express nectin4 and have been shown to be infected with CDV (Pratakpiriya et al., 2012). Although morbilliviruses commonly use SLAM as a receptor, each morbillivirus preferentially uses the SLAM of its host animals (Seki et al., 2003; Tatsuo et al., 2001). Tatsuo et al. (Tatsuo et al., 2001) reported that the HA7 strain of CDV isolated using B95a cells, which express marmoset SLAM, used human SLAM reasonably well, whereas the Onderstepoort CDV vaccine strain failed to use it. In the present study, we analyzed the ability of wild-type CDV strains to use human nectin4 and human SLAM as receptors. In addition, the genetic changes in CDV required for efficient growth in human epithelial cells were investigated.

Results and discussion

Ability of wild-type CDV strains to use human nectin4 and human SLAM as receptors

Our recent study showed that wild-type CDV strains use dog nectin4 as a receptor (Pratakpiriya et al., 2012). A human nectin4 cDNA was obtained from human lung carcinoma-derived NCI-H358 cells (Takeda et al., 2007), and Vero cells constitutively expressing human nectin4 (Vero/hNectin4) were generated using this cDNA. Flow cytometry analyses confirmed that Vero/hNectin4 cells expressed human nectin4, but the expression level was several-fold lower than that of dog nectin4 in Vero/dNectin4 cells (Pratakpiriya et al., 2012) (Fig. 1A). The MV H protein binds to the V domain of human nectin4 (Muhlebach et al., 2011; Noyce et al., 2011). We found six amino acid differences in the V domain between human nectin4 and dog nectin4 (DDBJ/Gen Bank accession numbers AB755430 and AB755429, respectively) (Fig. 2). The parental Vero, nectin4-expressing (Vero/dNectin4 (Pratakpiriya et al., 2012) and Vero/hNectin4) and SLAM-expressing (Vero/hSLAM (Ono et al., 2001) and Vero. DogSLAM tag (Seki et al., 2003)) Vero cells were infected with wild-type CDV strains (Ac96I, 82Con, 55L, M24Cr, and Th12). As reported previously (Pratakpiriya et al., 2012; Seki et al., 2003), no syncytia developed in the parental Vero cells infected with these CDV strains (Fig. 1B), although they may have had low levels of infection without causing syncytium formation, as shown for MV (Hashimoto et al., 2002). On the other hand, all the strains produced large syncytia in Vero/hNectin4 cells as efficiently as in Vero/dNectin4 cells (Fig. 1B). Syncytium formation was barely detectable in Vero/hSLAM cells, but clearly evident in Vero.DogSLAM tag cells (Fig. 1B). Thus, human SLAM seemed to be suboptimal as a wild-type CDV receptor. The growth kinetics of Ac96I in the parental Vero, Vero/dNectin4, and Vero/hNectin4 cells were analyzed. Ac96I did not replicate in Vero cells, but showed efficient replication in both Vero/dNectin4 and Vero/hNectin4 cells (Fig. 1C). The replication kinetics in Vero/dNectin4 and Vero/hNectin4 cells were comparable with one another (Fig. 1C). To show the direct contribution of the H protein to the syncytium formation, cell-to-cell fusion assays were performed using mammalian cell expression vectors. In all of the cell lines, no syncytia were observed when the F protein was expressed alone (Fig. 3). When the H protein of Ac96I was expressed together with the F protein, large syncytia developed in Vero/hNectin4 and Vero/dNectin4 cells, but not in the parental Vero cells (Fig. 3). The susceptibilities of Vero, Vero/dNectin4, and Vero/hNectin4 cells to Ac96I infection were analyzed and compared. The infectivity of Ac96I in Vero/hNectin4 cells determined by the 50% cell culture infectious dose ($CCID_{50}$) was 4.25 ± 0.00 $CCID_{50}$/ml, which was comparable to that in Vero/dNectin4 cells (4.58 ± 0.12 $CCID_{50}$/ml). The titer determined in Vero cells was less than 1.75 $CCID_{50}$/ml. These data demonstrate that human nectin4 functions as a CDV receptor as efficiently as dog nectin4, while human SLAM does not.

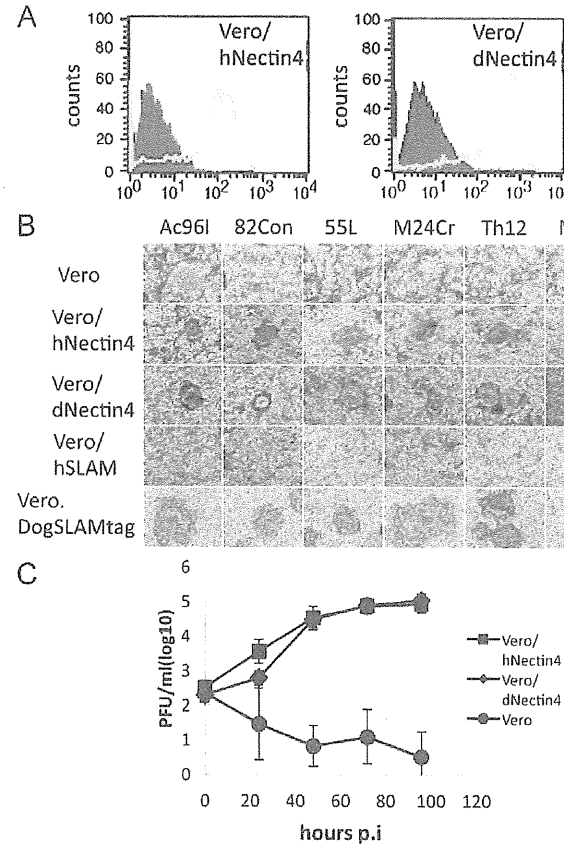


Fig. 1. Infection of the parental, nectin4-expressing, and SLAM-expressing Vero cells with wild-type CDV strains. (A) Vero/hNectin4 (left panel) and Vero/dNectin4 (right panel) cells were stained with a goat anti-human nectin4 polyclonal antibody (gray empty profile) or a control goat IgG (filled black profile), followed by staining with Alexa Fluor 488-conjugated anti-goat IgG. (B) Vero, Vero/hNectin4, Vero/dNectin4, Vero/hSLAM, and Vero.DogSLAMtag cells were infected with wild-type CDV strains (Ac96I-VDS, 82Con, 55L, M24Cr, and Th12) or mock-infected. At 48 h (Vero, Vero/hNectin4, Vero/dNectin4, and Vero/hSLAM) or 24 h (Vero.DogSLAMtag) post-infection, the cells were stained with the Giemsa solution, and observed under a phase-contrast microscope. (C) Replication kinetics of Ac96I. Vero/hNectin4, Vero/dNectin4, and parental Vero cells were infected with Ac96I at a MOI of 0.01. At various time intervals post-infection, the virus titers were determined by plaque assays.

The pathology of morbilliviruses is well documented for MV. MV is an airborne virus, and the infection starts via SLAM-mediated entry into alveolar macrophages and dendritic cells in the lung or respiratory tracts. After replicating in local lymph nodes, MV spreads to various lymphoid organs or tissues throughout the body using SLAM as a receptor (Takeda et al., 2011). Thus, the ability to use SLAM is critical for MV, and probably for all morbilliviruses, to spread and cause diseases in vivo. Conversely, nectin4 is used at the late stage of infection to shed progeny viruses into the respiratory tract (Takeda et al., 2011). Thus, the low capacity for using human SLAM would explain the inability of CDV to replicate in humans.

Potential of wild-type CDV strains to replicate in human epithelial cells

The host range of MV is determined by not only receptors but also intracellular factors (Iwasaki and Yanagi, 2011). NCI-H358 cells are highly susceptible and permissive to MV infection (Takeda et al., 2007). NCI-H358 cells were found to express human nectin4, although the expression level was significantly

```
human   32 GELETSDVVTVVLGQDAKLPCFYRGDSGEQVGQVAWARVDAGEGAQELALLHSKYGLHVS  91
dog     31 .......L.................P.................R.............  90
mouse   31 ..........................PD............PN..IR............N  90

human   92 PAYEGRVEQPPPPRNPLDGSVLLRNAVQADEGEYECRVSTFPAGSFQARLRLRVLV  147
dog     91 A............S....A.....................................  146
mouse   91 ....D.........D.................................M......  146
```

Fig. 2. Amino acid sequence comparison of the V domains of human, dog, and mouse nectin4. Dots indicate identical residues to those of human nectin4.

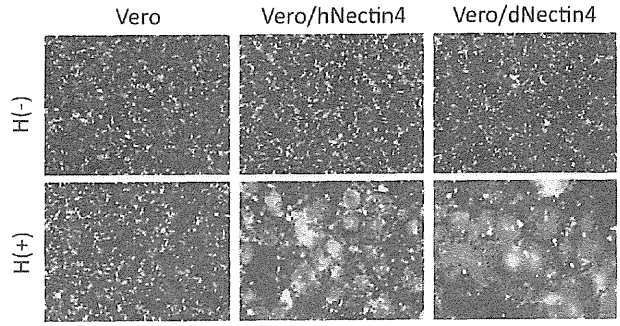


Fig. 3. Cell-to-cell fusion assays. Vero (left panels), Vero/hNectin4 (middle panels), and Vero/dNectin4 (right panels) cells were transfected with the mCherry-expressing plasmid and the F protein-expressing plasmid (upper panels) or the mCherry-expressing plasmid and both the F protein-expressing plasmid and H protein-expressing plasmid (bottom panels). At 48 h post-transfection, the cells were observed using an Axio Observer.D1 microscope.

lower than that in Vero/hNectin4 cells (Fig. 4A). Nevertheless, MV replicates in these cells in a nectin4-dependent manner (Muhlebach et al., 2011; Noyce et al., 2011). NCI-H358 cells were infected with wild-type CDV strains (Ac96I, 82Con, 55L, M24Cr, and Th12) at a multiplicity of infection (MOI) of 0.01, and the infectious virus titers were determined at 5 days post-infection (Fig. 4B). The data showed that the virus titer of the Ac96I strain was significantly lower than those of the other four wild-type CDV strains (Fig. 4B). The Ac96I strain was originally isolated from the large intestine of dogs with distemper using Vero.DogSLAMtag cells (Lan et al., 2006) and passaged 2–3 times in Vero.DogSLAMtag cells to obtain a sufficient amount of virus stocks for analysis. In the following experiments, the Vero.DogSLAMtag cell-grown Ac96I strain was designated Ac96I-VDS. NCI-H358 cells were infected with Ac96I-VDS, and the virus titers were determined at 1, 3, 5, and 7 days post-infection (Fig. 4C). The results confirmed that Ac96I-VDS replicated poorly in NCI-H358 cells (Fig. 4C). Nevertheless, at 7 days post-infection, some production of infectious viruses was observed in Ac96I-infected NCI-H358 cells (Fig. 4C). After eight passages of Ac96I-VDS in NCI-H358 cells, the CDV-infected cells were scraped into culture medium, subjected to three cycles of freezing-and-thawing, and centrifuged. The supernatants were collected. The NCI-H358 cell-grown CDV was designated Ac96I-H358. Fresh monolayers of NCI-H358 cells were infected with Ac96I-H358, and the replication kinetics were analyzed at various time intervals. The data showed that Ac96I-H358 replicated well in NCI-H358 cells and produced infectious virus particles efficiently (Fig. 4B). To confirm the growth ability of Ac96I-H358 in human epithelial cells, the replication kinetics of Ac96I-VDS and Ac96I-H358 were analyzed in another nectin4-positive human epithelial cell line, II-18 (Shirogane et al., 2010). Both Ac96I-VDS and Ac96I-H358 were able to replicate in II-18 cells (Fig. 4D). However, the replication ability of Ac96I-H358 was obviously greater than of Ac96I-VDS in II-18 cells, as observed in NCI-H358 cells (Fig. 4C and D). These findings confirmed that Ac96I-H358 exhibits restored ability to grow in human epithelial cells.

Genetic changes in the Ac96I-H358 strain during propagation in human epithelial NCI-H358 cells

Similar to other RNA viruses, CDV forms quasispecies. The nucleotide sequences of Ac96I-VDS and Ac96I-H358 were deeply analyzed by next-generation sequencing. Table 1 shows a list of the nucleotide substitutions acquired by Ac96I-H358, as well as selected preexisting nucleotides, during the eight passages of Ac96I-H358 in NCI-H358 cells. All of the nucleotide positions where more than 15% of the Ac96I-H358 genome acquired a nucleotide substitution or the proportion of preexisting nucleotides was increased by more than 15% are listed in Table 1. Unsurprisingly, these data showed no amino acid change in the H protein during the passages in NCI-H358 cells. These data strengthen the above conclusion that the CDV H protein possesses an intrinsic ability to use human nectin4 as a receptor. Our previous data for other wild-type strains also demonstrated that no amino acid changes occurred in the H protein during isolation from tissues of dogs with distemper and passages in Vero.DogSLAMtag cells (Lan et al., 2005a). Therefore, the ability to use human nectin4 is an intrinsic phenotype of wild-type CDV strains. The most striking change was observed in the P gene. The P gene encodes a polymerase cofactor protein, P, and two nonstructural proteins, C and V. Although the C and V proteins are nonessential for virus replication in cultured cells, they play crucial roles in counteracting the host innate immune responses (Devaux et al., 2007; Nakatsu et al., 2008; Rothlisberger et al., 2010; von Messling et al., 2006). The majority (91%) of Ac96I-VDS had a thymine at nucleotide position 2198, producing a truncated C protein, while the remaining 9% possessed the intact C protein (Table 1). The truncated C protein had a nonsense mutation at amino acid position 126 and became shorter by 49 amino acids, compared with the intact C protein (the site of the authentic stop codon was predicted by analyses of other wild-type CDV strains and previously reported CDV sequences (Fig. 5) (Wang et al., 2012)). The other four wild-type CDV strains (82Con, 55L, M24Cr, and Th12) possessed the intact C protein (Fig. 5). For MV, a truncation of the C protein at amino acid position 157 made the C protein defective in its ability to counteract the host interferon system (Nakatsu et al., 2008). The C protein sequence was perfectly (100%) restored in Ac96I-H358 (Table 1). These findings suggest that expression of the intact C protein was critical for replication in NCI-H358 cells, but not in Vero.DogSLAMtag cells. The nucleotide substitution in the P gene also caused a valine-to-alanine substitution at amino acid position 133 in the P protein (Table 1). P gene mutations accompanied by truncation or mutations in the C protein are often observed in the genome of Vero cell-grown MV strains (Miyajima et al., 2004; Takeda et al., 1998; Takeuchi et al., 2000). Vero cells are defective in the production of interferons (Chew et al., 2009; Emeny and Morgan, 1979), whereas NCI-H358 cells possess a functional interferon system (Ikegame et al., 2010). Thus, it is most likely that the truncation mutation for the C protein in Ac96I-VDS occurred in Vero.DogSLAMtag cells, rather than occurring in nature, since the C protein plays an important role for CDV pathogenesis in vivo (von Messling et al., 2006). Recently, Vero-based cell lines, e.g., Vero/hSLAM for MV,

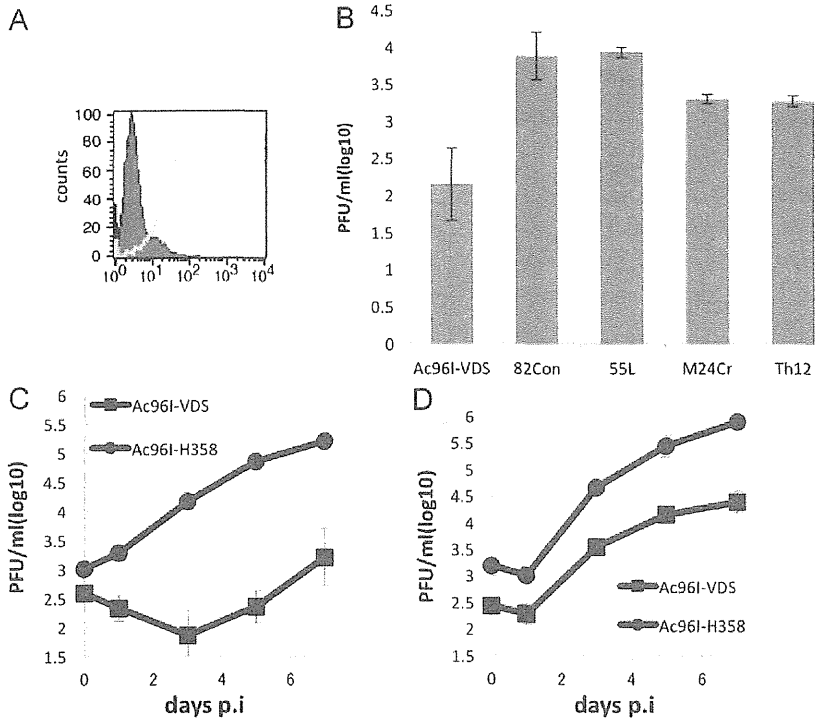


Fig. 4. Replication kinetics in NCI-H358 cells. (A) NCI-H358 cells were stained with a goat anti-human nectin4 polyclonal antibody (gray empty profile) or a control goat IgG (filled black profile), followed by staining with Alexa Fluor 488-conjugated anti-goat IgG. (B) NCI-H358 cells were infected with the Ac96I-VDS, 82Con, 55 L, M24Cr, or Th12 CDV strains at a MOI of 0.01. At 5 days post-infection, the virus titers were determined by plaque assays. (C, D) NCI-H358 (C) and II-18 (D) cells were infected with Ac96I-VDS or Ac96I-H358 at a MOI of 0.01. At 1, 3, 5, and 7 days post-infection, the virus titers were determined by plaque assays.

Table 1
Acquired and selected nucleotides during passages in NCI-H358 cells.

		Ac-96I-VDS					Ac-96I-H358						
			Proportion (%)					Proportion (%)					
Gene	Nucleotide position	Total no. of reads	A	C	G	T	Total no. of reads	A	C	G	T	Protein	Amino acid substitution
N	792	349	0	0	0	100	138	0	0	75[a]	25	N	L229V
N	974	541	0	1	0	99	148	0	32	0	68	n.a.[b]	no
N	995	340	0	0	0	100	116	0	0	43	57	N	I296M
N	1506	100	0	0	100	0	33	18	0	82	0	N	E467K
P/V/C	2198	1024	0	9	0	91	101	0	100	0	0	P and VC	V133A 126Q[c]
M	3681	364	98	0	2	0	73	78	22	0	0	M	T84P
M	3965	63	0	0	0	100	46	50	0	0	50	M	F178L
M	4047	48	100	0	0	0	37	73	0	27	0	M	N206D
M	4417	81	84	0	0	16	31	100	0	0	0	M	L329Q
F	5926	196	0	78	22	1	61	0	100	0	0	F	R331P
F	6074	167	26	0	74	0	37	97	0	3	0	n.a.	no
L	9107	194	2	0	98	0	23	17	0	82	0	n.a.	no
L	14271	39	72	0	28	0	50	100	0	0	0	L	D1748N

[a] Acquired and selected nucleotides are underlined.
[b] Not applicable.
[c] A termination codon at position 126 was replaced with a glutamine residue.

have been used for the isolation of many viruses. Indeed, the interferon-deficient phenotype of Vero cells is an advantage for virus isolation. However, it may lead to the selection of a minor clone with a low ability to counteract the host innate immune responses. In this regard, use of lymphocyte-based cell lines and epithelial cell lines with a functional interferon system is advisable for the isolation of viruses for pathogenesis studies. In either case, our data showed that the P gene in the 9% population of Ac96I-VDS, which became 100% dominant in Ac96I-H358 cells, encoded C and V proteins that were functional for counteracting the human interferon system.

Although the C protein appears to play a critical role for CDV replication in NCI-H358 cells, mutations in other genes or proteins may contribute to the Ac96I-H358 growth in these cells, since the virus titer of Ac96I-H358 at 5 days post-infection was ~10-fold greater than those of the other wild-type CDV strains possessing the intact C protein (Fig. 4B, C). The data obtained by next-generation sequencing also showed that certain proportions

```
Ac96I-H358     1 MSIKGWNASKPSERILLTLRRFKRSAVSEIKPATQAKRMEPQASRKKRSLRISMNHTSQQ   60
Ac96I-VDS      1 ............................................................   60
82Con          1 ............................................................   60
55L            1 ..A..L.....................A..T..T.........VC..R.............   60
M24Cr          1 ..A..L.....................A..T..T.........VC..R.............   60
Th12           1 ........................I.................C.................   60

Ac96I-H358    60 KDQTMSAMYSKIILDVERATLRLWRQSSPLKMMSNQDLEYDVIMFMITVVKRLRESKMLT   120
Ac96I-VDS     60 ............................................................   120
82Con         60 ............................................................   120
55L           60 ..............R..............L...T................A...........   120
M24Cr         60 ..............R..............L...T................A...........   120
Th12          60 ..............R..............L.....K.........................   120

Ac96I-H358   120 VSWYLQALSVIEDSREEKEALMIALRILAKIIPREMLHLTGDILSALNQTERLM         174
Ac96I-VDS    120 .....----------------------------------------------          125
82Con        120 ..................................................           174
55L          120 ........................K.......K.................QP.         174
M24Cr        120 ........................K.......K.................QP.         174
Th12         120 ..................................................           174
```

Fig. 5. Amino acid sequence comparison of the C protein. Dots indicate identical residues to the Ac96I-H358 strain. Bars indicate that there are no amino acid residues at these positions.

of Ac96I-H358 have nonsynonymous substitutions in the N and M genes (Table 1). These substitutions were leucine-to-valine, isoleucine-to-methionine, and glutamic acid-to-lysine at amino acid positions 129, 296, and 467 (L129V, I296M, and E467K), respectively, in the N protein, and threonine-to-proline, phenylalanine-to-leucine, and asparagine-to-aspartic acid at amino acid positions 84, 178, and 206 (T84P, F178L, and N206D), respectively, in the M protein (Table 1). Some mutations in the M protein of MV were shown to contribute to virus growth in unnatural host cells (Dong et al., 2009; Miyajima et al., 2004; Tahara et al., 2005, 2007). Therefore, it is of great interest to evaluate whether mutations in the M and N proteins are critical for CDV adaptation to be able to grow in human cells.

In addition, the M gene possessed a selected nonsynonymous substitution at position 4417 and both the F and L genes of Ac96I-H358 possessed a single selected nonsynonymous substitution (L329Q, R331P, and D1748N in the M, F, and L proteins, respectively) (Table 1). However, these mutations did not seem to be important for CDV to acquire the capacity to replicate well in NCI-H358 cells, since the major population (72–84%) of Ac96I-VDS already possessed these substitutions (Table 1). Since CDV possesses a nonsegmented RNA genome, some unnecessary mutations could be accompanied by significant changes, which exist on the same RNA genome.

Tatsuo et al. (2001) demonstrated that the CDV HA7 strain, but not the Onderstepoort CDV vaccine strain, used human SLAM reasonably well. However, our data showed that wild-type CDV strains used human SLAM poorly, similar to the case for the Onderstepoort vaccine strain. Since the HA7 strain was isolated using marmoset SLAM-expressing B95a cells, it may have adapted to use nonhuman primate SLAM as well as human SLAM. Thus, the Onderstepoort vaccine strain, rather than the HA7 strain, may have retained the original phenotype regarding the ability to use human SLAM. The data for the HA7 strain described in Tatsuo et al. (2001) may rather suggest that CDV easily adapts to the use of human SLAM. Seki et al. (2003) demonstrated that only a few amino acid changes in the H protein are sufficient for wild-type CDV strains to use marmoset SLAM efficiently, although they did not examine whether these changes conferred the ability to use human SLAM on the wild-type CDV strains. Unlike human SLAM, human nectin4 was fully functional as a wild-type CDV receptor. Despite this ability, Ac96I-VDS failed to spread in a human epithelial cell line. However, this phenotype is unlikely to be the original phenotype of wild-type CDV strains. Instead, they may have an intrinsic potential to replicate in human cells. In addition, the restoration of the C protein observed in the Ac96I-H358 strain implies that the CDV C protein is capable of counteracting human innate immune responses. Indeed, the CDV strains possessing the intact C protein showed reasonable degrees of ability to replicate in human epithelial cells. Nevertheless, the virus titers of these CDV strains in NCI-H358 cells were still lower than that of MV (data not shown) (Takeda et al., 2007). Some intracellular human host factors may not be optimal for the CDV replication machinery. Importantly, the Ac96I-H358 strain, which adapted to growth in NCI-H358 cells, showed a significantly higher replication potential than the other CDV strains in NCI-H358 cells, and its growth ability approached that of MV.

The critical factors that create the species barrier of CDV between susceptible animals and humans still remain unknown. However, some CDV strains appear to have already adapted to growth in nonhuman primates (Qiu et al., 2011; Sun et al., 2010). CDV is closely related to MV, and the major structural proteins of these viruses have some immunological cross-reactivity, such that immunity raised against MV proteins is partially, but insufficiently, effective against CDV infection (Giraudon and Wild, 1981; Norrby and Appel, 1980; Orvell and Norrby, 1980; Stephenson and ter Meulen, 1979; Taylor et al., 1991). Furthermore, some viral proteins of CDV and MV are even functionally exchangeable (Brown et al., 2005). The CDV genome forms an active ribonucleocapsid complex with the N, P, and L proteins of MV, and synthesizes viral RNAs using the MV proteins, and vice versa (Brown et al., 2005). Thus, immunity against MV may have protected humans against CDV infection. However, based on the present experimental data and the observation of large CDV outbreaks in monkeys (Qiu et al., 2011; Sun et al., 2010), we suggest that CDV has sufficient latent potential to adapt to humans, and may cause a serious disease in humans in the future. We thus propose that the preparation of a countermeasure against CDV needs to be seriously considered at the present time.

Materials and methods

Cells and viruses

Vero cells were maintained in DMEM supplemented with 5% fetal bovine serum (FBS). Vero.DogSLAMtag, Vero/hSLAM, and

Vero/dNectin4 cells were reported previously (Pratakpiriya et al., 2012; Seki et al., 2003) and maintained in DMEM supplemented with 5% FBS and 1 mg/ml geneticin (G418; Invitrogen Life Technologies, Carlsbad, CA). NCI-H358 cells (Takeda et al., 2007) and II-18 cells (Shirogane et al., 2010) were maintained in RPMI medium supplemented with 10% fetal calf serum. Total RNA obtained from NCI-H358 cells was used to synthesize the cDNA of human nectin4. The primers used for amplification of the human nectin4 cDNA were 5′-*GAATTC*AGTCTGCCTTTCAACCA-3′ and 5′-*GCGGCCGC*AGGCAGGCCTGGGTCA-3′ (sequences corresponding to EcoRI and NotI sites are shown in italics). The human nectin4 cDNA fragment was inserted into the pCXN2 vector, generating pCXN2-hNectin4. A Vero cell clone stably expressing human nectin4 (Vero/hNectin4) was generated by transfecting Vero cells with pCXN2-hNectin4. Vero/hNectin4 cells were maintained in DMEM supplemented with 5% FBS and 1 mg/ml geneticin. The wild-type Ac96I strain of CDV was isolated from the large intestine of dogs with distemper using Vero.DogSLAMtag cells (Lan et al., 2006), and the working stock of the Ac96I strain, which was passaged in Vero.DogSLAMtag cells 2–3 times in total, was designated Ac96I-VDS. The other wild-type CDV strains (82Con, 55L, M24Cr, and Th12) were isolated from morbid or dead dogs with distemper using Vero.DogSLAMtag cells, and reported previously (Lan et al., 2009; Lan et al., 2006; Lan et al., 2005b). To obtain an NCI-H358 cell-adapted Ac96I strain, Ac96I-VDS was inoculated into monolayers of NCI-H358 cells and passaged eight times in these cells. After the eight passages, the CDV-infected cells were scraped into culture medium, subjected to three cycles of freezing-and-thawing, and centrifuged. The supernatant was collected. The NCI-H358 cell-adapted Ac96I strain was designated Ac96I-H358.

Flow cytometry

The cell surface expression of nectin4 was analyzed using a goat anti-human nectin4 polyclonal antibody (R&D Systems, Minneapolis, MN) as the primary antibody and Alexa Fluor 488-conjugated anti-goat IgG (Molecular probes, Eugene, Oregon) as the secondary antibody. Normal goat IgG (PEPROTECH, Rocky Hill, NJ) was used as a control. The cell surface fluorescence of 1×10^4 cells was analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Replication kinetics

Monolayers of Vero, Vero.DogSLAMtag, Vero/dNectin4, and NCI-H358 cells in 24-well plates were infected with Ac96I-VDS or Ac96I-H358 at a MOI of 0.01 per cell. After various time intervals, the cells were scraped into the culture supernatants, and the virus titers (PFU) were determined by plaque assays in Vero.DogSLAMtag cells.

Plaque titration

Monolayers of Vero.DogSLAMtag cells in 6-well cluster plates were infected with serially diluted virus samples, incubated for 1 h at 37 °C, and overlaid with 3 ml of minimal essential medium containing 2% FBS and 0.8% agarose (0.8% agarose-MEM). At 7 days post-infection, 2 ml of 0.8% agarose-MEM containing 0.01% neutral red was overlaid on each well, and the PFU was determined by counting the number of plaques at 2 days after the procedure.

Cell susceptibility to CDV infection

To analyze the susceptibility of Vero, Vero/dNectin4, and Vero/hNectin4 cells to Ac96I-VDS infection, serially diluted virus samples were inoculated into monolayers of these cells, and the $CCID_{50}$ of Ac96I-VDS in these cells was determined.

Cell-to-cell fusion assay

DNA fragments encoding the H protein of Ac96I-VDS and the CDV F protein were amplified and cloned into the pCAGGS vector. Vero, Vero/dNectin4, and Vero/hNectin4 cells were seeded in 12-well plates, and transfected with the F protein-expressing plasmid (0.3 μg) with or without the H protein-expressing plasmid (0.3 μg) using the TransIT-LT1 reagent (Mirus Bio, Madison, WI). To clearly detect syncytia, a fluorescent protein-expressing plasmid (pCA7-FR-mCherry; 0.3 μg) was included into the transfection mixture, as reported previously (Seki et al., 2011). At 48 h post-transfection, the cell monolayers were observed using an Axio Observer.D1 microscope.

Next-generation sequencing

Culture supernatants of Vero.DogSLAMtag and NCI-H358 cells infected with Ac96I-VDS and Ac96I-H358, respectively, were collected, and the virus particles were centrifuged into pellets through 30% sucrose cushions (30% sucrose [wt/vol] in NTE [0.1 M NaCl, 0.01 M Tris pH 7.4, 0.001 M EDTA]) at 185,000 × *g* for 2 h in a Beckman 45 Ti rotor at 4 °C. Subsequently, viral RNA was purified from the virus particles using Isogen (Nippon Gene, Tokyo, Japan). An RNAseq library was prepared using a ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Illumina-compatible) (Epicentre Biotechnologies, Madison, WI) and the indexing method. Deep sequencing runs for pair-end short reads were performed with MiSeq and GAIIx systems (Illumina, San Diego, CA). To identify nucleotide variations based on the RNAseq analysis, the obtained short reads were mapped to the corresponding reference CDV genome sequence (Ac96I-VDS) by the BWA mapping tool (Li and Durbin, 2010). The obtained mapping data were visualized with GenomeJack viewer software (Mitsubishi Space Software, Tokyo, Japan).

Sequencing of the P gene of CDV strains

Viral RNAs were extracted from each virus stock using a High Pure Viral RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized using Super ScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA), and then amplified by PCR using Prime STAR GXL DNA polymerase (Takara Bio, Shiga, Japan). The primers used for amplification and sequencing are available upon request. The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen KK, Tokyo, Japan). The nucleotide sequences of the purified PCR products were determined using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and a capillary sequencer.

Nucleotide sequence accession numbers

The short reads obtained by the next-generation sequencing have been deposited in the DDBJ Sequence Read Archive (DRA) of Japan (accession number: DRA000586). The consensus genome nucleotide sequences of Ac96I-VDS and Ac96I-H358 have been deposited in DDBJ/GenBank with accession numbers AB753775 and AB753776, respectively. The nucleotide sequences for the P gene of 55L, Th12, M24Cr, and 82Con have been deposited in

DDBJ/GenBank with accession numbers AB755425, AB755426, AB755427, and AB155428, respectively.

Acknowledgments

We thank Dr. Yusuke Yanagi for providing the Vero.DogSLAM-tag and Vero/hSLAM cells. We also thank all the members of Department of Virology 3, NIID, Japan, for technical support and valuable suggestions. This work was supported, in part, by grants from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare of Japan, and a grant from The Takeda Science Foundation.

References

Appel, M.J., Yates, R.A., Foley, G.L., Bernstein, J.J., Santinelli, S., Spelman, L.H., Miller, L.D., Arp, L.H., Anderson, M., Barr, M., et al., 1994. Canine distemper epizootic in lions, tigers, and leopards in North America. J. Vet. Diagn. Invest.: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc. 6, 277–288.

Brown, D.D., Collins, F.M., Duprex, W.P., