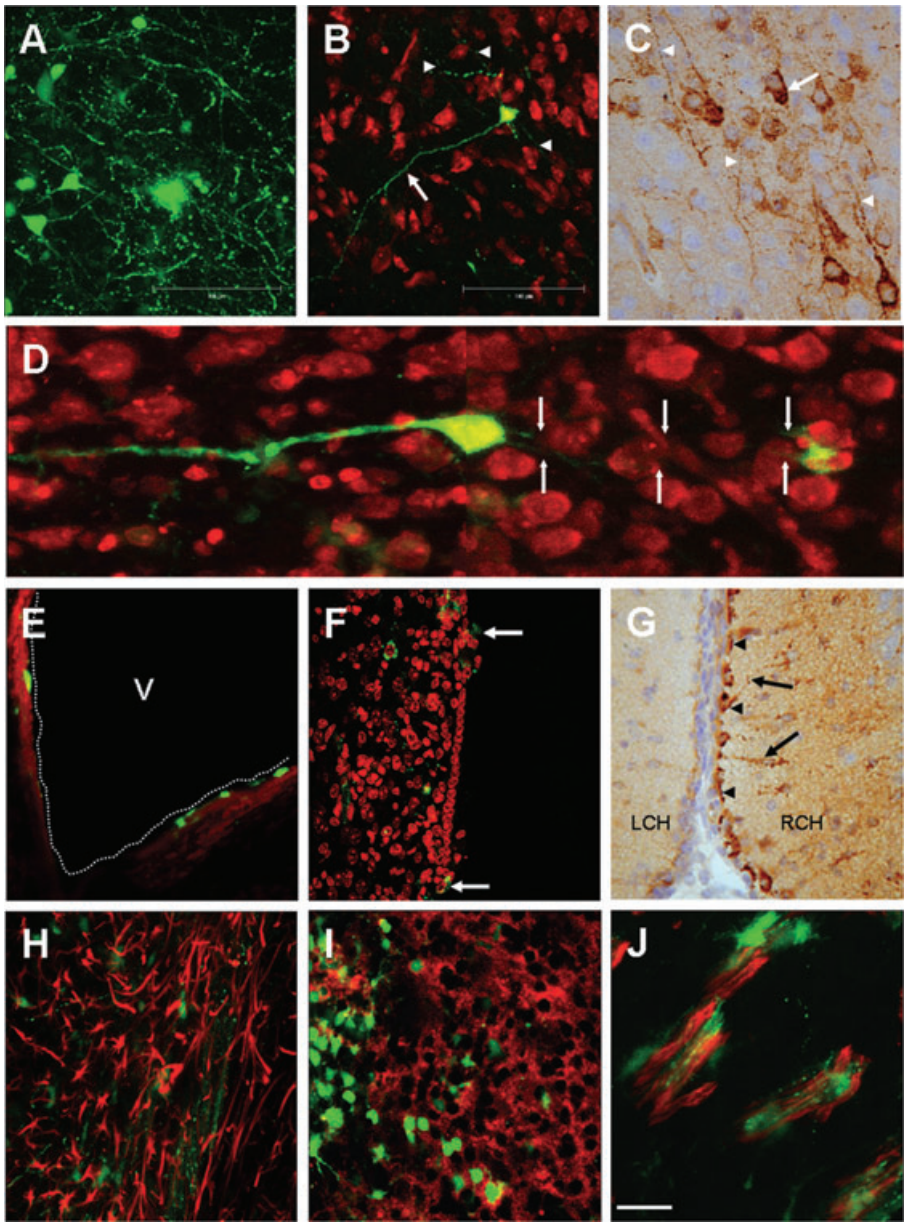

Figure 3. Detection of enhanced green fluorescent protein autofluorescence (EGFP) and measles virus (MV) antigen in vibratome-cut (A, B, D, E, H–J) and microtome-cut (C, F and G) coronal sections from MVeGFP- and Edtag-infected Ifnar^{ko}-CD46Ge transgenic mouse brains. Propidium iodide (red) was used as a cellular counterstain in Plates B, D, E and F. (**A**) Numerous interconnected fluorescent neurones are visible in the cortex of MVeGFP-infected mice. One or more neuronal processes emanates from the cell body of most fluorescent cells. (**B**) An axon (arrow) and multiple branched dendritic processes (arrowheads) emanate from the cell body of a single MVeGFP-infected neurone. (**C**) Many infected neurones with large cytoplasmic aggregates of N protein (arrow) and long neuronal processes (arrowheads) are visible in the outer cortex of an Edtag-infected mouse. (**D**) Two neuronal processes (arrows) connect MVeGFP-infected neurones in the outer cortex. (**E**) Detection of MVeGFP-infected ependymal cells lining the lateral ventricle (V). (**F**) Disruption of a layer of ependymal cells observed lining the lateral ventricle of an Edtag-infected mouse is associated with MV infection (arrows). (**G**) An infected submeningial cell layer (arrowheads) is visible in the right cerebral hemisphere (RCH) of an MVeGFP-infected mouse. No infected cells are visible in the left cerebral hemisphere (LCH). A number of infected neurones are also visible immediately adjacent to infected cells in the RCH (arrows). (**H–J**) Immunocytochemical staining was used to map the cellular distribution of EGFP to specific cell types in serial 50-μm vibratome-cut coronal brain slices from an MVeGFP-infected mouse brain. (**H**) No colocalization between EGFP and GFAP positive astrocytes is apparent. (**I**) A number of neurones contain both EGFP and synaptophysin vesicles. (**J**) A number of neuronal processes contain both EGFP and neurofilament protein. Bar: A–C, E–J = 43 μm: D = 27 μm.

