Cell-to-Cell Measles Virus Spread between Human Neurons Is Dependent on Hemagglutinin and Hyperfusogenic Fusion Protein

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ABSTRACT Measles virus (MV) usually causes acute infection but in rare cases persists in the brain, resulting in subacute sclerosing panencephalitis (SSPE). Since human neurons, an important target affected in the disease, do not express the known MV receptors (signaling lymphocyte activation molecule [SLAM] and nectin 4), how MV infects neurons and spreads between them is unknown. Recent studies have shown that many virus strains isolated from SSPE patients possess substitutions in the extracellular domain of the fusion (F) protein which confer enhanced fusion activity. Hyperfusogenic viruses with such mutations, unlike the wild-type MV, can induce cell-cell fusion even in SLAM- and nectin 4-negative cells and spread efficiently in human primary neurons and the brains of animal models. We show here that a hyperfusogenic mutant MV, IC323-F(T461I)-EGFP (IC323 with a fusion-enhancing T461I substitution in the F protein and expressing enhanced green fluorescent protein), but not the wild-type MV, spreads in differentiated NT2 cells, a widely used human neuron model. Confocal time-lapse imaging revealed the cell-to-cell spread of IC323-F(T461I)-EGFP between NT2 neurons without syncytium formation. The production of virus particles was strongly suppressed in NT2 neurons, also supporting cell-to-cell viral transmission. The spread of IC323-F(T461I)-EGFP was inhibited by a fusion inhibitor peptide as well as by some but not all of the anti-hemagglutinin antibodies which neutralize SLAM- or nectin-4-dependent MV infection, suggesting the presence of a distinct neuronal receptor. Our results indicate that MV spreads in a cell-to-cell manner between human neurons without causing syncytium formation and that the spread is dependent on the hyperfusogenic F protein, the hemagglutinin, and the putative neuronal receptor for MV.

IMPORTANCE Measles virus (MV), in rare cases, persists in the human central nervous system (CNS) and causes subacute sclerosing panencephalitis (SSPE) several years after acute infection. This neurological complication is almost always fatal, and there is currently no effective treatment for it. Mechanisms by which MV invades the CNS and causes the disease remain to be elucidated. We have previously shown that fusion-enhancing substitutions in the fusion protein of MVs isolated from SSPE patients contribute to MV spread in neurons. In this study, we demonstrate that MV bearing the hyperfusogenic mutant fusion protein spreads between human neurons in a cell-to-cell manner. Spread of the virus was inhibited by a fusion inhibitor peptide and antibodies against the MV hemagglutinin, indicating that both the hemagglutinin and hyperfusogenic fusion protein play important roles in MV spread between human neurons. The findings help us better understand the disease process of SSPE.

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Measles, characterized by high fever, conjunctivitis, and a maculopapular rash, is caused by the measles virus (MV), a highly contagious human pathogen (1). Effective live vaccines have greatly reduced its morbidity and mortality, but measles is still prevalent in certain developing countries (2). MV rarely establishes persistent infection in the central nervous system (CNS), and several years after acute infection, 6.5 to 11 cases per 100,000 cases of measles develop subacute sclerosing panencephalitis (SSPE) (3); the risk of developing SSPE is much higher for children contracting measles infection below 5 years of age (4). Patients with SSPE exhibit characteristic clinical manifestations such as personality changes, myoclonus, and dementia, and there is currently no effective treatment for the disease (5).

MV is a member of the genus *Morbillivirus* of the family *Paramyxoviridae* and possesses a nonsegmented, negative-sense RNA genome with six genes encoding the nucleocapsid (N), phospho- (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins (1). The P gene also encodes nonstructural proteins V and C. The N protein encapsidates the viral genome, forming the nucleocapsid, which is bound by the RNA-dependent RNA polymerase comprised of the L and P proteins. This ribonucleoprotein (RNP) complex binds to the M protein, which interacts with cytoplasmic tails of the H and F proteins and is responsible for the assembly of virus particles. The H and F proteins are envelope glycoproteins and mediate receptor binding and membrane fusion, respectively. Binding of the H protein to a cellular receptor induces conformational changes of the F protein, leading to virus-cell fusion and virus entry into the cell (6, 7). In addition, the expression of the H and F proteins on the cell surface causes cell-cell fusion between infected and adjacent cells, producing multinucleated giant cells (syncytia).

The cellular receptors for MV are signaling lymphocyte activation molecule (SLAM) expressed on immune cells (8, 9) and nectin 4 expressed on epithelial cells (10, 11). Since human neurons, an important target affected in SSPE, express neither SLAM nor nectin 4 (12, 13), MV is thought to infect neurons differently from nonneuronal target cells. Furthermore, free virus particles are not usually detected in the brains of SSPE patients although viral RNA and proteins are present (14–17). The Edmonston strain of MV (a laboratory-adapted strain) can use ubiquitously expressed CD46 as an additional receptor through mutations in the H gene (18, 19). It has been shown that the Edmonston strain can infect and spread between primary hippocampal neurons from CD46 transgenic mice that express human CD46 on neurons (20). The transmission of the Edmonston strain between neurons was blocked by the fusion inhibitor peptide (FIP) Z-D-Phe-Phe-Gly (21), and the virus could spread from CD46⁺ to CD46[−] neurons in a cell-cell contact-dependent manner (20). The authors of the previous study proposed that only the F protein mediates the spread of MV between neurons and that the H protein is dispensable for the process (21, 22). The Edmonston strain was also shown to spread between rat hippocampal neurons (23).

MVs recovered from brain tissues of SSPE patients (SSPE strains) contain characteristic features in their genomes. The function and expression of the M protein are impaired in most SSPE strains by such mechanisms as A-to-G hypermutations and read-through between the P and M genes (24–26). Because these mutations lead to lower production of virus particles and possible evasion of recognition by the host immune system, the defect of the M protein has been thought to contribute to neurovirulence (26, 27). The defective nature of the M protein also affects its ability to modulate viral RNA synthesis, allowing enhanced transcription of the genome (28, 29). Furthermore, most SSPE strains have alterations in the cytoplasmic tail of the F protein (30, 31). Mutations causing the defect of the M protein or producing shortened cytoplasmic tails of the F and H proteins were shown to endow MV with hyperfusogenicity and facilitate MV spread in the brains of genetically modified mice (27).

FIG 1 Neuronal differentiation of NT2 cells. (A) Phase-contrast images of undifferentiated NT2 cells and postmitotic NT2 neurons (NT2N). For neuronal differentiation, NT2 cells were treated with RA for 2 weeks and with mitotic inhibitors for an additional 1 week. NT2 neurons tended to form clusters (arrow). There were a small number of glia-like cells in the postmitotic cell population (arrowhead). Scale bar, 250 μ m. (B) Relative gene expression levels of neuronal (MAP2, MAPT, RBFOX3, and TUBB3) and astrocytic (GFAP and GLUL) markers in NT2 and NT2N cells were quantified by RT-qPCR. Data were normalized to those of GAPDH and are presented as means $\pm$ standard errors of the means of three independent experiments. Asterisks indicate statistically significant increases compared with NT2 levels ($P < 0.05$).

More recent studies have shown that many SSPE strains possess amino acid substitutions in the extracellular domain of the F protein that confer enhanced fusion activity in SLAM- or nectin 4-expressing cells (32, 33). Importantly, recombinant MVs possessing these substitutions in the F protein, but not the wild-type MV, cause syncytium formation even in SLAM- and nectin 4-negative cells, including human neuroblastoma cell lines, and spread efficiently in the brains of mice with knockout of the type I interferon receptor subunit 1 (IFNAR1) and of suckling hamsters as well as in human primary neurons (33, 34). The H protein of SSPE strains was also shown to be partly responsible for neurovirulence (32, 35, 36).

In the present study, by using cells and viruses relevant to MV infection in human brains, we aimed to examine how MV is transmitted between neurons. To this end, we employed NTERA-2cl.D1 (NT2) cells, a human embryonal carcinoma cell line, which can be differentiated into postmitotic neurons following the treatment with retinoic acid (RA) (37–40). Removal of undifferentiated cells by mitotic inhibitors increases the proportion of differentiated neurons up to 95% (40). These cells express several neurotransmitters (41), exhibit neuronal electrophysiological properties (42), and have been used as human model neurons in the fields of basic sciences, drug screening, and clinical applications (43). SSPE strains accumulate many mutations during persistence but generally do not have those in the H gene that would allow them to utilize CD46 as a receptor (18, 44–46). Thus, we used for this study the wild-type strain-based recombinant MV and its mutant possessing an SSPE strain-derived substitution in the F protein.

RESULTS

Spread of the hyperfusogenic MV between NT2N. Enhanced green fluorescent protein (EGFP)-expressing recombinant MVs possessing a fusion-enhancing substitution (T461I) in the F protein [e.g., IC323-F(T461I)-EGFP], but not the parental MV possessing the wild-type F protein (IC323-EGFP), efficiently spread in human primary neurons (34). To investigate mechanisms of MV spread between human neurons in detail, we used human NT2 neurons (NT2N), which are more tractable than primary neurons. Undifferentiated NT2 cells were differentiated into postmitotic neurons using the cell aggregate method (40) (Fig. 1A). NT2N cells had small, phase-bright cell bodies and long axons and tended to form clusters, as previously reported (40). Expression levels of several neuronal marker genes were greatly increased in NT2N cells compared

FIG 2 Spread of recombinant MVs in NT2N cells. (A) NT2N cells were infected with IC323-EGFP or IC323-F(T461I)-EGFP at an MOI of 2. NT2N cells were also infected with VSVΔG*-G at an MOI of 0.05. The cells were observed under light and fluorescence microscopes at 1 and 2 dpi. Representative images are shown. The same areas were photographed each day. Arrowheads indicate the same infected cell in IC323-EGFP- or VSVΔG*-G-infected NT2N cells. Scale bar, 250 μ m. (B) NT2N cells were infected with IC323-EGFP or IC323-F(T461I)-EGFP at an MOI of 2, and cell lysates were collected at the indicated time points. The samples were subjected to SDS-PAGE and Western blotting using an antibody against the N protein (MV-N). Actin was used as a loading control. Relative expression levels of the N protein normalized to the level of actin are indicated for each time point. The value at 3 h p.i. was set to 1 for each virus. —, uninfected.

to levels in undifferentiated cells (Fig. 1B). A small number of larger glia-like cells were also present in the postmitotic cell population, but expression levels of astrocytic marker genes were not elevated significantly compared to those of undifferentiated cells (Fig. 1A and B).

To examine the effect of the fusion-enhancing substitution T461I in the F protein on the spread of MV between neurons, NT2N cells were infected with IC323-EGFP or IC323-F(T461I)-EGFP at a multiplicity of infection (MOI) of 2 (Fig. 2A). NT2N cells were also infected with VSVΔG*-G (where VSV is vesicular stomatitis virus), which contains the GFP gene in its genome and does not produce infectious particles because it lacks the glycoprotein (G) gene (47), at an MOI of 0.05. Single infected cells were observed 1 day after infection with any of the three viruses. At 2 days postinfection (dpi), expression of EGFP was largely restricted to cells originally infected with IC323-EGFP and hardly spread further. In contrast, expression of EGFP spread efficiently from neurons infected with IC323-F(T461I)-EGFP to adjacent cells, without syncytium formation (Fig. 2A). The spread of GFP expression was never observed in VSVΔG*-G-infected neurons, indicating that GFP *per se* cannot be transmitted between neurons. In addition, a larger increase in the expression of the MV N protein was observed over time in neurons infected with IC323-F(T461I)-EGFP than in those infected with IC323-EGFP (Fig. 2B). Taken together, these results indicate that the fusion-enhancing substitution in the F protein is critical for efficient spread of MV in NT2N cells and that the viral genome is indeed transmitted from neurons infected with the hyperfusogenic MV to adjacent neurons. After MV infection, we continued to observe EGFP-positive infected NT2N cells, which appeared to be damaged and died at 4 to 6 dpi (data not shown).

The hyperfusogenic MV spreads between NT2 neurons in a cell-to-cell manner.

To examine how the hyperfusogenic MV spreads between NT2N cells, we next performed confocal time-lapse photography (Fig. 3A; see also Movie S1 in the supplemental material). NT2N cells were infected with IC323-F(T461I)-EGFP and observed under a confocal microscope. At 24 h postinfection (p.i.), a small number of NT2N cells were EGFP positive. During observation, expression of EGFP efficiently spread from originally infected neurons to those connected via axons, and the number of EGFP-positive neurons increased over time.

FIG 3 Cell-to-cell spread of the hyperfusogenic virus in NT2N cells. (A) NT2N cells were infected with IC323-F(T461I)-EGFP at an MOI of 2 and observed by confocal time-lapse imaging. Arrowheads indicate the spread of EGFP expression from an infected neuron to an adjacent cell. See also Movie S1 in the supplemental material. (B) Growth kinetics of IC323-F(T461I)-EGFP in NT2N cells. NT2N cells were infected as described for panel A. Supernatants and cells were harvested at 3, 24, 48, 72, and 96 h p.i. The virus titer (combined titer of cell-associated and cell-free viruses) at each time point was determined by plaque assay. Data are shown as means $\pm$ standard deviations of triplicate samples. The dotted line indicates the detection limit.

IC323-F(T461I)-EGFP was reported to propagate in SLAM- or nectin 4-expressing cells at levels comparable to those of the wild-type MV until 48 h p.i. (thereafter its titers decreased sharply due to strong cytopathic effects) (34). In contrast, IC323-F(T461I)-EGFP produced only low virus titers in NT2N cells for 4 days after infection (Fig. 3B), despite its efficient spread between the cells. The results, together with the above morphological findings, indicate that the hyperfusogenic virus spreads between NT2N cells mainly, if not exclusively, in a cell-to-cell manner.

Membrane fusion is involved in the spread of the hyperfusogenic MV between NT2N cells. While IC323-F(T461I)-EGFP induced syncytium formation in SLAM- and nectin 4-negative nonneuronal cells (e.g., Vero cells), it did not in human primary neurons and NT2N cells (33, 34; also this study). However, it is possible that a small amount of membrane fusion occurs between infected and adjacent cells, contributing to the spread of MV. To test this idea, we used the FIP, which is known to inhibit membrane fusion induced by MV (48). Vero cells expressing human SLAM (Vero/hSLAM) and NT2N cells were infected with IC323-F(T461I)-EGFP at an MOI of 0.1 for 1 h and then incubated in the presence of FIP or the solvent dimethyl sulfoxide (DMSO). While Vero/hSLAM cells treated with DMSO formed extensive syncytia at 1 dpi, FIP strongly inhibited cell-cell fusion (Fig. 4). Remarkably, FIP completely blocked the spread of IC323-F(T461I)-EGFP between NT2N cells, while DMSO had no effect on the spread. The results suggest that membrane fusion is indeed involved in the cell-to-cell spread of the hyperfusogenic MV between NT2N cells. We also tested substance P (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂), which was reported to inhibit membrane fusion induced by MV (49) and neuronal spread of the Edmonston strain (21). Substance P inhibited neither syncytium formation in Vero/hSLAM cells nor the spread of IC323-F(T461I)-EGFP between NT2N cells (data not shown).

The H protein is required for the cell-to-cell spread of the hyperfusogenic virus between NT2N cells. To examine whether the H protein is involved in MV spread between NT2N cells, we generated anti-H monoclonal antibodies (MAbs) that can block SLAM- and nectin 4-dependent MV infection. Vero/hSLAM cells and Vero cells expressing human nectin 4 (Vero/hNectin4) were infected with IC323-EGFP, and NT2N cells

FIG 4 Spread of the hyperfusogenic virus is inhibited by FIP. Vero/hSLAM and NT2N cells were infected with IC323-F(T461I)-EGFP at an MOI of 0.1. At 1 h p.i., FIP or the solvent DMSO was added to the culture medium at a final concentration of 200 μ M. The cells were observed under light and fluorescence microscopes at the indicated days after infection. The panels show representative images. Scale bar, 250 μ m.

were infected with IC323-F(T461I)-EGFP at an MOI of 0.1 for 1 h. The infected cells were then incubated in the presence of the previously reported anti-H MAb 2F4 (50) or anti-H MAbs that we generated (7C6, 8F6, and 10B5) and observed under a fluorescence microscope (Fig. 5A). The extents of syncytium formation or viral spread were also determined by quantitating EGFP-positive areas (Fig. 5B). All four MAbs examined inhibited, albeit to different extents, syncytium formation in IC323-EGFP-infected Vero/hSLAM and Vero/hNectin4 cells (Fig. 5A and B). When these cells were infected with the hyperfusogenic IC323-F(T461I)-EGFP, all the MAbs also exhibited inhibitory activity although to a lesser degree (data not shown). MAbs 2F4 and 8F6 were also able to inhibit the spread of IC323-F(T461I)-EGFP between NT2N cells, whereas 7C6 failed to do so (Fig. 5A and B). 10B5 exhibited a weaker ability to inhibit syncytium formation in Vero/hSLAM and Vero/hNectin4 cells than the other three MAbs, but it completely blocked the spread of IC323-F(T461I)-EGFP between NT2N cells (Fig. 5A and B).

DISCUSSION

In this study, we demonstrated that the hyperfusogenic IC323-F(T461I)-EGFP, but not the parental wild-type IC323-EGFP, spread efficiently between human neuronal NT2N cells. The finding is consistent with our previous results obtained with this and other hyperfusogenic recombinant MVs in human primary neuron culture and in the brains of suckling hamsters and IFNAR1 knockout mice (33, 34). Thus, enhanced fusion activity of the F protein appears to be essential for efficient spread of MV in human and rodent neurons.

On the other hand, the Edmonston strain of MV has been shown to spread between mouse (20) and rat neurons (23), without fusion-enhancing substitutions in the F protein. The Edmonston strain has many substitutions in the receptor-binding H protein, including those allowing the use of CD46 as a receptor (18, 19). However, CD46 is not critically involved in the spread of the Edmonston strain because neurons from rats and from CD46 nontransgenic mice do not express CD46. At present it is unknown whether the molecule involved in the spread of the Edmonston strain between rodent neurons is the same as the one used for MV spread between human neurons. Furthermore, expression of CD46 on human neurons makes it difficult to interpret the

FIG 5 Spread of the hyperfusogenic virus is inhibited by anti-hemagglutinin antibodies. Cells were seeded in 96-well plates. Vero/hSLAM and Vero/hNectin4 cells were infected with IC323-EGFP, and NT2N cells were infected with IC323-F(T461I)-EGFP at an MOI of 0.1 in triplicate. At 1 h p.i., the indicated MABs against the MV H protein were added to the culture medium. As a negative control, anti-H MAB 5G7, which has no ability to neutralize MV, was used. EGFP fluorescence was observed under a fluorescence microscope at 48 h p.i. (A) A fluorescence image of a representative well is shown for each sample. Ab(-), no antibody. (B) Relative areas of EGFP-expressing cells in each well were quantified by imaging software. Data are shown as means $\pm$ standard deviations of triplicate samples. The value of Ab(-) was set to 1 for each cell type.

results obtained with the Edmonston strain. To avoid these problems and study what indeed occurs in the human brain, we employed human neurons derived from NT2 cells and wild-type MV-based recombinant viruses.

Because SLAM and nectin 4 are not expressed on human neurons, it is likely that MV uses some other molecule(s) to enter and spread between neurons. Given that fusion-enhancing substitutions in the F protein strongly promote MV spread between human neurons, only the F protein, and not the H protein, may play a role in MV spread, as previously proposed (21, 22). However, most of anti-H MABs (neutralizing infection via SLAM or nectin 4) examined blocked the spread of IC323-F(T461I)-EGFP between NT2N cells. One MAB neutralized SLAM- and nectin 4-dependent MV infections but did not inhibit the spread of IC323-F(T461I)-EGFP between NT2N cells. Another MAB only weakly inhibited syncytium formation in Vero/hSLAM and Vero/hNectin4 cells but strongly blocked the spread of IC323-F(T461I)-EGFP between NT2N cells. These results suggest that the H protein is also required for the spread of the hyperfusogenic MV between NT2N cells and that the region on the H protein involved in MV spread between neurons overlaps but is different from that involved in the interaction with SLAM or nectin 4. Furthermore, these findings indicate the presence of the neuronal receptor interacting with the H protein of MV. In the sera and cerebrospinal fluids of SSPE patients, high levels of anti-MV antibodies (Abs) are present (5). This means that MV can somehow escape from neutralization by these Abs in SSPE patients. Detailed analyses of amino acid substitutions in the H protein from SSPE strains may reveal the mechanisms by which MV spreads in the CNS in the presence of anti-H Abs.

In SSPE patients, MV persistently infects neurons without producing virus particles (14). The lack of virus production is attributed to the defect of the M protein (26, 27). Thus, it is thought that the cell-to-cell MV transmission occurs between neurons in the brains of SSPE patients. By using confocal time-lapse imaging, we observed the cell-to-cell spread of IC323-F(T461I)-EGFP between NT2N cells. The virus was found to

spread from originally infected neurons to those connected to them via axons. Furthermore, little virus production was detected in NT2N cells although IC323-F(T461I)-EGFP possesses the intact M gene, unlike most SSPE strains. Similarly, the Edmonston strain (possessing the intact M protein) was reported to grow well in undifferentiated NT2 cells (presumably using CD46 as a receptor) but not in NT2 neurons (20). Thus, mutations in the M gene, a hallmark of SSPE strains, may partly result from the dispensability of the M protein for their survival in neurons.

IC323-F(T461I)-EGFP did not induce syncytia in NT2N cells as in human primary neurons (34). However, its spread between NT2N cells was prevented by FIP, indicating that cell-cell fusion does occur when the virus is transmitted. This finding is consistent with the clinical observation that syncytia are not present in the brains of SSPE patients (5). In the human brain, the cell-cell contacts between neurons may be hindered by other supporting cells and myelinated nerve fibers and limited to small areas such as synapses. This spatial arrangement may be a reason why neurons do not form syncytia in SSPE patients. In our culture of NT2N cells, nonneuronal cells are very few, and the cell-cell contact appears to occur between cell bodies of different neurons. Thus, in NT2N cells, membrane fusion may occur only at synapses where virus (RNP complex) transmission takes place between neurons but may not occur in other parts of cells. Recently, it has been shown that peptides derived from the heptad repeat regions of the F protein can inhibit entry and cell fusion by MV and protect model mice from MV-induced encephalitis (51–53). A fusion-enhancing substitution in the F protein was also found in MV genomes from the brains of human immunodeficiency virus-infected patients with measles inclusion body encephalitis (54). Blocking of membrane fusion caused by mutant F proteins might be a good strategy to inhibit progression of fatal MV infection in the CNS.

In conclusion, the H protein and the mutant F protein possessing fusion-enhancing substitutions play crucial roles in membrane fusion and subsequent MV transmission between neurons. Our data also suggest that a molecule(s) other than SLAM and nectin 4 acts as a neuronal receptor for MV. We envisage that this putative receptor is highly concentrated at synapses.

MATERIALS AND METHODS

Cells. Vero/hSLAM (9) and Vero/hNectin4 cells (55) were maintained in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries) supplemented with 7.5% fetal bovine serum (FBS; Sigma) and 1% penicillin-streptomycin (Gibco). NTERA-2cl.D1 (NT2) cells were purchased from the American Type Culture Collection and maintained in Opti-MEM (Gibco) supplemented with 5% FBS and 1% penicillin-streptomycin. We slightly modified a previously described protocol for neuronal differentiation of NT2 cells (40). Briefly, NT2 cells were suspended in DMEM supplemented with 10% FBS and seeded in bacteriological-grade petri dishes (Eiken Chemical) at a density of 4×10^6 to 5×10^6 cells per dish. On the next day, all-*trans*-RA (Sigma) was added to the culture medium at a final concentration of 10 μ M. Every 2 to 3 days the medium and dishes were changed. After 7 to 8 days, the cells were seeded in 10-cm cell culture dishes (Nippon Genetics) and cultured for another 6 to 8 days. The cells were detached by trypsin (MP Biomedicals), transferred to 15-cm cell culture dishes (Nunc), and cultured without RA for 2 days. The cells were trypsinized again, seeded in 10-cm cell culture dishes precoated with 10 μ g of poly-D-lysine (PDL; Sigma) per ml, 10 μ g of laminin (LAM; Sigma) per ml, and 0.1% gelatin (Sigma) and supplied with medium containing a final concentration of 40 μ M 1- β -D-arabinofuranosylcytosine (ara-C; Sigma) and 4 μ M uridine (Sigma). After 7 to 10 days, differentiated neurons (NT2N) were detached by brief trypsinization and seeded in plates precoated with PDL, LAM, and gelatin for further experiments.

Viruses. IC323-EGFP is a recombinant MV expressing EGFP based on the wild-type IC-B strain (56, 57). IC323-F(T461I)-EGFP was generated, based on IC323-EGFP (33). The recombinant MVs were prepared as described previously (58) and titrated on Vero/hSLAM cells by plaque assay. VSV Δ G*-G was prepared and titrated on 293T cells as previously described (47, 59).

Confocal time-lapse imaging. NT2N cells seeded in glass-bottom dishes (Matsunami Glass Ind., Ltd.) were infected with IC323-F(T461I)-EGFP at an MOI of 2. EGFP fluorescence was observed under a confocal microscope (Radiance 2100; Bio-Rad). Images were taken every 15 min from 24 h p.i. to 40 h p.i.

Inhibition of virus spread by FIP. FIP (Z-D-Phe-Phe-Gly; Peptide Institute) and substance P (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂; Peptide Institute) were dissolved in DMSO (Sigma). Vero/hSLAM and NT2N cells seeded in 24-well plates were infected with IC323-F(T461I)-EGFP at an MOI of 0.1. At 1 h p.i., DMSO, FIP, or substance P was added to the culture medium at a final concentration of 2% (DMSO) or 200 μ M (FIP and substance P).

Inhibition of virus spread by anti-H MAbs. MAbs 2F4 is a previously described antibody against MV H protein (50). To generate anti-H MAbs, the expression plasmid encoding soluble histidine (His)-tagged

MV H was transiently transfected into HEK293S GnTI(-) cells (60, 61). One week after transfection, culture medium containing the secreted MV H was collected. MV H was purified by using a Ni²⁺-nitrilotriacetic acid (NTA) affinity column (cOmplete His-tag purification resin; Roche) and Superdex 200 GL 10/300 gel filtration chromatography (GE Healthcare). Six-week-old BALB/c mice were immunized with the purified MV H protein four times at intervals of 1 week. Three days after the last immunization, the mice were euthanized, and spleen cells were harvested. All animal experiments were reviewed by the Institutional Committee of Ethics on Animal Experiments and carried out according to the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University, Japan. We screened hybridoma cells for anti-H MABs possessing neutralizing ability for MV. Vero/hSLAM and Vero/hNectin4 cells were infected with IC323-EGFP mixed with supernatants of hybridomas, and at 1 h p.i., FIP was added to the culture medium. At 24 to 36 h p.i., EGFP fluorescence was observed under a fluorescence microscope (Axiovert 200; Carl Zeiss). MV-neutralizing MABs were purified by protein G affinity chromatography. We verified by enzyme-linked immunosorbent assay that all purified MV-neutralizing MABs could bind to MV H. Vero/hSLAM, Vero/hNectin4, and NT2N cells were seeded in 96-well plates and infected with IC323-EGFP or IC323-F(T461I)-EGFP at an MOI of 0.1. At 1 h p.i., anti-H MABs were added to the culture medium at a final concentration of 10 μ g/ml. Fluorescence images of cells were taken with a BZ-X710 microscope (Keyence) at 48 h p.i. Relative areas of EGFP-expressing cells in triplicate samples were determined using a BZ-X Analyzer (Keyence).

Reverse transcription-quantitative PCR (RT-qPCR). RNA was extracted with TRIzol (Invitrogen) from undifferentiated NT2 cells and postmitotic neurons (NT2 cells treated with RA for 2 weeks and with mitotic inhibitors for another 1 week). The RNA samples were treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed using a PrimeScript RT reagent kit (TaKaRa Bio). Quantification of mRNAs of neuronal and astrocytic markers and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out using SYBR Premix Ex Taq II (TaKaRa Bio) and a LightCycler, version 1.5, instrument (Roche). For microtubule-associated protein 2 (MAP2), microtubule-associated protein tau (MAPT), RNA binding protein Fox-1 homolog 3 (RBFOX3), class III beta-tubulin (TUBB3), glial fibrillary acidic protein (GFAP), and glutamate-ammonia ligase (GLUL), we used the following primer pairs, respectively: 5'-TTTGGGCACACTCTTGTTC-3' and 5'-TTGCTTCCGTTGGCATTTCG-3'; 5'-CAGACCTGAAGAATGTCAAGTCC-3' and 5'-ACACTTGGAGGTCACCTTGC-3'; 5'-GCAAATGTTCCGGGCAATTTCG-3' and 5'-ATCGTCCCATTCAGCTTC-3'; 5'-TCATCAGTGATGAGCATGGC-3' and 5'-TCGTTGTAGTAGACGCTGATCC-3'; 5'-ACTCAATGCTGGC-3' and 5'-AGCGAACCCTCTCGATGATGC-3'; and 5'-ATGCTGGAGTCAAGATTGCG-3' and 5'-AGTCTTCACACACGATGC-3'. Data were analyzed by a two-tailed Student's *t* test.

Western blotting. NT2N cells seeded in 12-well plates were infected with IC323-EGFP or IC323-F(T461I)-EGFP at an MOI of 2. The cells were washed by phosphate-buffered saline (PBS) and lysed in 1× sodium dodecyl sulfate (SDS) loading buffer (40 mM Tris-HCl, pH 6.8, 1.6% SDS, 8% glycerol, 0.05% bromophenol blue, 0.1 M dithiothreitol). Proteins in the lysate were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with PBS containing 0.05% Tween 20 and 5% skimmed milk and subsequently incubated with anti-MV serum for detection of MV N (kindly provided by M. B. A. Oldstone) (62) or mouse anti-actin MAB (sc-8432; Santa Cruz Biotechnology). After three washes with PBS-Tween 20 (PBS-T), the membranes were incubated with goat anti-human IgG-horseradish peroxidase (HRP) or goat anti-mouse IgG-HRP (Jackson ImmunoResearch), washed with PBS-T three times again, and treated with Chemi-Lumi One Super (Nacalai Tesque). Chemiluminescent signals were detected by VersaDoc 5000 (Bio-Rad).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.02166-17>.

SUPPLEMENTAL FILE 1, MP4 file, 6.0 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

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