表6　Hibワクチンの効果
（5歳未満人口10万人あたりの罹患率のワクチン導入前後での推移）

国・地域	対症とした5歳未満人口（千人）	Hib髄膜炎罹患率（導入前→導入後）	侵襲性Hib疾患罹患率（導入前→導入後）
米国	20,524	54 → <1	88 → 1.6
英国	3,831	24 → 0.6	36 → 1
ドイツ	4,115	23 → 0.9	46 → 1.3
スカンジナビア	1,581	31 → <1	51 → 1
オーストラリア	1,360	25 → 6	59 → 16
イスラエル	566	18 → <1	34 → <1
チリ	1,500	40 → <2	—

（中野：文献[4]，Peltola文献[7]より）

同時接種の利点はいくつかある。第1に，複数の疾患に対する免疫を早くつけることができる。たとえば，百日咳やHib髄膜炎・肺炎球菌髄膜炎は，乳児期早期の罹患頻度が高く，重症化のリスクも高い。これらの疾患に対して1つひとつ免疫をつけていくのも1つの方法だが，免疫をつける前に罹ってしまう可能性がある。同時接種を行うと，一度に複数の疾患に対する免疫をつけることができ，早期に複数の疾患に対する抵抗力が備わる。また，一度の受診で複数のワクチンを接種することができるので，児や家族の負担を軽減することができる。さらに，基礎疾患を有する児は，各種疾患が重症化する可能性も高いため早くワクチンを済ませたい一方で，当日の体調によっては副反応への懸念から接種を見合わせる機会も多い。同時接種は，体調のよい時期を見計らって，複数の免疫をつけることができる。

一方，同時接種には，1本ずつ接種する単独接種にはない懸念事項も存在する。まずは，接種後に起こる有害事象は，予防接種による副反応以外に，たまたまその時期に偶発的に発生するものもある。複数のワクチンの同時接種を行っていた場合は，因果関係の解析に関して複雑な考察が必要となる可能性がある。単独接種のほうが，どのワクチンにより起こったものかが明確に議論できる。また，定期接種と任意接種のワクチンを同時接種して重篤な有害事象が発生した場合，どのような規定や法律にもとづいて対処されるのかに関する具体的な指針や過去の判例が明示されていない。そして，基礎疾患を有する児は「接種要注意者」に分類され，予防接種の副反応に対する注意が健常児よりも必要である。同時接種が副反応の程度や頻度を増強しないかという心理的な懸念が保護者，医療者の双方にある。

国内外のデータでは，同時接種は，単独接種に比べて，発熱や接種局所の発赤などの軽い副反応が起こりやすいという研究報告があるが，両群で差がないとする報告もある。同時接種により，重篤な副反応が起こりやすくなるという報告は現状ではない。肺炎球菌，Hibワクチン，ともに生後2か月から接種が可能である。細菌性髄膜炎などの重篤な侵襲性感染症は，母親からの移行抗体が低下する生後6か月頃から急増し，1歳前後で患者発生のピークを迎えるため，できるかぎり早期に接種を完了することが大切である。乳児期早期からの高い接種率を達成するためには，世界標準の同時接種がさらに普及してゆくことが望ましいと考える。ま

た，後に述べるロタウイルスワクチンも，接種時期も，肺炎球菌やHibワクチンと重複する。ワクチンによる予防を積極的に心がけるならば，やはり同時接種をうまく活用することが必要であろう。

小児用肺炎球菌ワクチンとHibワクチンの接種一時見合わせについて

2011年3月4日，小児用肺炎球菌やHibワクチンなど同時接種後の死亡症例報告を受けて，厚生労働省がこれらワクチンの接種一時的見合わせを決定した。死亡と接種の因果関係については，専門家を含むメンバーによる検討が重ねて行われ，剖検所見も含めて死亡症例と両ワクチンの間には直接的な明確な因果関係は認められないと判断された。接種後に発生した突然死の頻度は，両ワクチンとも対10万接種あたり0.1～0.2程度であり，海外での頻度との間に大きな隔たりはなく，乳児突然死症候群(sudden infant death syndrome；SIDS)などの紛れ込み有害事象を含めた頻度と考えられた。また，ワクチン製剤の検定結果や安全性にも問題はなかった[8]。

これら結果にもとづき，肺炎球菌とHibワクチンの接種は4月1日から再開された[8]。同時接種についても，単独接種も選択肢として考慮することは必要であるが，児の状態を確認して実施することが可能と判断された。基礎疾患を有する児も含めて，髄膜炎などの重症感染症予防のためには，ワクチンによる予防が大切であることが再確認されたわけであり，漠然とした不安からせっかくの接種機会を逃すことのないように啓発することが大切である。

ロタウイルスワクチン

ロタウイルス胃腸炎は，激しい嘔吐と水様性の下痢により，急激に脱水症状が進行する。また，脳症やけいれんなどの合併症を引き起こすこともあり，小児が頻繁に罹る一般的な疾患のなかでは，重症の部類に属する代表的なものである。ロタウイルス胃腸炎は生後6か月くらいから急増する。また，乳児など年少児では重症化しやすく，ロタウイルス胃腸炎による入院は，2歳未満児が全体の7～8割を占めるとされる[9]。ロタウイルスは感染力が強く，衛生水準の向上だけでは流行を制御できないため，ワクチンによる予防が重要である。

ロタウイルスワクチンは，弱毒生ウイルスを接種することにより，自然感染に類似した免疫を誘導し，疾患に対する防御効果を期待するものである。ロタウイルス感染症では，年少児が経験する初感染が最も重篤な病態につながるので，それを弱毒ワクチンで予防するという理論にもとづいている。

2011年7月にわが国で承認されたロタウイルスワクチン(商品名：ロタリックス®)は，経口投与の弱毒生ワクチンで，生後6～24週に，4週間以上の間隔をあけて2回投与する。より多くの子どもを重症ロタウイルス胃腸炎から守るために，ワクチンスケジュールの早期に組み込むことが必要と思われる。また，過去に米国で1990年代後半に使用されたことのあるロタウイルスワクチンは，月齢依存的に腸重積のリスクが高まったため，安全性の面からも，早期に接種を完了することが推奨される。

日本での臨床試験の成績では，プラセボと比較して重症ロタウイルス胃腸炎の発症を約92％予防し，重症軽症を問わないロタウイルス胃腸炎の発症全体でも約79％を予防した。最も多く報告された副反応は，易刺激性(約7％)

であったが，その他の副反応も含めて，程度や頻度の点で大きく問題となるものは認められなかった。

おわりに

本項で紹介した3種類のワクチンは，海外の先進諸国では定期接種としてすべての子どもたちに接種されており，その予防効果には素晴らしいものがある。ぜひとも普及に努め，子どもたちを感染症の脅威から守りたいものである。

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Amino Acid Substitutions in Matrix, Fusion and Hemagglutinin Proteins of Wild Measles Virus for Adaptation to Vero Cells

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Key Words
CD46 · Fusion protein · Hemagglutinin protein · Matrix protein · Measles virus · SLAM

Abstract
Background: Wild-type measles virus (MV) is isolated in B95a but not in Vero cells. Through an adaptation process of wild-type MV to Vero cells, several amino acid substitutions were reported. ***Methods:*** Six strains were adapted to Vero cells and membrane (M), fusion (F) and hemagglutinin (H) genes were sequenced. Cell fusion was assessed and recombinant MVs were constructed, having wild-type H or M gene with or without mutations. ***Results:*** No F gene substitution was noted. Amino-acid substitutions at positions 481 from Asn to Tyr (N481Y) and 546 from Ser to Gly (S546G) were observed in the H protein. Glu at position 89 of the M protein was substituted for Gly (E89G) and two mutations were noted at positions 62 (S62R) and 83 (S83P) in M protein. Recombinant viruses with mutation(s) detected in Vero-adapted strains induced a cytopathic effect and grew well in Vero cells, but those with the wild type did not. Recombinant viruses with mutation(s) demonstrated lower viral growth in B95a cells. ***Conclusions:*** Substitutions of E89G, S62R and S83P of the M protein were newly observed through adaptation to Vero cells, besides the mutations described in previous reports, with varying adaptation for each strain.

Introduction

Measles virus (MV) is a member of the genus *Morbillivirus*, family Paramyxoviridae, order Mononegavirales, characterized by non-segmented, negative-strand RNA virus. The genome consists of 15,894 nucleotides, coding six structural proteins: nucleoprotein (N), phospho (P), membrane (M), fusion (F), hemagglutinin (H) and large (L) proteins. The genomic RNA is encapsidated with N protein and consisted of ribonucleoprotein, together with RNA-dependent RNA polymerase complex of L and P proteins [1, 2]. MV has two envelop glycoproteins, F and H, which play an important role in virus attachment and subsequent virus cell fusion as well as cell-to-cell fusion [3–5]. The Edmonston strain was isolated in 1954 using a primary culture of human kidney cells and, thereafter, MV was isolated using Vero cells, but the sensitivity of Vero cells was poor, and two or three blind passages were required. MV was isolated more efficiently in B95a cells, marmoset lymphoblastic cell lines transformed by Epstein-Barr virus [6]. CD46 was initially reported to act as a cellular receptor for laboratory-adapted, vaccine strains of MV. CD46 is a member of the regulators of the complement activation gene cluster and is widely expressed on epithelial cells, but not on lymphocytic cells [7, 8]. In 2000, human signaling lymphocyte activation molecule (SLAM; CD150) was reported as the receptor of wild-type MV and it was expressed on lymphocytic cells [9, 10]. The

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Edmonston strain and relevant vaccine strains were found to use both SLAM and CD46 as receptors and circulating wild-type strains utilize SLAM as a receptor, but not CD46 [9, 10].

Several amino-acid changes in H, M, L and P proteins and/or accessory V and C proteins were responsible for attenuation through adaptation to Vero cells. Some authors reported that the majority of MV strains using CD46 as a receptor have tyrosine at position 481, whereas wild-type strains have asparagine at this position [11–17]. When the wild-type MV strains became adapted to grow in Vero cells, the substitution at position 481 of H protein from asparagine to tyrosine (N481Y) was often observed after several passages [16, 18]. In some Vero cell-adapted strains, a substitution at position 546 of the H protein from serine to glycine (S546G) was observed instead of the N481Y substitution [19–22]. A single substitution of N481Y or S546G enabled the H protein of wild-type MV strains to utilize CD46, without influencing their ability to use SLAM. In addition, two amino-acid differences were observed in Edmonston-derived strains in comparison with wild-type strains at positions 64 and 89 of M protein (P64S and E89K), which allowed an interaction of M protein with the cytoplasmic tail of H protein, thereby enhancing cell fusion and assembly of infectious particles in Vero cells [23–25].

In this report, amino-acid substitutions were investigated in M, F and H proteins of Vero-adapted strains from six wild-type MV strains isolated in B95a cells, in comparison with the original wild types. Mutations of N481Y or S546G of the H protein region were observed as previously reported. In addition, mutations in S62R, S83P and E89G of the M protein region were noted, being different from the mutations described in previous reports. Recombinant MV strains with mutations in the H and M genes were constructed to conduct a functional analysis of the mutations.

Materials and Methods

Cells and Viruses

Six strains of the wild type were used in this study.

