Characteristics of Viruses Derived from Nude Mice with Persistent Measles Virus Infection

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Measles virus (MV) isolates from patients with subacute sclerosing panencephalitis (SSPE) differ from wild-type MV virologically. However, few animal models have reported viruses with characteristics of the SSPE virus. The MV Edmonston strain was inoculated into the subarachnoid space of nude mice. All nude mice displayed weight loss and required euthanasia, with a mean survival duration of 73.2 days. The viral load in the brain was 4- to 400-fold higher than the inoculated load, and brain infection was confirmed by immunostaining. Gene sequencing of the viruses revealed that amino acid mutations occurred more frequently in matrix proteins. The most common mutation was a uridine-to-cytosine transition. The virus exhibited lower free virus particle formation ability than the Edmonston strain. When nude mice were challenged with 2 × 10^4 PFU of the brain-derived virus, the mean survival duration was 34.7 days, which was significantly shorter than that of the mice challenged with 4 × 10^3 PFU of the Edmonston strain (P < 0.01). This study indicated that MV in a nude mouse model of persistent infection exhibited characteristics of the SSPE virus. This model may prove useful in elucidating the pathogenic mechanism of SSPE and developing potential therapeutics.

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Measles virus (MV) is a negative-strand RNA virus of the paramyxovirus family. The MV RNA sequence comprises 15,894 nucleotides and six transcription units, the N, P, M, F, H, and L genes, which encode nucleocapsid (N) protein, phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin (H) protein, and large polymerase (L) protein, respectively. The P gene also encodes the nonstructural proteins V and C. The MV genome includes the ribonucleoprotein (RNP) complex comprising the N, P, and L proteins. The H protein is associated with virus receptor attachment, and the F protein is associated with membrane fusion when the RNP complex invades and infects a cell. The M protein connects with the RNP complex, F protein, and H protein and is associated with virus particle formation and maturation (1, 2). MV has two identified receptors: CD46 and the signaling lymphocyte activation molecule (SLAM; CD150) (3). In addition, the tumor cell marker PVRL4 (nectin 4) was recently reported to associate with MV through the epithelial cell receptor (4). However, it was reported that CD46 does not participate in interneuronal transmission of MV (5) and that SLAM does not exist in neurons (3). In the central nervous system, MV spreads to neighboring neurons transsynaptically (5, 6).

Subacute sclerosing panencephalitis (SSPE) is a fatal degenerative disease caused by MV infection in the central nervous system that persists for several years after acute MV infection (7). The incidence of SSPE varies according to the prevalence of MV in a country and ranges from 4 to 11 per 100,000 cases of measles (7, 8). It is an extremely rare but important disease because its prognosis is poor and there is no effective treatment. MV strains isolated from patients with SSPE, the so-called SSPE virus, differ from the wild-type MV strain virologically, including the inability to become a cell-free virus, the ability to spread from cell to cell, and strong neurovirulence (2, 9, 10). The SSPE virus has gene mutations compared with the wild-type MV genome. The M gene is more frequently mutated than other genes. The mutations in the M gene lead both to defects in the start and stop codons and to the generation of mutant proteins. In addition, the M protein is associated with the virological characteristic and pathogenesis of the SSPE virus (1, 11, 12).

Although MV infection has been investigated experimentally in immunocompetent mice, it does not result in a persistent infection such as SSPE (1). Ohuchi et al. reported an SSPE murine model in which nude mice were infected with the MV Edmonston strain intracranially (13). All mice died 59 to 140 days after infection, and the homogenates of the brains of the dead mice exerted cytopathic effects in cell culture. Recently, Oldstone et al. reported that CD46 transgenic mice died at approximately 46 days after intracerebral inoculation of the Edmonston strain (14). Another study reported that C57BL/6 mice survived over 50 days after infection with MV possessing an H gene with an affinity for hamster neurons (15), thus confirming the development of an adequate immunocompetent mouse model. Although there are reports that viruses derived from the brains of persistently MV-infected immunocompetent mice had mutations in the N, F, and M genes, particularly the M gene (14), there are no reports about the virological characteristics of these viruses.

To elucidate the pathogenic mechanisms of SSPE, nude mice were infected with the Edmonston strain by intracerebral inoculation and the brain-derived viruses were analyzed for gene mutations to determine their virological characteristics.

MATERIALS AND METHODS

Cell, medium, and viruses. African green monkey kidney (Vero) cells expressing human SLAM (Vero/SLAM cells) were provided by Yusuke Yanagi at Kyushu University (16). The cells were grown in minimum
essential medium supplemented with 10% fetal bovine serum (FBS), 0.4 mg/ml Geneticin, 100 IU/ml penicillin, and 0.3 mg/ml l-glutamine (10% FBS–Vero/SLAM cell medium). The cells were cultured in an incubator at 37°C.

The Edmonston strain of MV was inoculated into Vero/SLAM cells and harvested after freeze-thawing of the infected cells. The SSPE Yamagata-1 strain (17) was cultured in Vero/SLAM cells, harvested by trypsinization, and stocked as an infected cell suspension in 10% FBS–Vero/SLAM cell medium. The harvested brain fluids of infected nude mouse brains were used in gene sequence analysis and inoculated into Vero/SLAM cell monolayers in flasks. The cytopathic effects appeared after 24 to 48 h. The infected cells were then harvested after 96 h by trypsinization and again inoculated into Vero/SLAM cell monolayers. The three virus-infected passage cells were stocked as an infected cell suspension in 10% FBS–Vero/SLAM cell medium supplemented with 10% dimethyl sulfoxide. The infected cell fluids were stored at −80°C. The viral load of the stock-infected cell fluids was determined by a plaque assay.

Animal inoculation. On the basis of a previous report (13), 4-week-old female BALB/c nude mice (CLEA Japan Inc., Tokyo, Japan) were used for all experiments. The mice were inoculated with MV at the 100% lethal dose indicated in the report (13). The MV Edmonston strain (4.0 × 10^4 PFU/30 µl) was inoculated into the subarachnoid space of the nude mice with a 27-gauge needle to a depth of 2 mm while the mice were under isoflurane anesthesia. Thereafter, the mice were weighed daily. The ethical endpoint was defined as a more than 20% weight loss relative to the maximum weight. At the ethical endpoint, euthanasia was performed by cervical dislocation while the mouse was under isoflurane anesthesia. The brain of each mouse was harvested. However, even though the mice had less than 20% weight loss, the brains of two mice were harvested after euthanasia 56 days after inoculation to investigate MV mutations before weight loss. All experiments were performed in accordance with national ethical guidelines and with the approval from the Institutional Animal Care and Use Committee of Fukushima Medical University.

Pathological samples. The harvested brains were fixed for 48 h in 10% formalin. Paraffin blocks were made and cut into 5-µm sections. The sections were stained with hematoxylin-eosin (H&E) stain. Immunohistochemical detection was performed on paraffin sections using the enzyme-labeled indirect method. Polyclonal antibody against the measles N protein (catalog number pab0035; Covalab, Lyon, France), which was diluted 500-fold with phosphate-buffered saline (PBS) containing 1% bovine serum albumin, was used as the primary antibody. Histofine Max PO (R) (Nichirei Bioscience, Tokyo, Japan), which is a peroxidase-conjugated rabbit anti-mouse IgG, was used as the secondary antibody. Endogenous peroxidase activity was blocked with 0.3% H$_2$O$_2$ in methanol for 30 min at room temperature. Then, the sections were incubated with primary antibody overnight at 4°C. Next, the sections were incubated with secondary antibody for 30 min at room temperature. The sections were reacted in 3,3' diaminobenzidine tetrachloride (DAB) before H&E nuclear staining. Control sections were created from the brains of mice injected with medium only.

Viral quantification. The homogenized fluids of six nude mouse brains were additionally diluted 10-fold with 10% FBS–Vero/SLAM cell medium and inoculated into Vero/SLAM cell monolayers growing in 12-well microtiter plates. The plates were then incubated for 1 h at 37°C, and 2% FBS–Vero/SLAM cell medium containing 0.75% methylcellulose was gently added to each well. After incubation at 37°C for 96 h, the plates were fixed in 10% formalin and stained with H&E, and the number of typical plaques was counted.

Gene sequence analysis. RNAs of persistent virus in nude mice were extracted from the six mouse brain homogenized fluids derived directly using the ISOGEN-LS reagent (Nippon Gene, Tokyo, Japan). RNA of the Edmonston strain that passed into Vero/SLAM cells was extracted before inoculation. cDNA was synthesized using a reverse transcription kit (PrimeScript reverse transcription-PCR kit; TaKaRa, Shiga, Japan), and PCR was performed using TaKaRa Ex Taq HS DNA polymerase. Primers targeting the coding regions of the N, P, M, F, and H genes were synthesized as described previously (18). The cDNA step consisted of 10 min at 30°C, 30 min at 42°C, and 5 min at 95°C. The PCR cycling conditions were as follows: initial denaturation at 94°C for 1 min, followed by 50 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min. PCR products were separated by 2% agarose gel electrophoresis, and bands of interest were excised. DNA was purified using a QIAquick gel extraction kit (Qiagen) and sequenced using GeneTify software (version 10; Genetyx Corporation, Tokyo, Japan) and compared with those of the Edmonston strain that was passaged into Vero/SLAM cells before inoculation.

Free virus particle formation ability of persistent virus strains in the nude mouse brain. The persistent virus stock was prepared by very low passage into Vero/SLAM cells. Fifty microliters of the persistent virus stock cell fluids was inoculated into Vero/SLAM cell monolayers in 12-well microtiter plates (multiplicity of infection, 0.001) and incubated for 1 h. One milliliter of 2% FBS–Vero/SLAM cell medium was added to each well, and the cells were incubated at 37°C. Supernatant was harvested after 24 and 48 h. Concurrently, cells were harvested by trypsinization and centrifuged at 1,500 rpm for 10 min. The supernatant was removed, and 500 µl of 2% FBS–Vero/SLAM cell medium was added to the cells. The viral loads of the supernatants and cell suspensions were determined as described in the “Viral quantification” section above.

Inoculation of persistent virus strains in nude mice. Cell fluids infected with persistent viruses (2.0 × 10^3 PFU) were inoculated into 10 nude mice, as described in the “Animal inoculation” section above. Furthermore, the mice were also infected with 2.0 × 10^3 PFU of an Edmonston strain-infected cell suspension. The nude mice were also inoculated with 2.0 × 10^2 PFU of the SSPE Yamagata-1 strain. The viral load of the SSPE Yamagata-1 strain was the lower limit of the 100% lethal dose in our experiment (data not shown). The log-rank test was used for statistical analysis of the survival curves.

RESULTS

Percent survival after intracerebral inoculation. After inoculation with the Edmonston strain, the nude mice exhibited weight loss approximately 56 days after inoculation. All nude mice died naturally or required euthanasia due to weight loss before 100 days after inoculation (Fig. 1). The mean survival duration was 73.2 days. The typical symptoms in the nude mice were sudden jumps, hypersensitivity to sound, and gait disorder after weight loss. The affected mice displayed a gradual loss of mobility before dying.

Pathology of harvested brains. No abnormal findings were detected in low-power fields after hematoxylin-eosin (H&E) staining. In high-power fields, neuronal degeneration was noted in the cortex. No inflammatory change was detected. A polyclonal antibody against the N protein revealed the presence of diffuse measles virus antigens in brain regions such as the cortex, hippocampus, and cerebellum. In addition, in high-power fields, measles virus antigens filled the neuronal cell bodies and axons (Fig. 2). No measles virus antigen was detected in neuroglia.
Viral quantification. The plaques of brain-derived virus were mixed with the plaques of the original Edmonston strain and the plaques of a strain larger than the Edmonston strain. All their plaques were counted. The MV load in nude mice increased by 4- to 400-fold over time after inoculation (Table 1). The mouse numbers are the same in Tables 1 to 3.

Amino acid mutations. The brain-derived virus with the highest load was from mouse 6 and was used to determine amino acid mutations. Ten clones of the coding regions for the N, P, M, F, and H genes were analyzed, and the resulting amino acid mutations were clarified (Fig. 3). The N, P, M, F, and H proteins consist of 525, 507, 335, 553, and 617 amino acids, respectively, and 5, 5, 57, 11, and 12 amino acid mutations were detected in these five proteins, respectively. The M protein had the greatest number of amino acid mutations, and defects in the start and stop codons were detected only in the M gene.

Base mutations in the M gene and amino acid mutations in the protein product. There were 97 point mutations in the 1,008-base sequence of the M gene in the 10 clones. In total, 92 of the 97 point mutations were uridine (U)-to-cytosine (C) transitions. There were variations among individuals and clones from the same brain. The mean number of base mutations for the 10 clones of each mouse ranged from 8.7 to 86.4, and the mean number of amino acid mutations of each mouse ranged from 2.3 to 58.6 (Table 2). Between 0 and 9 defects in the start and stop codons were detected in the clones. In MV strains displaying more than 10 base mutations, approximately 90% of the base mutations were U-to-C transitions (Table 3). U-to-C transitions in the start codon in all clones resulted in mutations from methionine to threonine, whereas U-to-C transitions in the stop codon generally resulted in mutations to glutamine. A mutation to tryptophan due to a mutation from adenine to guanine was observed in one clone.

Free virus particle formation ability of persistent viruses in nude mouse brain. Viruses with the highest number of M-gene mutations from mouse 2 were used to determine the free virus particle formation ability of persistent viruses in the nude mouse brain. Within 24 h of inoculation, no plaque was detected in the supernatant of the Edmonston strain- and persistent virus isolate-

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Time of euthanasia after inoculation (days)</th>
<th>Wt loss</th>
<th>Viral load in brain (PFU)*</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>56</td>
<td>-</td>
<td>$4.2 \times 10^5$</td>
<td>10.6</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>-</td>
<td>$7.4 \times 10^5$</td>
<td>18.5</td>
</tr>
<tr>
<td>3</td>
<td>69</td>
<td>+</td>
<td>$1.6 \times 10^5$</td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>+</td>
<td>$8.5 \times 10^5$</td>
<td>211.6</td>
</tr>
<tr>
<td>5</td>
<td>83</td>
<td>+</td>
<td>$4.8 \times 10^6$</td>
<td>120.0</td>
</tr>
<tr>
<td>6</td>
<td>88</td>
<td>+</td>
<td>$1.6 \times 10^7$</td>
<td>400.0</td>
</tr>
</tbody>
</table>

* The viral load in 50 μl of homogenized brain fluid was used to determine the amount in the brain.

Table 1: Viral loads in the brains of inoculated mice
infected brains *in vitro*. In cell suspensions, $3.2 \times 10^2$ PFU/well was detected for the Edmonston strain, whereas 50 PFU/well was detected for the persistent virus (Fig. 4). At 48 h after inoculation, $1.3 \times 10^3$ PFU/well was detected in the supernatant for the Edmonston strain, whereas 2.3 $\times 10^2$ PFU/well was detected for the persistent virus isolate. On the other hand, in cell suspensions, $3.0 \times 10^3$ and $4.5 \times 10^3$ PFU/well were detected for the Edmonston strain and persistent virus isolate, respectively. At 48 h after inoculation, the ratio of the number of PFU in the cell suspension to the number of PFU in the supernatant was 22:1 for the Edmonston strain, whereas it was 1,900:1 for the persistent virus isolate.

**Inoculation of persistent virus isolates into nude mice.** The uninfected nude mice that were intracranially inoculated with $2.0 \times 10^2$ PFU of the Edmonston strain did not result in weight loss or death in the mice. In contrast, the mean survival duration after the inoculation of the nude mice with the SSPE Yamagata-1 strain was 17.4 days.

**DISCUSSION**

In the brain of an SSPE patient or SSPE hamster model, defects in neurons, infiltration of plasma cells and lymphocytes into the perivascular space, increases in the number of astrocytes and microglia, or inclusion bodies were observed (19, 20). Although degenerated neurons were detected in this study, none of the above-mentioned findings were observed in this immunodeficient mouse model. However, neuronal infection was revealed by staining with a polyclonal antibody against the measles virus N protein.

**TABLE 2** Nucleotide mutations in the M-gene sequence and resulting amino acid mutations in the M protein

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Mean no. of mutations</th>
<th>No. of defects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Base</td>
<td>Amino acid</td>
</tr>
<tr>
<td>1</td>
<td>13.3</td>
<td>8.6</td>
</tr>
<tr>
<td>2</td>
<td>86.4</td>
<td>58.6</td>
</tr>
<tr>
<td>3</td>
<td>8.8</td>
<td>4.9</td>
</tr>
<tr>
<td>4</td>
<td>58.8</td>
<td>2.3</td>
</tr>
<tr>
<td>5</td>
<td>8.7</td>
<td>6.2</td>
</tr>
<tr>
<td>6</td>
<td>26.7</td>
<td>10.2</td>
</tr>
</tbody>
</table>

The 10 clones were analyzed for each brain from six mice. The mean number of nucleotide and amino acid mutations was calculated by averaging the total number of mutations detected in 10 clones.

In the brains of the nude mice because the harvested brains displayed a higher viral load than the inoculum.

**TABLE 3** Base mutations in M gene

<table>
<thead>
<tr>
<th>Mouse</th>
<th>No. (%) of the following mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U to C</td>
</tr>
<tr>
<td>1</td>
<td>116 (87.2)</td>
</tr>
<tr>
<td>2</td>
<td>849 (98.6)</td>
</tr>
<tr>
<td>3</td>
<td>59 (67)</td>
</tr>
<tr>
<td>4</td>
<td>574 (97.7)</td>
</tr>
<tr>
<td>5</td>
<td>73 (83.9)</td>
</tr>
<tr>
<td>6</td>
<td>253 (94.8)</td>
</tr>
</tbody>
</table>

The base mutations in the M gene in each infected mouse are shown. The mutation numbers denote the total numbers of mutations in 10 clones. The percentage of total mutations in each mouse is shown in parentheses.

In addition to mutations in MV, it is believed that the immune state of the host is also associated with the onset of SSPE (1). MV infection in young children, such as those less than 1 year old, is a risk factor for SSPE because the immune system of young children is not completely mature (8, 21, 22). The association of host genes with immunity has been investigated. Several recent reports of polymorphisms in SSPE patients have suggested that the genes for interleukin-2 (IL-2), IL-4, IL-12, myxovirus resistance A, and angiotensin-converting enzyme are related to the onset of SSPE (23–26). In particular, T-cell immunity is an important factor for the elimination of MV infection (1, 27). Therefore, conditions that result in T-cell abnormalities, such as AIDS, increase the severity of MV infection (28, 29). In this study, the nude mice exhibited T-cell deficiencies and MV inoculation resulted in persistent infection.

Cattaneo and colleagues reported that the genomes of viruses isolated from two patients with SSPE and one patient with measles inclusion body encephalitis displayed numerous U-to-C transitions in the M gene (biased hypermutation) (30, 31). Double-stranded RNA adenosine deaminase (previously known as double-stranded RNA unwinding/modifying activity), which is ubiquitously expressed (32), is believed to cause biased hypermutation since adenosine residues would be deaminated to yield inosine (30, 33, 34). Adenosine-to-inosine conversions were identified in human neuroblastoma and glioblastoma cells *in vitro* (35). Biased hypermutation was detected in a CD46 transgenic mouse.
model (14). In this study, 67 to 98.6% of the mutations in the M gene of the persistent viruses in nude mice at 56 days after inoculation were U-to-C conversions, a finding which indicates biased hypermutation.

Ayata and colleagues reported that biased hypermutation caused a start codon defect due to the mutation of AUG to ACG in the SSPE Yamagata-1 strain, resulting in the production of a defective M protein (11, 12). The MV Edmonston strain in which the M gene was replaced with the M gene of the SSPE Biken strain did not produce free virus particles (10). In comparison with standard MV, MV with a defective M protein induced cell-to-cell fusion more efficiently and formed a plaque different from that formed by standard MV, although it exhibited 250-fold lower viral titers in the supernatant than standard MV (36). Consequently, it is believed that persistent MV strains exhibit mutations in the M gene due to biased hypermutation, resulting in M-protein mutations due to start and stop codon defects and the absence of cell-free virus particle formation. In this study, the N, P, M, F, and H genes were analyzed, and numerous mutations were detected in the M gene as well as in virus strains isolated from patients with SSPE. Furthermore, there were clones that displayed start and stop codon defects only in the M gene. Regarding the ability of persistent viruses to form free virus particles in the nude mouse brain in this study, the viral titers of the persistent viruses in the supernatant cells were approximately 100-fold lower than those of the Edmonston strain before inoculation, and the clones were less likely to display particle formation under cell-free conditions. Therefore, it is suggested that some of the persistent virus strains in nude mice expressed mutated M proteins due to start and stop codon defects, resulting in reduced cell-free virus particle formation.

MV generally does not induce neurological disease in experimental small animals such as hamsters and ferrets, whereas the SSPE virus induces lethal neurological diseases in these animal models (1, 37). However, the mutations responsible for the neurovirulence of the SSPE virus have not been identified. Ayata et al. reported that a recombinant virus containing the F gene of the SSPE Osaka-2 strain alone induced lethal encephalopathy in a hamster model. Furthermore, a single T461I substitution in the F protein transformed the nonneuropathogenic wild-type MV into a lethal virus (37). However, it is unknown whether the F-gene mutation is common among all SSPE virus strains. It is possible that each SSPE virus strain has unique F-gene mutations that are associated with its respective neurovirulence. In our study, the survival duration of the nude mice inoculated with the persistent virus strains was significantly shorter than that of the nude mice inoculated with the Edmonston strain. Thus, the persistent virus strains had stronger neurovirulence. Although these strains had some mutations in the F gene, the T461I substitution was not detected. Other mutations in the F gene or other genes may be associated with their strong neurovirulence. Analysis of persistent virus strains may clarify this question.

In this study, persistent MV strains inoculated into nude mice exhibited two characteristics of the SSPE virus, an inability to become a cell-free virus and strong neurovirulence, which had not previously been demonstrated in an animal model. This model may clarify the pathogenic mechanism of SSPE onset and reveal the utility of potential therapeutics against SSPE.

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