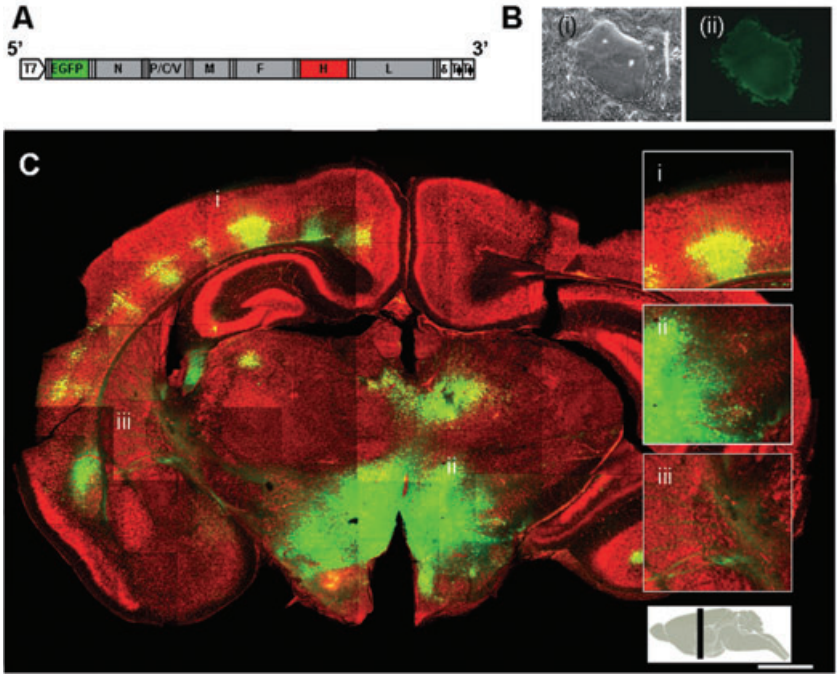

Figure 4. (**A**) Construction of full-length pMVeGFP^{CAMH}. Enhanced green fluorescent protein (EGFP)FP (green) is expressed from an additional transcription unit inserted prior to the N gene. The hemagglutinin gene (red) is derived from the rodent adapted strain CAM/RB. The remaining genes (grey) are derived from the recombinant Edtag strain of measles. (**B**) An MVeGFP^{CAMH} syncytium observed by phase (i) and UV (ii) microscopy at 24 h post infection in an infected Vero cell monolayer. (**C**) Examination of the distribution of MV-infected fluorescent (green) cells in a single 200-μm vibratome-cut brain slice from an MVeGFP^{CAMH}-infected C57/BL/6 animal. Propidium iodide (red) was used as a counterstain in vibratome-cut sections in order to obtain a neuroanatomical assessment of regions of MV infection. A number of intensely fluorescent foci of MV infection are visible in the cortex (i) and hypothalamus (ii) of the right cerebral hemisphere (RCH). A number of fluorescent axon tracts connect the foci of MV infection in the hypothalamus and cortex of the RCH (iii). The inset shows the approximate position of this brain slice in a schematic representation of a mouse brain (Harvard Mouse Brain Atlas). Bar: B = 750 μm; C = 100 μm; inserts = 50 μm.

ing EGFP, were visualized at different levels in 200-μm vibratome-cut sections by confocal microscopy, made possible three-dimensional imaging of infected cells and gave enhanced information about the degree and anatomy of viral spread within the brain. This approach is technically straightforward, enables the rapid assessment of MV-infected brain slices and allows immunohistochemical resources to be used selectively to answer specific questions.

Knowledge of viral cellular tropism and the anatomy of viral spread are of great value in experimental viral neuropathogenesis in elucidating viral–host structural and molecular interactions. Thus, it has been shown that many viruses spread transneuronally and often cross synapses, using host mechanisms of transport [24]. The importance of this approach in hypothesis formulation can be illustrated by classical neuroanatomical and electron microscopical studies, from which postulates on the mechanism of viral transport have emanated, to be tested later *in vitro* [25,26]. Such studies, using conventional microtome-cut brain slices and light microscopy, are by their nature labour-intensive, requiring serial sections, extensive immunocytochemistry and sometimes electron microscopy. It is not surprising therefore that, despite the many studies of measles neuropathogenesis, few document the precise anatomy of MV spread within the nervous system.

An initial concern in the present study was that the recombinant viruses may have altered neuropathogenicity *in vivo*. Therefore, in order to validate the use of EGFP as a sensitive and efficient method of examining MV neurovirulence, it was first necessary to demonstrate that EGFP expression does not affect either the distribution or the extent of MV infection in the brains of animals infected with recombinant MVs. It was found that the expression of EGFP from an ATU prior to the N gene did

not affect the capacity of MVeGFP^{CAMH} and MVeGFP to attain high titres *in vitro* or to rapidly induce lethal encephalitis in infected animals. Thus, it is clear that the presence of an ATU in the promoter proximal position of an MV vaccine strain does not adversely modulate neuropathogenicity in this model. This is in contrast to a recent study in which a recombinant wild-type strain of canine distemper virus which also expressed EGFP from an ATU prior to N gene was found to be attenuated in infected ferrets [27]. The detection of MV antigen in analogous regions of the olfactory bulb, cerebral cortex, hippocampus, basal ganglia, thalamus and SVZ in both MVeGFP- and Edtag-infected animals provided further confirmation that the expression of EGFP did not affect MV cell-to-cell spread in the murine CNS.

The high expression level of EGFP in recombinant MV-infected cells meant that these viruses were intrinsically fluorescent and therefore self-tracing and could be tracked through multiple layers of 200-μm vibratome sections by confocal microscopy. The stacked images could then be reconstructed to give a three-dimensional view of virus infection of cell bodies, associated processes and fibre tracts. By contrast, only very short sections of virus-infected tracts could be seen in microtome sections (7 μm) stained by immunoperoxidase against the N protein of the virus. Figure 2 illustrates this comparison, where the detailed anatomy of MV spread within the hippocampus is much more easily observed in 200-μm vibratome-cut sections than in 7-μm immunoperoxidase-stained microtome-cut sections.

These experiments were designed to prove the validity of the technical approach, and observations were not made in a timed sequence. It is not therefore possible to comment on specific mechanisms of MV axonal or synaptic transport. However, some conclusions as to MV spread in the murine CNS can be made. The presence of numerous foci of adjacent MV-infected neurones in both strains of infected mice suggests that the trans-neuronal spread of MV antigen is the predominant local mechanism of MV spread in the suckling murine CNS. Comparison can be made with experimental Japanese encephalitis [28] and with Semliki Forest virus infection in suckling mice [29,30]. In both models, it is clear that the ability to establish infection is inversely related to neuronal maturity rather than immune responses. This is probably the case in this model as the adult CD46Ge mouse is resistant to MV infection of the CNS [21]. The adult Ifnar^{ko}CD46Ge mouse is susceptible to MV infection of the CNS only if their type 1 interferon response is defective [31]. In the suckling mouse, neuronal development and myelination are incomplete and ongoing, thereby facilitating infection. Such factors are less important in humans, where MV infection occurs predominately in the neuronally mature CNS. The presence of MV-infected axonal tracts and MV-infected ependymal and SVZ cells indicates that additional mechanisms may contribute to the spread of MV in these animals. Ependymal infection raises the possibility of dissemination of MV to the SVZ and through cerebrospinal fluid pathways to more distant sites. The extensive infection of cells, presumed to be progenitors, in the SVZ indicates that these cells, through their migratory role, might induce infection at distal sites.

Immunofluorescent labelling of EGFP-positive vibratome-cut sections with GFAP, an astrocyte-specific marker, did not indicate infection of astrocytes, but EGFP fluorescence was strongly associated with the neuronal markers NFP and synaptophysin. The presence of MV-infected neuronal cell bodies, adjacent to large infected axonal tracts, but distant from the site of inoculation, raises the possibility of neuronal infection secondary to axonal tract infection. Although the functional connections of these cells with the infected axonal tract were not known and synapses were not visualized, it is possible that synaptic connections are the means of the neuronal perikaryon infection.

In other instances, for example in the hippocampus, the pattern of individual neuronal infection and of axonal tract infection followed known anatomical pathways. The striking demonstration in the vibratome-cut sections of the infected anterior commissure, extending from a region of the piriform cortex to deep within the cerebral hemisphere, emphasizes paradoxically both the extent and restriction of intrahemispheric spread. The demonstration by immunoperoxidase staining of MV infection of the corpus callosum illustrates one mechanism of transhemispheric spread, although clearly other mechanisms may operate.

The use of the novel technical approaches described in this study could in future experiments help to resolve some of the conflicting results from detailed anatomical studies already undertaken. Thus, Urbanska *et al.* (1997) found that MV spread through axonal pathways in both the anterograde and retrograde direction [11]. Their experiments suggest slow axonal transport of the virus, determined by the slow transfer of cytosolic nucleoproteins (K. Kristensson, pers. comm.). Oldstone *et al.*, while con-

firming the spread of MV by axons, suggested that the mechanism is fast axonal transport [32]. Ehrengruber *et al*., in a hippocampal slice model of MV infection, described only retrograde axonal transport of the virus [16]. Such conflicting results may be the result of the use of different viral strains, in different hosts, and different neuronal populations may respond differently to MV infection. Nevertheless, it is important to understand these mechanisms at a molecular level, if only in the hope that they point to future therapy for the devastating complications of measles, that is, SSPE and MIBE. The use of self-tracing MV, viewed in thick vibratome-cut sections by confocal microscopy, may contribute to this goal.

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