MVi/Tokyo.JPN/17.07-AN/B4, MVi/Mie.JPN/19.07-OY/B4, MVi/Mie.JPN/23.07-TY/B3, MVi/Mie.JPN/41.07-MA/B3 and MVi/Mie.JPN/03.08-KU/B4 were genotype D5 isolated in 2007/2008 outbreaks in Japan, using B95a cells after three or four passages. MVi/Aichi.JPN/44.06/B3 was genotype D9 [26]. Through several passages in Vero cells, eight Vero-adapted strains were obtained. AN-V4 was obtained after four passages of MVi/Tokyo.JPN/17.07-AN/B4 in Vero cells. OY-V4 and OY-V22 were obtained after four and 22 passages of MVi/Mie.JPN/19.07-OY/B4 in Vero cells, respectively. TY-V4, TY-V22, MA-V15, KU-V4 and D9-V4 strains were obtained after passages of respective strains in Vero cells. MVAT7 pol., non-replicative vaccinia virus expressing T7 RNA polymerase (a kind gift from Dr. G. Sutter), was used for fusion analysis and the recovery of infectious viruses.

B95a cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), and Vero cells in MEM supplemented with 5% FCS. 293 T cells were cultured in MEM supplemented with 10% FCS.

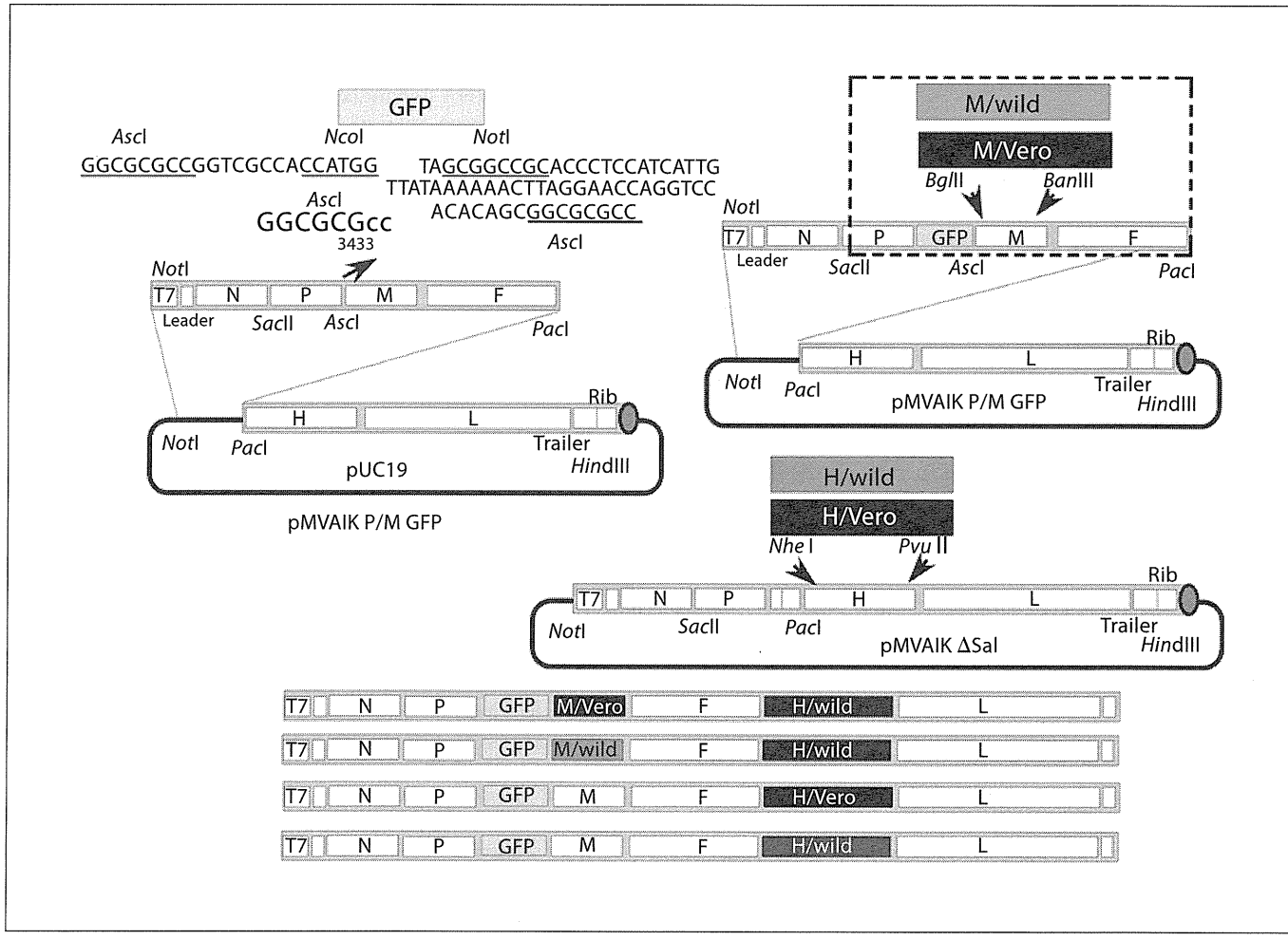
Construction of F and H Expression Plasmids and Fusion Experiment

The H protein expression plasmids were constructed from Vero-adapted strains (AN-V4, OY-V4, OY-V22, TY-V22, MA-V15, KU-V4 and D9-V4 strains) and their original wild-type isolates and AIK-C strain. The H gene was amplified by RT-PCR using the set of primers H-ATG (5′-GTTGAATTCATGTCACCACAACGAGACCGGA-3′) and H-TAG (5′-AATGCGGCCGCCTATCTGCGATTGGTTCCA-3′), containing restriction enzyme sites of *Eco*RI and *Not*I (underlined). The amplified DNA fragments were cloned into multicloning sites located downstream of the T7 promoter of pBluescript SK II–. Several clones were sequenced to analyze the frequency of mutations. Constructed H expression plasmid (0.2 μg) was co-transfected together with the AIK-C F expression plasmid into a monolayer of B95a or Vero cells infected with recombinant vaccinia virus, MVAT7 pol., using Mirus Superfect III (Invitrogen Life Technologies, Carlsbad, Calif., USA) [27]. Cells were fixed with cooled acetone and further subjected to indirect immunofluorescent (IF) staining.

Construction of Recombinant MV Strains with Mutations

The infectious recombinant MV strains were recovered from the infectious cDNA clone based on the AIK-C measles vaccine strain, expressing wild-type measles H protein cloned from the wild-type MV [27, 28], and the recombinant infectious cDNA constructions are shown in figure 1. Briefly, full-length cDNA of the AIK-C strain was divided into two parts: the first half consisted of AIK-C cDNA from the leader to the *Pac*I site at nucleotide position 7238 of the AIK-C genome, and the second half of the AIK-C cDNA consisted of H and L regions from the 7239 *Pac*I site to the trailer sequence. The *Asc*I site (GGCGCGCC) was artificially introduced by adding a GGCGCG sequence upstream of the genome position 3433 in the P/M junction, and R1 and R2 sequences were added. The green fluorescent protein (GFP) sequence was inserted using *Nco*I and *Not*I restriction enzyme sites in accordance with the rule of six of the genome length [29, 30], designated as pMVAIK P/M-GFP. The H gene of the wild-type or Vero-adapted strains was cloned at *Nhe*I (genome position 7426) and *Pvu*II (genome position 9082) of the second half of the AIK-C cDNA and combined with the first half of the cDNA. The M gene of the wild-type or Vero-adapted strains was cloned at *Bgl*II (genome position 3445) and *Ban*III (genome position 4312) sites of the first half of the AIK-C cDNA clone and combined with the second half to construct full-length cDNA.

For the recovery of the recombinant MV, 293 T cells were infected with MVAT7 pol., and then 0.5 μg of pAIK-N, 0.25 μg of pAIK-P, 0.1 μg of pAIK-L and 1.5 μg of full-length recombinant cDNA, using Mirus Superfect III (Invitrogen Life Technologies) modified from previous reports [27, 28]. After 2 days of culture,



Color version available online

Fig. 1. Construction of recombinant MV. Full-length AIK-C cDNA was divided into two parts at the *Pac*I restriction enzyme site. The H genes of the wild-type and Vero-adapted strains were amplified and cloned using *Nhe*I and *Pvu*II restriction enzyme sites. The M gene was inserted at *Bgl*II and *Ban*III restriction enzyme sites. The *Asc*I site (GGCGCGCC) was artificially introduced by adding a GGCGCG sequence upstream of the genome position 3433 in the P/M junction, and R1 and R2 sequences were added. The GFP sequence was inserted at the P/M junction.

293 T cells were co-cultured with B95a cells. Infectious virus particles were rescued through two blind passages in B95a cells at 32.5° in 5% CO_2.

Sequence Analysis

The M, F and H genes of the wild-type isolates and Vero-adapted strains were amplified by RT-PCR and sequenced by dye terminator methods using ABI 3130 (Applied Bio Systems Japan, Tokyo). Consensus sequence was defined as the dominant sequence determined by direct sequence and respective gene cloning.

Indirect IF Staining and GFP Expression

B95a or Vero cells were cultured in 8-well LabTek Glass slides (Nalge Nunc International, Rochester, N.Y., USA) and infected with recombinant MV strains. GFP expression was confirmed and cells were fixed with cooled acetone and further subjected to indirect IF staining using 1:100 dilution of monoclonal antibody against measles H protein (kindly supplied by Dr. T. A. Sato, National Institutes for Infectious Diseases) and that against N protein (Chemicon, Temecula, Calif., USA). They were stained with 1:100 dilution of anti-mouse IgG monoclonal antibody labeled with FITC (Sigma-Aldrich, St. Louis, Mo., USA).

Virus Growth and GFP Expression

B95a or Vero cells were infected with recombinant MV strains at MOI = 0.01 and culture supernatant was obtained on days 1, 3, 5 and 7 of culture. Virus infectivity was calculated by the Reed-Muench method using B95a cells, and GFP expression was monitored with a microplate fluorescent reader, FLx 800 (Bio-Tek Instruments, Winooski, Vt., USA).

Table 1. Amino-acid substitutions of wild-type isolates after adaptation to Vero cells

MV strains	Genotype	Passage in Vero cells	H original		M original		
			481Asn	546Ser	62Ser	83Ser	89Glu
MVi/Tokyo.JPN/17.07-AN/B4	D5	AN-V4	–[1]	Gly	–	–	–
MVi/Mie.JPN/19.07-OY/B4	D5	OY-V4	Tyr	–	–	–	–
MVi/Mie.JPN/19.07-OY/B4	D5	OY-V22	Tyr	Gly	Arg	Pro	–
MVi/Mie.JPN/23.07-TY/B3	D5	TY-V4	–	–	–	–	–
MVi/Mie.JPN/23.07-TY/B3	D5	TY-V22	–	Gly	–	–	–
MVi/Mie.JPN/41.07-MA/B3	D5	MA-V15	Tyr	–	–	–	–
MVi/Mie.JPN/03.08-KU/B4	D5	KU-V4	–	Gly	–	–	–
MVi/Aichi.JPN/44.06/B3	D9	D9-V4	–	–	–	–	Gly

AN-V4 was obtained after four passages of MV/Tokyo.JPN/17.07-AN/B4 in Vero cells. H original/M original = Amino acids of the original strain.

[1] No amino-acid substitution after adaptation to Vero cells.

Table 2. Sequence diversity of M and H protein-encoding plasmids derived from Vero-adapted virus strains

	H gene				M gene				
	N481Y	S546G	481/546	H-wt	S62R	S83P	62/83	E89G	M-wt
OY-V4	4	0	0	6					
OY-V22	3	6	1	2	2	0	22		0
TY-V22	0	6	0	1					
MA-V15	6	0	0	0					
KU-V14	0	7	0	0					
D9-V4								4	2

Results

Amino-Acid Substitutions of MV for Adaptation to Vero Cells

Eight Vero-adapted strains belonging to the D5 and D9 genotypes were obtained after 4–22 passages: AN-V4, OY-V4, OY-V22, TY-V4, TY-V22, MA-V15 and KU-V4 from five wild-type D5 strains and D9-V4 after four passages of wild-type D9 strain. Original wild-type strains did not show any cell fusion in Vero cells and eight Vero-adapted strains were obtained, demonstrating syncytia formation. No mutation was noted in the F gene in the strains studied and amino-acid substitutions in the M and H proteins are shown in table 1. Regarding the H gene of the Vero-adapted strains, an amino-acid substitution at position 481 from Asn to Tyr (N481Y) was noted in OY-V4 and MA-V15. An amino-acid substitution at position 546 from Ser to Gly (S546G) was observed in AN-V4, TY-V22 and KU-V4. OY-V22 showed substitutions at positions 481 and 546. No mutation was observed for TY-V4 and D9-V4. With respect to the M gene, substitutions were observed at position 62 of the M protein from Ser to Arg (S62R) and at position 83 from Ser to Pro (S83P) of OY-V22, and at position 89 from Glu to Gly (E89G) of D9-V4. The mutation site(s) for adaptation to Vero cells was different from strain to strain.

For each Vero-adapted MV strain, H and M expression plasmids were constructed, and the results of sequence analyses are shown in table 2. Among ten H expression plasmids derived from the OY-V4 strain, four had N481Y and the remaining six were wild type. Among 12 H expression plasmids of OY-V22, three had N481Y substitution, six had S546G, one had both N481Y and S546G, and the remaining two were the original wild type. Six H expression plasmids of MA-V15 showed N481Y substitution. Six of seven plasmids derived from

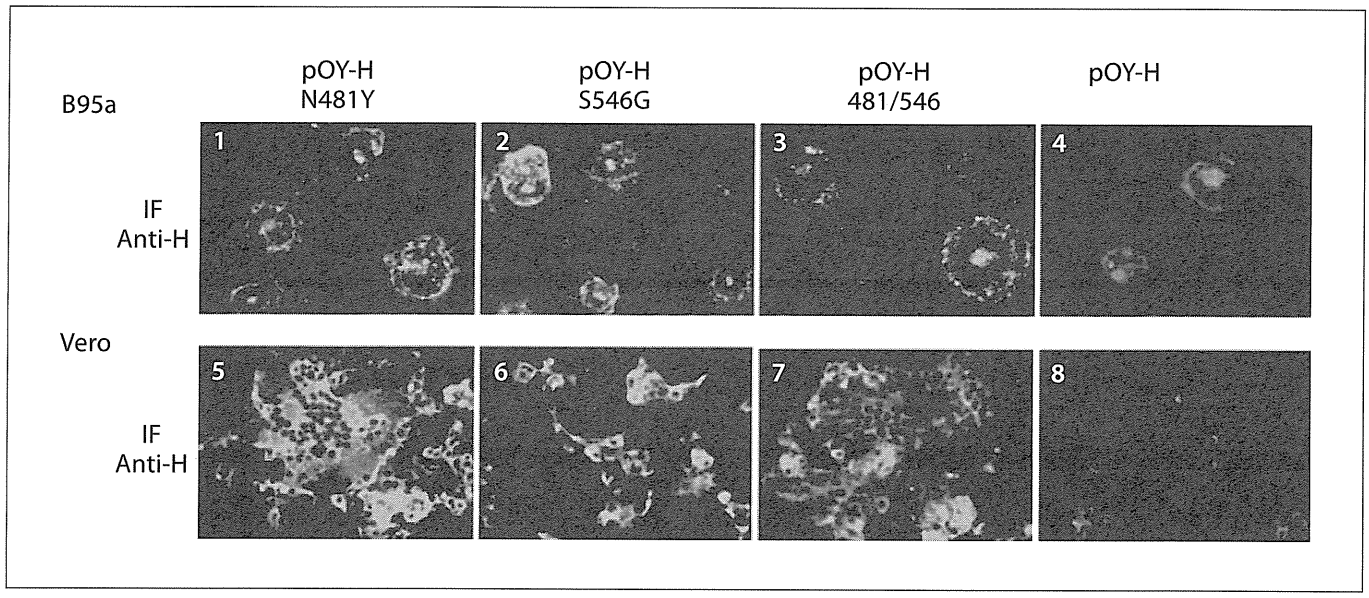


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Fig. 2. Fusion experiment involving H protein expression plasmids. The H protein expression plasmids were constructed: pOY-H was constructed from MVi/Mie.JPN/19.07, pOY-H N481Y, pOY-H S546G and pOY-H 481/546 from OY-V22 (Vero-adapted strain from MVi/Mie.JPN/19.07-OY). They were co-transfected with the F expression plasmid of the AIK-C in B95a cells (upper panels) and Vero cells (lower panels). The appearance of cell fusion and the results of IF using monoclonal antibody against H protein.

TY-V22 and all seven from KU-V4 showed S546G substitution. For the M gene, substitutions of S62R and S83P were observed in OY-V22 and that of E89G in D9-V4. Among 24 M gene clones derived from OY-V22, there were two clones with S62R and the remaining 22 had both S62R and S83P substitutions. As for the D9-V4 strain, four plasmids out of six M gene clones showed E89G substitution.

Expression Experiments Involving F and H Proteins

Through cloning experiments, the H protein expression plasmid was constructed from the original MVi/Mie.JPN/19.07-OY strain (pOY-H), and three expression plasmids with mutation(s) derived from OY-V22 (Vero-adapted strain of MVi/Mie.JPN/19.07-OY strain) were constructed: pOY-H N481Y (with an amino-acid substitution at position 481 of H protein), pOY-H S546G (with an amino-acid substitution at position 546) and pOY-H 481/546 (with two substitutions). pAIK-F was used as an F expression partner constructed from the AIK-C vaccine strain. The H expression plasmids were co-transfected with pAIK-F as the F expression partner in B95a or Vero cells, and the results of indirect IF staining are shown in figure 2. They induced a similar level of cell fusion in B95a cells, but the original wild-type pOY-H did not induce cell fusion in Vero cells (panel 8). The plasmids with an amino-acid substitution of N481Y (pOY-H N481Y) or S546G (pOY-H S546G) induced cell fusion in both B95a and Vero cells, and plasmid with both N481Y and S546G substitutions (pOY-H 481/546) induced more prominent cell fusion in Vero cells (panel 7).

Construction of Recombinant MV Strains with H Mutations

Recombinant cDNAs having wild-type original H gene of MVi/Mie.JPN/19.07-OY and mutated H gene from the Vero-adapted strains (OY-V22) were constructed based upon AIK-C cDNA, and infectious viruses were recovered: rMV-OY H without amino-acid substitution, rMV-OY H N481Y with amino-acid substitution of N481Y, rMV-OY H S546G with amino-acid substitution of S546G and rMV-OY H 481/546 with both substitutions. These recombinant MV strains were designed to express GFP, and the expression of GFP and IF staining against N protein are shown in figure 3. rMV-OY H did not show cell fusion in Vero cells similar to the original wild-type MVi/Mie.JPN/19.07-OY (panels 13, 18), but the Vero-adapted strain OY-V22 induced cell fusion in Vero as well as B95a cells (panels 5, 14). rMV-OY H N481Y and rMV-OY H S546G induced cell fusion in Vero cells to a

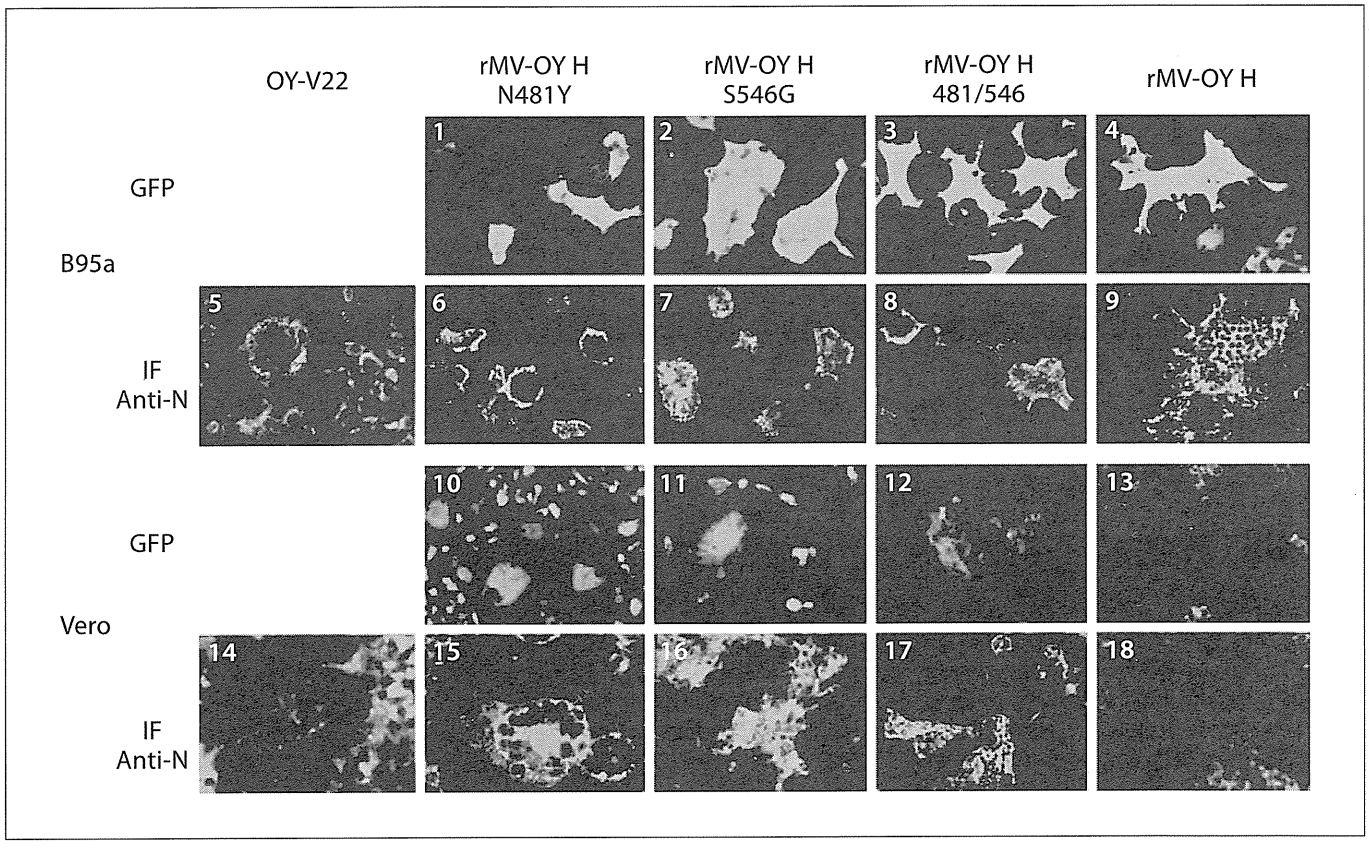

Fig. 3. GFP expression and IF staining of B95a and Vero cells infected with recombinant MV strains with substitutions of H protein. OY-V22 was a Vero-adapted strain after 22 passages in Vero cells. Four recombinant MV strains were constructed: rMV-OY H had no mutation, and rMV-OY H N481Y, rMV-OY H S546G and rMV-OY H 481/546 had mutations at the respective positions. They were used to infect B95a and Vero cells, and the results of GFP expression and IF using a monoclonal antibody against measles N protein are shown.

similar extent, and rMV-OY H 481/546 led to slightly more extensive fusion in Vero cells.

Construction of Recombinant MV Strains with M Gene Mutations of OY-V22

Two amino-acid substitutions were noted in the M gene of the OY-V22 strains, S62R and S83P. The AIK-C vaccine strain has Tyr (Y) at position 481 of the H region, and mutation was introduced to generate Asn (N) at 481 (rMVAIK H481N). The M gene of the AIK-C was replaced by that of OY-V22 in the pMVAIK H481N cDNA, and two recombinant MV strains were recovered: rMVAIK H481N/M S62R and rMVAIK H481N/M 62/83. These M gene mutations were introduced into the cDNA of rMV-OY H and rMV-OY H/M S62R and rMV-OY H/M 62/83 were recovered. GFP expression and expression of measles N protein are shown in figure 4. rMVAIK H481N did not induce cell fusion in Vero cells (panels 11, 16), but rMVAIK H481N/M S62R and rMVAIK H481N/M 62/83 strains induced cell fusion (panels 12, 13, 17, 18). rMV-OY H/M S62R and rMV-OY H/M 62/83 strains induced cell fusion in Vero cells (panels 14, 15, 19, 20).

Virus Growth of Recombinant MV Strains with H and M Gene Mutations

All recombinant MV strains were designed to express GFP and virus growth was monitored by GFP expression, measured as fluorescence units (FU). The cell-free infectious virus titer was examined on days 1, 3, 5 and 7 after infection. The results of virus growth of recombinant MV strains with mutations in the H gene are shown in figure 5. Baseline GFP expression was <400 FU. rMV-OY H (Hwt) failed to produce syncytia in Vero cells without GFP expression, similar to the fusion experiment using

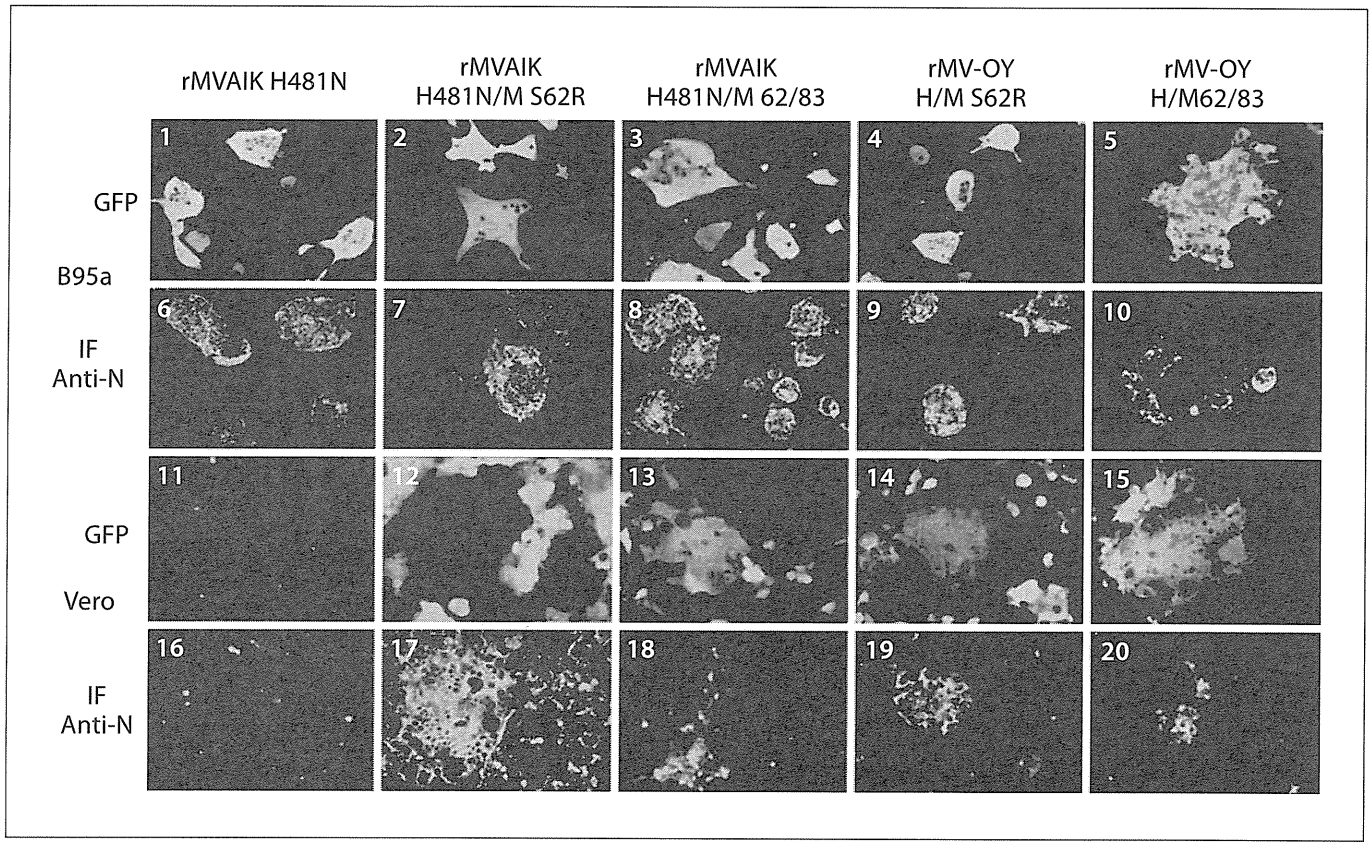

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Fig. 4. GFP expression and IF staining of B95a and Vero cells infected with recombinant MV strains with M protein substitutions. The M gene of AIK-C was replaced with that obtained from OY-V22 with mutation of S62R, S62R/S83P, and the H gene was also replaced by OY-H wild-type (rMV-OY H/M S62R, rMV-OY H/M 62/83). The M gene mutations were also introduced into rMVAIK H481N, rMVAIK H481N/M S62R and rMVAIK H481N/M 62/83. The results of GFP expression and IF using a monoclonal antibody against measles N protein are shown.

the H expression plasmids, and no infectious virus was observed in the supernatants. rMV-OY H 481/546 (H481/546) grew better on day 5 or 7 after infection with a high infectious titer of 10^5 TCD_{50} and high GFP expression over 2,500 FU in Vero cells, and induced more marked fusion in Vero cells than in the other recombinant MV strains with N481Y or S546G substitution. rMV-OY H S546G (H546) induced higher GFP expression than rMV-OY H N481Y (H481), but there were no significant differences in the production of infectious virus particles. In B95a cells, four recombinant MV strains demonstrated similar infectious virus production, but the rMV-OY H S546G strain led to a lower expression of GFP than rMV-OY H, rMV-OY H481N and rMV-OY H 481/546 (fig. 5).

GFP expression and the production of infectious virus in Vero and B95a cells infected with recombinant MV strains with M gene mutation(s) are shown in figure 6. M gene mutation(s) was introduced into rMVAIK-H481N or rMV OY-H. rMV OY-H (Hwt) did not express GFP and no infectious virus particle was produced in Vero cells. rMV-OY H/M S62R (M62/Hwt) and rMV-OY H/M 62/83 (M62/83/Hwt) induced GFP expression, and an infectious virus titer of $10^{2.0-3.0}$ TCD_{50} was obtained in the supernatants on day 7 of Vero cell culture. rMV-OY H induced extensive cell fusion in B95a cells with higher level of GFP expression and particle formation, but four other recombinant MV strains with M gene mutation(s) induced lower GFP expression with a lower production of infectious particles. Although recombinant MV stains with mutated M protein produced cell fusion in Vero cells, they induced lower cell fusion with lower numbers of fusion foci in comparison with those produced in B95a cells.

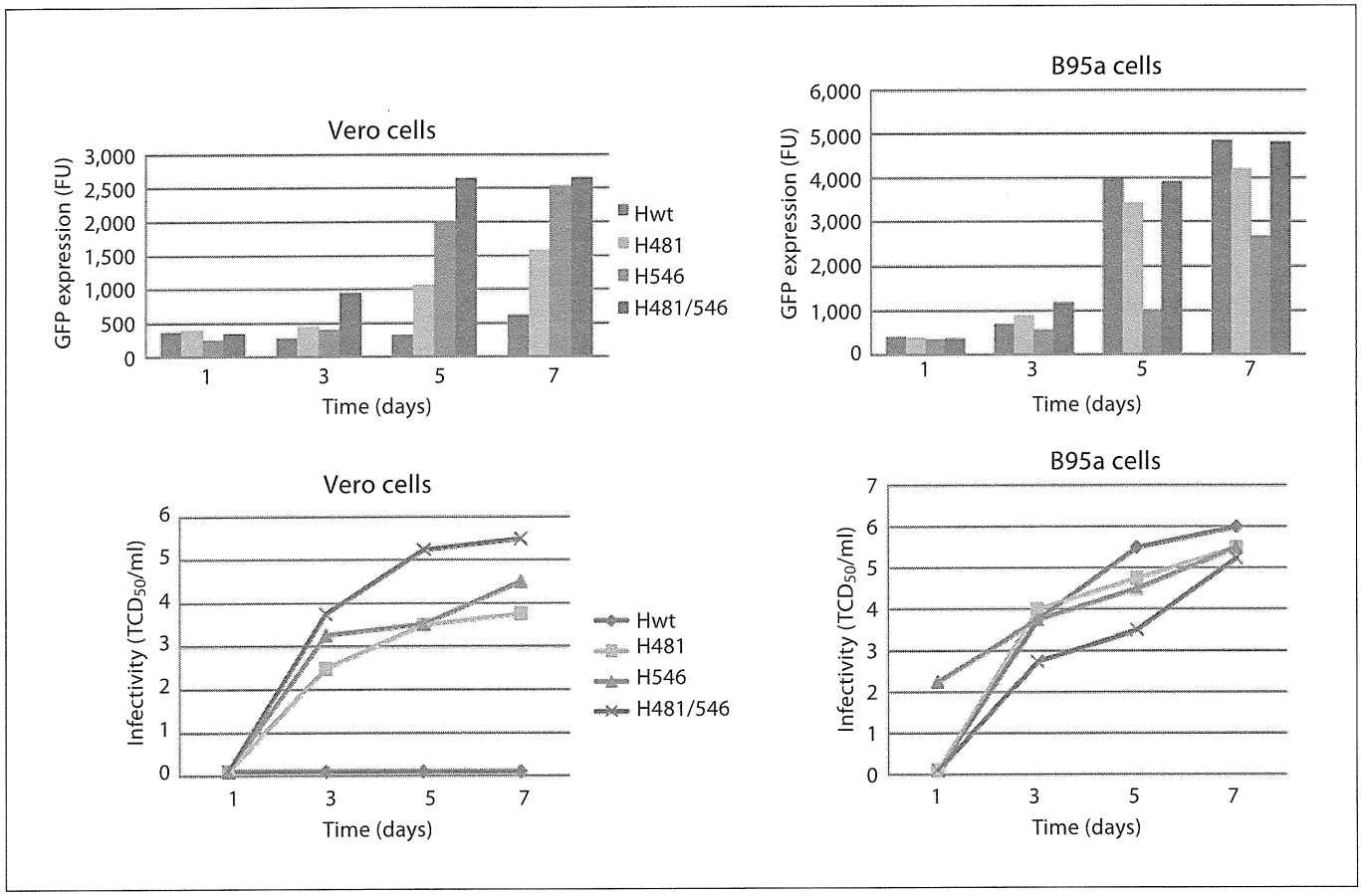

Fig. 5. GFP expression and virus growth of recombinant MV strains with substitution of H protein. Vero and B95a cells were infected with rMV-OY H (Hwt), pOY-H N481Y (H481), pOY-H S546G (H546) and pOY-H 481/546 (H481/546) strains at MOI = 0.01. GFP expression was monitored on days 1, 3, 5 and 7 after infection, and infectivity in culture supernatants was assayed (baseline GFP expression: 200–300 FU).

Recombinant MV Strains Derived from D9 Strain

D9-V4 was a mixture of M gene mutation. Four clones showed mutation at position 89 of the M gene from Glu to Gly, and the remaining two clones showed no mutation. The H and M genes of AIK-C cDNA were replaced with those amplified from the D9-V4 strain. rMV-D9 H/M has the original wild-type H and M genes, and rMV-D9 H/M E89G has wild-type H gene and E89G mutation of the M gene, similar to the D9-V4 strain. The results of cell fusion, GFP expression and infectivity in culture fluids are shown in figure 7. Two recombinant MV strains showed similar cell fusion in B95a cells, with similar infectious virus production. Whereas rMV-D9 H/M E89G induced cell fusion and produced infectious virus particles ($10^{3.1}$ TCD_{50}/ml) in Vero cells, rMV-D9 H/M did not induce cell fusion and showed no virus growth in Vero cells.

Discussion

MV induces extensive syncytium formation with cell fusion, and the appearance of a syncytium is a positive indicator of virus isolation. Binding of the H protein to the receptor induces the conformational changes of H and F proteins required for the protrusion of the fusion domain into lipid bilayers of the cell membrane [3–5]. At present, two MV receptors have been identified: CD46 and SLAM (CD150). CD46 is expressed widely on the surface of epithelial cells, including Vero cells, whereas CD150 is a lymphocyte-stimulating factor expressed on the surface of lymphoreticular cells [10, 31]. In the past, MV was isolated after three or more blind passages in Vero cells, and the isolation rate was low. Otherwise, current wild-type MV strains were isolated in B95a cells. The

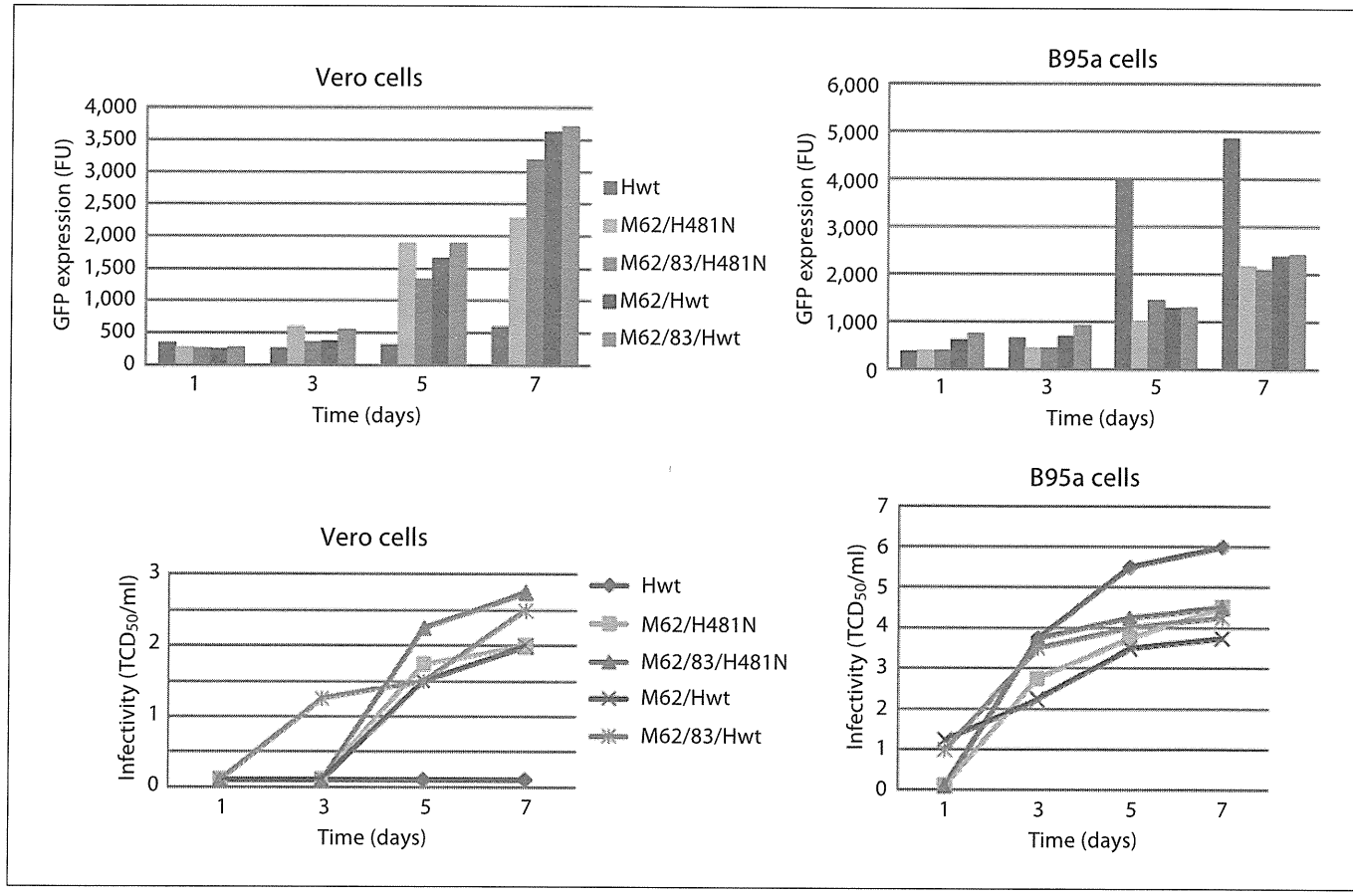

Fig. 6. GFP expression and virus growth of recombinant MV strains with substitution of M protein. Vero and B95a cells were infected with rMV-OY H (Hwt), rMVAIK H481N/M S62R (M62/H481), rMVAIK H481N/M 62/83 (M62/83/H481N), rMV-OY H/M S62R (M62/Hwt) and rMV-OY H/M 62/83 (M62/83/Hwt). GFP expression was monitored on days 1, 3, 5 and 7 after infection, and infectivity in culture supernatants was assayed (baseline GFP expression: 200–300 FU).

adaptation of the current wild MV to Vero cells led to amino-acid substitution(s) in the H gene that increased the binding capacity of the measles H protein to CD46 [19]. Lecouturier et al. [12] reported that substitutions of two amino-acid positions of 451 and 481 in H protein of the Halle strain abrogated the fusion inducibility of the functional domain(s) of the measles H protein. Hsu et al. [14] reported that a single amino-acid change at position 481 determined the ability of H protein to bind CD46. Xie et al. [15] reported that Asn at position H481 of the wild-type expression plasmid was replaced by various amino acids, and the mutant plasmid with Tyr, similar to the Edmonston strain, induced cell fusion, but this substitution did not cause the down-regulation of CD46 expression, unlike the Edmonston strain. Thus, Tyr at position 481 was indispensable for measles H protein to interact with CD46, similar to the other reports [16, 21]. A single substitution of N481Y of the wild H protein was not sufficient to use CD46, suggesting that further substitutions were required for efficient virus growth in Vero cells [22]. From the comparison of the H gene of the Edmonston and current circulating strains, three substitutions (N309I and E492G, plus N416D or T446S) were necessary for efficient virus growth in Vero cells [32]. Li and Qi [20] examined the amino-acid substitutions of MV H protein when three hemadsorption-negative strains were passaged >20 times in Vero cells. They reported that amino-acid substitution at position 546 of the measles H protein from Ser to Gly was critical for hemadsorption and CD46 binding besides the amino-acid change at position 481. In the three-dimensional surface representation of the structural model, three of these residues (D505, D507 and

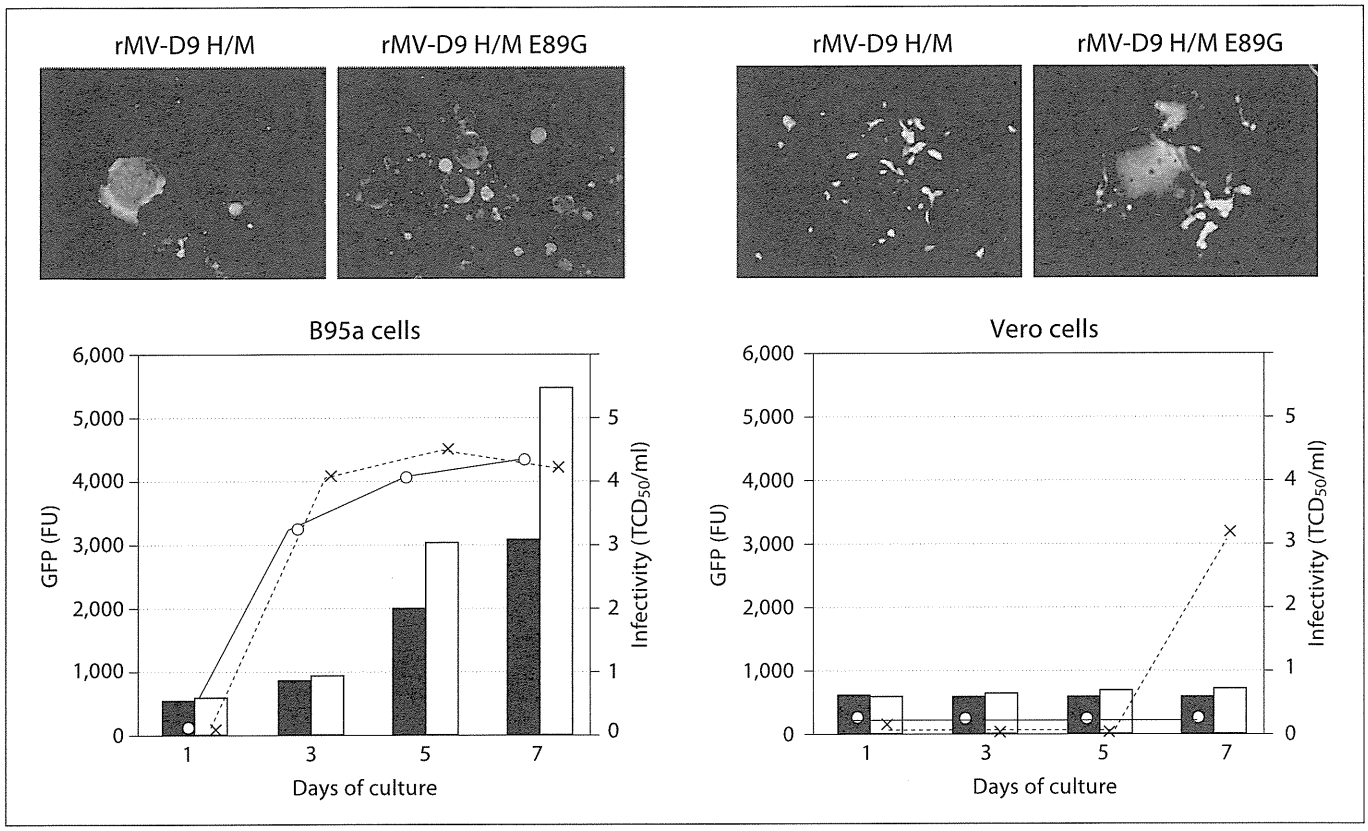

Fig. 7. Characteristics of recombinant MV strains with a mutation in M protein observed in the D9-V4 Vero-adapted strain. The M and H genes of AIK-C were replaced with those of MV/Aichi.JPN/44.06 (rMV-D9 H/M), and the mutated M gene was introduced (rMV-D9 H/M E89G). GFP expression is shown in the upper panels. Vero and B95a cells were infected, and GFP expression (■; □) and virus infectivity (——; - - -) were monitored. ——○——/■ = rMV-D9 H/M; - - × - -/□ = rMV-D9 H/M E89G.

R533) align along the rim on one side of the cavity on the top surface of the measles H globular head, and form the basis of a single continuous site that overlaps with the 546-548-549 CD46 binding site. Mutations at position 481 or 546 induce conformational changes in the measles hemagglutinin globular head and influence the affinity for CD46 binding [33]. The MV H protein three-dimensional approach suggested that the SLAM- and CD46-relevant residues are located in contiguous areas in propeller β sheets 5 and 4, respectively, and several CD46-relevant amino acids may be shielded from direct receptor contact [34–36]. Using the Edmonston H protein, the association rate for SLAM binding to H protein was very low; about 20 times lower than CD46. However, SLAM bound to H protein more tightly than CD46, as revealed by a 5-fold lower dissociation rate [37].

The Vero-adapted MV strains showed several amino-acid changes in the other genes: two in the P, V and C proteins, three in the H gene (Ala H14 Gly, Leu H423 Pro and Ser H546 Gly) and two in the L gene [13]. Only two nucleotide differences were reported at 2331 genome position of P/V/C and at the 3628 genome position (amino acid position 64 of M protein), and none in the H gene between the MV genome isolated in B95a cells and that isolated in Vero cells from the same patient [38]. Through comparative studies of the M gene sequence of wild-type and Edmonston strains, substitutions of P64S and E89K from wild-type M protein were reported to be responsible for the fusion inducibility and efficient virus growth in Vero cells [23]. These two substitutions (P64S and E89K) allowed the interaction of the M protein with the cytoplasmic tail of H protein, thereby enhancing cell fusion and the assembly of infectious particles in Vero cells [24]. M protein also had binding activity to the cytoplasmic domain of H protein together with F protein and ribonucleoprotein complex, and was transported to the mem-

brane raft fraction [25, 39, 40]. In this report, no substitution was noted in the F protein for adaptation to Vero cells but substitutions were reported at positions 439 and 464 of the F protein, as well as those in the N, P/V/C, H and L proteins after adaptation to Vero cells [41].

In this report, cell fusion was observed after four passages of MVi/Mie.JPN/3.07-TY/B3 (TY-4 strain) in Vero cells but TY-4 had no amino-acid substitution in the M, F and H proteins. There was a possibility of mutation(s) in the P and/or L genes and, after 22 passages, substitution of H546G was detected. Amino-acid substitutions for adaptation to Vero cells were different from strain to strain. All D5 genotype strains showed a substitution at position 481 or 546 of the H protein region at an early stage of passage. These were mutated through the adaptation process, and no substitution except for those at these positions was observed on direct sequencing analysis. Thus, the substitutions of N481Y or S546G were essential for adaptation to Vero cells, and the other regions were not changeable. OY-V4 showed a mixed population of N481Y and original wild-type clones, whereas OY-V22 was a mixture of the N481Y substitution, S546G, substitutions of both N481Y and S546G, and the original sequence. Through the results of expression experiments involving H plasmids and GFP expression of recombinant MV strains, rMV-OY H 481/546 induced more extensive cell fusion in Vero cells than MV strains with either of the substitutions. Double mutants were predicted to use CD46 more efficiently, leading to efficient infectious virus production and growth in Vero cells.

Further repeated passages in Vero cells accumulated mutations in the M gene in addition to the H gene. Two strains adapted to Vero cells showed substitutions in M protein. Substitution of E89G was observed in D9-V4, and those of S62R and S83P in OY-V22. rMV-OY H/M S62R and rMV-OY H/M 62/83 strains induced cell fusion in Vero cells. These recombinant MV strains decreased virus growth and particle formation in B95a cells and the single substitution of S62R was sufficient. These positions were different from those in previous reports, demonstrating the fusogenicity of combined substitutions of P64S and E89K in Vero cells [23, 24, 32]. The backbone of our reverse genetics is the AIK-C vaccine strain and rMV H481N and rMV-OY H have K at position 89 of M protein, but these two strains did not induce cell fusion without infectious virus production. Thus, the single mutation of E89K would not be a critical region for interaction between M and H proteins for efficient virus growth in Vero cells. Substitution of E89G of the M protein was observed in D9-V4. rMV-D9 H/M (E at position 89 of M protein) did not induce cell fusion, with no virus growth, but GFP was demonstrated in cells without fusion. rMV-D9 H/M E89G induced a small fusion with a low level of infectious virus production, even though it had wild-type H protein. This may suggest the presence of another unidentified receptor for MV and, recently, the possibility of a molecule related to tight junctions on the basolateral sides of epithelial cells was reported [42, 43].

Adaptation would occur in a different manner depending on the strains or experimental condition. Three strains showed mutations in the H gene within four passages, and repeated passages added additional mutations to the M and H genes. One strain of D9 showed a substitution in the M protein, even without H protein substitution at N481Y or S546G which enables the virus to enter cells efficiently. Thus, we supposed that the amino acids at positions 481 and 546 of H protein are critical for the different tropisms based on the results of expression experiments. From the results of recombinant MV strains with M protein mutations, substitution in the M protein promoted efficient MV growth and particle formation in Vero cells, and would influence efficient receptor usage of the wild-type H protein to induce cell fusion irrespective of H gene mutation.

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The SI Strain of Measles Virus Derived from a Patient with Subacute Sclerosing Panencephalitis Possesses Typical Genome Alterations and Unique Amino Acid Changes That Modulate Receptor Specificity and Reduce Membrane Fusion Activity▽‡

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Subacute sclerosing panencephalitis (SSPE) is a fatal sequela associated with measles and is caused by persistent infection of the brain with measles virus (MV). The SI strain was isolated in 1976 from a patient with SSPE and shows neurovirulence in animals. Genome nucleotide sequence analyses showed that the SI strain genome possesses typical genome alterations for SSPE-derived strains, namely, accumulated amino acid substitutions in the M protein and cytoplasmic tail truncation of the F protein. Through the establishment of an efficient reverse genetics system, a recombinant SI strain expressing a green fluorescent protein (rSI-AcGFP) was generated. The infection of various cell types with rSI-AcGFP was evaluated by fluorescence microscopy. rSI-AcGFP exhibited limited syncytium-forming activity and spread poorly in cells. Analyses using a recombinant MV possessing a chimeric genome between those of the SI strain and a wild-type MV strain indicated that the membrane-associated protein genes (M, F, and H) were responsible for the altered growth phenotype of the SI strain. Functional analyses of viral glycoproteins showed that the F protein of the SI strain exhibited reduced fusion activity because of an E300G substitution and that the H protein of the SI strain used CD46 efficiently but used the original MV receptors on immune and epithelial cells poorly because of L482F, S546G, and F555L substitutions. The data obtained in the present study provide a new platform for analyses of SSPE-derived strains as well as a clear example of an SSPE-derived strain that exhibits altered receptor specificity and limited fusion activity.

Measles is an acute highly contagious disease characterized by high fever and a maculopapular rash. Acute measles is accompanied by temporary and severe immunosuppression, and pneumonia caused by secondary bacterial infections is a major cause of measles-related death in children. Subacute sclerosing panencephalitis (SSPE) is a fatal sequela associated with measles. It occurs at a mean latency period of 7 to 10 years after the acute measles stage of development (3, 52). SSPE is caused by persistent infection of the central nervous system (CNS) with measles virus (MV), and suffering from acute measles at an early age is a risk factor for developing SSPE (17). A recent analysis indicated that the risk of developing SSPE was 22 cases per 100,000 reported cases of acute measles (3).

The causative agent, MV, is an enveloped virus that belongs to the genus *Morbillivirus* in the family *Paramyxoviridae*. MV possesses a nonsegmented, negative-sense RNA genome that includes six linked tandem genes, N, P/V/C, M, F, H, and L. The genome is encapsidated by the nucleocapsid (N) protein and is associated with a viral RNA-dependent RNA polymerase composed of phosphoproteins (P proteins) and large proteins (L proteins) that form a ribonucleoprotein (RNP) complex (12). Two types of glycoprotein spikes, the hemagglutinin (H) and fusion (F) proteins, are expressed on the viral envelope. The H protein is responsible for binding to cellular receptors on the target host cells. The signaling lymphocyte activation molecule (SLAM) expressed on immune system cells functions as the principal receptor for MV (62, 69). We and another group recently demonstrated that certain epithelial cells that form tight junctions express an unidentified receptor for MV that is designated the epithelial cell receptor (ECR) (25, 50, 59). Binding of the H protein to a receptor triggers F protein-mediated membrane fusion of the virus envelope and the host cell plasma membrane (12). These proteins are also expressed on the cell surface and cause cell-to-cell fusion. The matrix (M) protein plays crucial roles in the process of virus assembly via its interaction with both the RNP and the cytoplasmic tails of the glycoproteins. MV strains derived from patients with SSPE (SSPE strains) generally do not express a functional M protein, becoming defective in producing infectious virus particles, and thus spread via cell-to-cell fusion (10, 14–16, 18). In addition, SSPE strains usually have a deletion or an alteration in the cytoplasmic tail of the F protein (4, 9, 31, 44).

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The SI strain was isolated in 1976 from a patient with SSPE by cultivating brain tissue biopsy samples with Vero cells (29). The patient was 8 years of age and had suffered from acute measles at 4 years of age (29). The SI strain was found to show neurovirulence, and all animals (mice, hamsters, and guinea pigs) inoculated intracerebrally with the SI strain showed neurological manifestations at 3 to 6 days after inoculation and eventually died (29). Despite these significant characteristics, molecular analyses of the SI strain have been poorly conducted. In the present study, we identified unique characteristics of the SI strain and identified substitutions responsible for the modulated receptor specificity and reduced membrane fusion activity. The present study also obtained data using a genetic engineering system of the SI strain expressing a fluorescent protein. This system could be a new platform for analyses of the molecular bases and pathogenesis of SSPE.

MATERIALS AND METHODS

Cells. BHK/T7-9 cells constitutively expressing T7 RNA polymerase (20) were maintained in Dulbecco's minimum essential medium (DMEM; Sigma, St. Louis, MO) supplemented with 7% fetal bovine serum (FBS). Vero/hSLAM (36) and CV1/hSLAM (58), which constitutively express human SLAM (hSLAM), were maintained in DMEM supplemented with 7% FBS and 0.5 mg/ml Geneticin (G418; Invitrogen Life Technologies, Carlsbad, CA). CHO cells and A549 cells constitutively expressing human SLAM, CHO/hSLAM (62), and A549/hSLAM (57), respectively, were maintained in RPMI medium (Invitrogen) supplemented with 7% FBS and 0.5 mg/ml G418. Vero cells and IMR-32 cells were maintained in DMEM supplemented with 7% FBS and 10% FBS, respectively. H358 (59) and II-18 (49) cells were maintained in RPMI supplemented with 10% FBS. SH-SY5Y cells were maintained in DMEM/F12 (Invitrogen) supplemented with 10% FBS (49, 59).

Plasmid constructions. The first-strand cDNA of the SI strain antigenome was synthesized by reverse transcription of total RNA isolated from Vero/hSLAM cells infected with the SI strain. Eight DNA fragments covering the entire region of the SI strain genome were then generated by PCR. These fragments were cloned into pBluescriptII KS(+) vector (Agilent Technologies, Inc., Santa Clara, CA) in a stepwise manner, generating a plasmid carrying the full-length antigenomic cDNA of the SI strain (detailed procedure provided upon request). A hammerhead ribozyme sequence (HHRz) was added between the T7 promoter sequence and the MV genome cDNA by a combination of PCR procedures using the synthesized DNA (5′-**GTGAATTGTAATACGACTCACTATAGGG***TGTTTG GTCTGATGAGGCCGAAAGGCCGAAACTCCGTAAGGAGTC*ACCAAACA AA-3′; the T7 promoter and HHRz sequences are shown in boldface and italics, respectively, and the MV genome cDNA sequence is underlined). To generate an additional transcriptional unit for a green fluorescent protein (GFP) derived from *Aequorea coerulescens* (AcGFP; Clontech, Palo Alto, CA), a fragment containing the open reading frame (ORF) of AcGFP was amplified by PCR using primer pair 5′-*GGCGCGCC***ATG**GTGAGCAAG-3′ and 5′-*GACGTC***TT A**CTTGTACAGCTCGT-3′ (sequences corresponding to the AscI and AatII sites are shown in italics; sequences corresponding to the initiation and termination codons are shown in boldface). The fragment was combined with the synthesized cDNA fragments containing the region between the H and L protein open reading frames of the IC-B strain by a combination of PCR procedures. The nucleotide sequences of the synthesized cDNA fragments were 5′-**ACTAGT**GAAATAGACA TCAGAATTAAGAAAAACGTAGGGTCCAAGTGGTTTCCCGTT*GGCGCG CC*-3′ and 5′-*GACGTC*TGCCAGTGAACCGATCACATGATGTCACCCAGAC ATCAGGCATACCC**ACTAGT**-3′ (sequences corresponding to SpeI sites are shown in boldface, sequences corresponding to AscI and AatII sites are shown in italics, and sequences corresponding to the gene end [GE] of the H gene and gene start [GS] of the L gene are underlined). The fragment containing the transcriptional unit for AcGFP was then inserted into the SpeI site between the H and L genes. The generated construct was named pHHRz-SI-AcGFP. Using a similar procedure, the additional transcriptional unit for AcGFP was also inserted into the p(+)MV323 plasmid, which carries the full-length antigenomic cDNA of the IC-B strain (60). The resulting plasmid was named p(+)MV323-AcGFP. A SalI-AatII fragment containing a region of the M, F, and H genes of p(+)MV323-AcGFP was replaced with a corresponding fragment of pHHRz-SI-AcGFP, and the generated construct was named p(+)MV323/SI-MFH-AcGFP. A SalI-BstEII fragment containing a region of the M gene of the pHHRz-SI-AcGFP was replaced with a corresponding fragment of p(+)MV323, and the generated construct was named pHHRz-SI/ICM-AcGFP. To generate support plasmids for the rescue of recombinant MVs from cloned cDNAs, DNA fragments encoding the N, P, and L proteins of the wild-type (wt) MV strains (IC-B or 9301B) were cloned into the pCITE vector (Novagen, Madison, WI), generating pCITE-IC-N, pCITE-IC-PΔC, and pCITEko-9301B-L, respectively. DNA fragments encoding the M proteins of the IC-B and SI strains fused with a red fluorescent protein, mCherry (Clontech), at the carboxyl-terminal end were generated by a combination of PCR procedures and inserted into a mammalian expression vector, pCA7 (32, 57). The resulting plasmids were named pCA7-FR-IC-M-mCherry and pCA7-FR-SI-mCherry, respectively. DNA fragments encoding the F proteins of the IC-B and SI strains were also amplified by PCR and cloned into pCAGGS (32), generating pCAGGS-IC-F and pCAGGS-SI-F, respectively. Similarly, DNA fragments encoding the H proteins of the IC-B and SI strains were amplified and cloned into pCAGGS, generating pCAGGS-IC-H and pCAGGS-SI-H, respectively. By replacing the SalI-XhoI, EcoRI-SalI, KpnI-XhoI, and SalI-KpnI regions of pCAGGS-IC-F with the corresponding region of pCAGGS-SI-F, four plasmids encoding chimeric F proteins between the IC-B and SI strains, designated pCAGGS-IC/SI-F-1, -F-2, -F-3, and -F-4, respectively, were generated. An amino acid substitution, G300E, was introduced into pCAGGS-SI-F, and five other amino acid substitutions, N390M, L482F, S546G, F555L, and I564L, were introduced independently into pCAGGS-IC-H by site-directed mutagenesis using complementary primer pairs.

Antibodies. A mouse monoclonal antibody (MAb) against CD46 (M75) was kindly provided by T. Seya (46). Mouse MAbs against the proteins encoded by MV H (B5), F (C527), and M (A23, A24, A27, A154, A157, A177, B46, A39, A41, A42, A51, and A133) were kindly provided by T. A. Sato (42).

Viruses. BHK/T7-9 cells were transfected with full-length genome plasmids carrying the antigenomes of MV and three support plasmids, pCITE-IC-N, pCITE-IC-PΔC, and pCITEko-9301B-L, by the use of Lipofectamine LTX Plus reagent (Invitrogen). After 2 days, the transfected cells were cocultured with Vero/hSLAM cells. IC323-AcGFP, SI-AcGFP, IC/SI-MFH-AcGFP, and SI/ICM-AcGFP were generated from p(+)MV323-AcGFP, pHHRz-SI-AcGFP, p(+)MV323/SI-MFH-AcGFP, and pHHRz-SI/ICM-AcGFP, respectively. The generated MVs were propagated in Vero/hSLAM cells. Infectious virus-like particles of SI-AcGFP and IC/SI-MFH-AcGFP were prepared by incubating the cells with 5 μg/ml cytochalasin D (Sigma) at 35°C for 30 min, as described previously (19). The infectious virus-like particles were concentrated using PEG-it precipitation solution (System Biosciences Inc., Mountain View, CA). The cell infectious units (CIUs) of the recombinant MVs expressing a fluorescent protein were determined using Vero/hSLAM cells, as described previously (51). To analyze the cytopathic effects (CPEs), monolayers of cells in 6-well cluster plates were infected with 500 CIUs of MV and the cells were observed daily using an Axio Observer.D1 microscope (Carl Zeiss, Jena, Germany).

Virus growth. Monolayers of Vero/hSLAM cells in 24-well plates were infected with recombinant MVs at a multiplicity of infection (MOI) of 0.01 per cell. At various time intervals, cell-free virus was obtained from the culture supernatants, and cell-associated virus was recovered from infected cells in 0.5 ml of DMEM-supplemented 7% FBS by freezing and thawing.

Virus titration. Monolayers of Vero/hSLAM cells in 6-well cluster plates were infected with serially diluted virus samples, incubated for 1 h at 37°C, and overlaid with DMEM containing 7% FBS and 1% agarose. PFU numbers were determined by counting the number of plaques.

Phylogenic tree analysis and Ka/K_s calculation. Nucleotide and amino acid sequence alignments and a phylogenic distance analysis were performed with the ClustalW program (63) at the genomeNet website maintained by the Kyoto University Bioinformatics Center. A phylogenic tree constructed using SI, IC-B, 9301B, WA.USA/17.98, and reference strains (66) was drawn using FigTree software. Ka/K_s calculations were performed using KaKs Calculator version 2.0 software (64). Briefly, using the two nucleotide sequences of each protein-coding region, the nonsynonymous and synonymous substitution rates (Ka and K_s, respectively) were calculated by counting the numbers of nonsynonymous and synonymous sites (NA and NS, respectively) and the numbers of nonsynonymous and synonymous substitutions (MA and MS, respectively). MA/NA and MS/NS represent the Ka and K_s substitution rates, respectively.

Immunofluorescence staining. Monolayers of Vero/hSLAM cells were seeded in 24-well plates or on coverslips in six-well cluster plates. Some monolayers were transfected with expression plasmids encoding M protein tagged with mCherry or not tagged. Other monolayers were infected with recombinant MVs and incubated with 50 μg/ml of a fusion-blocking peptide, Z-D-Phe-Phe-Gly (Peptide Institute Inc., Osaka, Japan), as described previously (41). At 24 h posttransfection or at 2 or 5 days postinoculation (p.i.) (using IC323-AcGFP or SI-AcGFP,

respectively), the cells were fixed and permeabilized with phosphate-buffered saline containing 2.5% formaldehyde and 0.5% Triton X-100. The cells were then stained with a mouse MAb against the M protein for 1 h at room temperature, followed by incubation with an Alexa Fluor 488- or 594-conjugated secondary antibody (Molecular Probes, Eugene, OR) for 1 h at room temperature. The nuclei of the infected cells were stained with 4′,6′-diamidino-2-phenylindole (DAPI; Nacalai Tesque, Kyoto, Japan) at 0.2 μg/ml. The cells were observed using a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan).

Cell-to-cell fusion assay. CHO/hSLAM, CV1/hSLAM, Vero, H358, or II-18 cells were seeded in 24-well plates, transfected with the H protein-expressing plasmid (0.5 μg) together with the F protein-expressing plasmid (0.5 μg), and incubated in the presence or absence of an anti-CD46 antibody (M75). At 1, 2, or 3 days posttransfection, the cells were fixed with methanol and stained with Giemsa solution (Sigma). The stained cells were observed under an Axio Observer.D1 microscope. To quantify cell-to-cell fusion, monolayers of cells were transfected with H protein-expressing plasmid (0.3 μg) and F protein-expressing plasmid (0.3 μg) together with a red fluorescent protein (mCherry)-expressing plasmid (0.3 μg). At 48 h posttransfection, areas expressing mCherry autofluorescence were measured using an Axio Observer.D1 microscope and ImageJ software (http://rsbweb.nih.gov/ij/index.html). Statistical analyses were performed using Microsoft Excel version 14.1.2 software.

Flow cytometry. CHO/hSLAM cells were transfected with the H or F protein-expressing plasmid (0.5 μg). At 24 h posttransfection, the cells were incubated with mouse MAbs B5 and C527 specific for the H and F proteins, respectively, followed by incubation with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Molecular Probes). The cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Minigenome assay. BHK/T7-9 cells were transfected with 0.2 μg of p18MGFLuc01 minigenome plasmid (23) together with 0.2 μg of pCITE-IC-N and various amounts of pCITE-IC-PΔC and pCITEko-9301B-L. At 48 h posttransfection, the enzymatic activity of firefly luciferase was measured using a Dual Glo luciferase assay system (Promega, Madison, WI) and a Mithras LB 940 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Nucleotide sequence accession number. The nucleotide sequence of the SI strain is available under GenBank accession number JF791787.

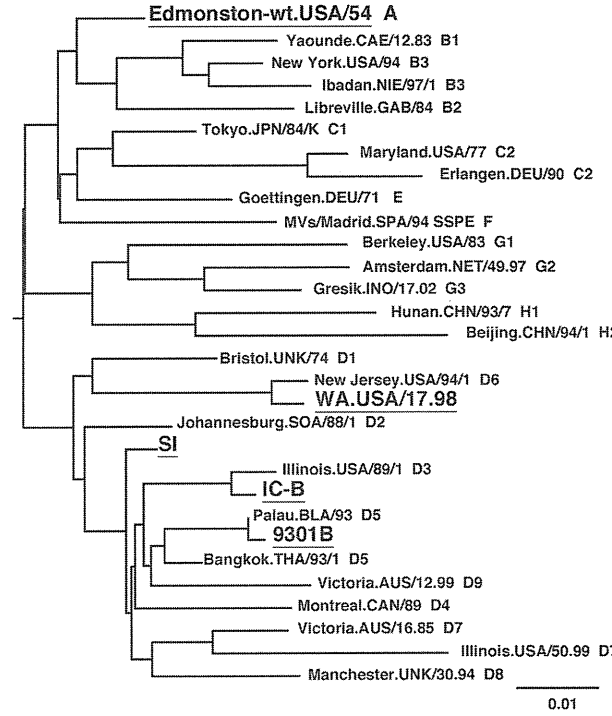

FIG. 1. Phylogenic tree drawn on the basis of the 450-nucleotide sequence that encodes the carboxyl-terminal 150 amino acids of the N protein. The names of the strains used for sequence comparisons in this study (Edmonston-wt, SI, IC-B, 9301B, and WA.USA/17.98) are underlined.

RESULTS

Characterization of the genome of the SI strain. We determined the entire genome nucleotide sequence of the SI strain. A phylogenic tree drawn on the basis of the 450-nucleotide sequence that encodes the carboxyl-terminal 150 amino acids of the N protein showed that the SI strain was classified into clade D but did not belong to a specific genotype (Fig. 1). Genotype analyses performed using a program at a website for measles nucleotide surveillance (MeaNS) (http://www.hpa-bioinformatics.org.uk/Measles/Public/Web_Front/main.php) confirmed the data for the phylogenic tree analysis (see Table S1 in the supplemental material). The entire genome nucleotide sequence of the SI strain was compared with those of three other strains in clade D, strain IC-B (genotype D3; GenBank accession number NC_001498), strain 9301B (genotype D5; GenBank accession number AB012948), and strain WA.USA/17.98 (genotype D6; GenBank accession number DQ227321) (2, 54, 61). The nucleotide sequences of the regulatory regions (i.e., the gene start, gene end, and integenic sequences) (38) were highly conserved in the SI strain genome. As indicated in previous reports (9, 11, 68), highly biased uracil-to-cystine substitutions were observed in the M gene (see Table S2 in the supplemental material). As also observed for other SSPE strains, nonsynonymous substitutions were accumulated in the M protein reading frame of the SI strain (see Fig. S1 in the supplemental material). The data for the comparison between the SI and IC-B strains are shown in the present paper, but similar results were obtained in the comparisons between the SI and other clade D MV strains. The Ka/K_s ratios were analyzed to reveal differences between the SI and clade D MV strains (IC/SI, 9301/SI, and WA98/SI) and between the Edmonston wild-type (wt) strain (genotype A; GenBank accession number AF266288) and clade D MV strains (IC/Edwt, 9301/Edwt, and WA98/Edwt) (37) (the phylogenic tree in Fig. 1 shows the relationships among the SI, IC-B, 9301B, WA.USA/17.98, and Edmonston wt strains). The data of comparisons between the Edmonston wt and clade D MV strains mostly reflect the selection pressure that operated during the natural evolution of wt MVs, while the data showing comparisons between the SI and clade D MV strains reflect the selection pressure that operated during persistent infection in the brain in addition to the natural evolution of MV. Previously, a similar study was performed by Woelk et al. (67). For the M protein reading frame, the Ka/K_s ratios in the comparisons between the Edmonston wt and clade D MV strains were ~0.03, whereas the ratios in the comparisons between the SI and clade D MV strains were 11 to 12 times greater than those observed in comparisons between the Edmonston wt and clade D MV strains (Table 1), confirming that a dynamic selection or a reduced stabilizing selection pressure operated for the M protein of the SI strain, as observed for other SSPE strains (67). Similarly, although the amino acid sequence of the F protein was highly conserved during the natural evolution of MV (Ka/K_s = 0.0000 ~ 0.0359), this was not the case during persistent infection in the brain (Ka/K_s = 0.1825 ~ 0.2504) (Table 1). Compared with those of IC-B, 12 amino acid changes were found in the F protein of the SI strain, including

TABLE 1. K_s, Ka, and Ka/K_s values from comparisons of Edmonston wild-type, IC-B, 9301B, WA.USA/17.98, and SI strains[a]

Protein reading frame	Gene region(s)	Nucleotides[b]	K_s		Ka		Ka/K_s	
			IC(D3)/Edwt, 9301(D5)/Edwt, WA98(D6)/Edwt	IC(D3)/SI, 9301(D5)/SI, WA98(D6)/SI	IC(D3)/Edwt, 9301(D5)/Edwt, WA98(D6)/Edwt	IC(D3)/SI, 9301(D5)/SI, WA98(D6)/SI	IC(D3)/Edwt, 9301(D5)/Edwt, WA98(D6)/Edwt	IC(D3)/SI, 9301(D5)/SI, WA98(D6)/SI
N		1–1578	0.0790, 0.0960, 0.1033	0.0512, 0.0703, 0.1218	0.0117, 0.0113, 0.0092	0.0046, 0.0050, 0.0075	0.1486, 0.1178, 0.0892	0.0898, 0.0712, 0.0618
P	P	1–1524	0.0416, 0.0443, 0.0330	0.0246, 0.0273, 0.0330	0.0114, 0.0176, 0.0132	0.0079, 0.0141, 0.0096	0.2740, 0.3981, 0.3982	0.3195, 0.5150, 0.2918
	P/C	22–582	0.0222, 0.0223, 0.0297	0.0073, 0.0074, 0.0147	0.0191, 0.0239, 0.0215	0.0024, 0.0071, 0.0047	0.8596, 1.0739, 0.7241	0.3217, 0.9645, 0.3212
	P/V	691–903	0.0178, 0.0177, 0	0.0177, 0.0176, 0	0, 0.0064, 0.0129	0.0195, 0.0262, 0.0327	0, 0.3643, NA[c]	1.0991, 1.4842, NA
	P′	1–21 + 583–690 + 904–1524	0.0648, 0.0704, 0.0465	0.0405, 0.0461, 0.0585	0.0088, 0.0160, 0.0071	0.0088, 0.0160, 0.0071	0.1361, 0.2272, 0.1516	0.2179, 0.3472, 0.1206
C		1–561	0.0464, 0.0705, 0.0543	0.0075, 0.0305, 0.0151	0.0119, 0.0095, 0.0143	0.0024, 0, 0.0047	0.2561, 0.1345, 0.2629	0.3132, 0, 0.3127
V	V trans[d]	690–902	0, 0.0217, 0.0439	0.0434, 0.0667, 0.0902	0.0063, 0, 0	0.0063, 0, 0	NA, 0, 0	0.1449, 0, 0
M		1–1008	0.0842, 0.0936, 0.0892	0.2135, 0.2134, 0.2141	0.0026, 0.0026, 0.0026	0.0758, 0.0758, 0.0772	0.0310, 0.0279, 0.0293	0.3551, 0.3552, 0.3606
F		1–1653	0.0566, 0.0627, 0.0675	0.0355, 0.0459, 0.0621	0, 0.0024, 0.0024	0.0089, 0.0113, 0.0113	0, 0.0359, 0.0357	0.2504, 0.2470, 0.1825
H		1–1854	0.0902, 0.0877, 0.0724	0.0675, 0.0651, 0.0907	0.0114, 0.0100, 0.0092	0.0085, 0.0071, 0.0135	0.1263, 0.1135, 0.1276	0.1262, 0.1089, 0.1490
L		1–6549	0.0801, 0.0927, 0.0822	0.0601, 0.0687, 0.0781	0.0047, 0.0051, 0.0049	0.0050, 0.0054, 0.0058	0.0584, 0.0548, 0.0594	0.0828, 0.0782, 0.0739

[a] Edwt, Edmonston wild type; IC(D3), IC-B; 9301(D5), 9301B; WA98(D6), WA.USA/17.98.
[b] The first nucleotide of the initiation codon for each open reading frame is taken as 1.
[c] NA, not applicable.
[d] V trans is the C-terminal region unique to the V protein.

a nonsense mutation at amino acid position 532 (Table 2). These changes in the F protein are typical of SSPE strains (4, 9, 31, 44). For the N, H, and L protein reading frames, in contrast, the Ka/K_s ratios revealed by the comparisons between the SI and clade D MV strains were similar to those observed between the Edmonston wt and clade D MV strains (Table 1). These data indicated that similar levels of stabilizing selection pressure operated for the N, H, and L protein reading frames of the SI strain during the persistent infection in the brain. For the P gene, it was not simple to assess the data for the Ka and K_s values, since the gene contains overlapping reading frames. Nonetheless, it was evident that both the C and V nonstructural proteins were highly conserved during the persistent infection in the brain. For the C protein-reading frame, the Ka values for the IC/SI and WA93/SI comparisons were as much as 3 to 5 times lower than those for the IC/Edwt and WA93/SI comparisons (Table 1). Indeed, no amino acid substitution was found in the C protein of the SI strain compared with that of the 9301B strain. Similarly, no amino acid substitution was found in the V protein-unique region of the SI strain compared with that of the WA.USA/17.98. strain. The V protein-unique region of the 9301B strain also had the same amino acid sequence as those of the SI and WA.USA/17/98 strains except that the 9301B V protein possessed an additional single amino acid at the carboxyl-terminal end, because it terminated one codon later (since this additional codon was not included in calculation, the Ka of 9301/SI comparison was zero [Table 1]). These data suggested that both the C and V proteins played important roles in the survival of the SI strain in the brain.

Generation of a recombinant SI strain expressing a fluorescent protein by establishment of an efficient MV rescue system. The SI strain did not produce cell-free infectious particles and spread poorly in cell cultures (data not shown). In addition, a CPE was barely detectable in some cultured cells, although the SI strain replicated in them (data not shown). Many studies have shown that the use of recombinant viruses genetically engineered to express a fluorescent protein is greatly advantageous for monitoring virus infections, especially when the virus infection shows a small or weak CPE. Therefore, we decided to generate a recombinant SI strain expressing a fluorescent protein. A full-length genome cDNA of the SI strain possessing an additional transcriptional unit encoding AcGFP between the H and L genes was generated and inserted into the pBluescript vector downstream of the T7 promoter (Fig. 2). The T7 promoter was followed by three guanines that enhance the transcription efficiency (Fig. 2). Since these guanines produce extra guanine residues at the 5′ end of the synthesized MV antig-

TABLE 2. Amino acid substitutions in the F proteins among the IC, SI, and Edmonston strains

Amino acid no.	Amino acid substitution(s) or category		
	IC	SI	Ed
78	R	G	R
165	R	K	R
167	A	T	A
187	I	V	I
242	I	T	I
246	L	F	L
247	E	K	E
268	G	D	G
300	E	G	E
487	M	I	M
532	R	Stop	R
533–550	18 aa[a]	Deletion	18 aa

[a] aa, amino acids.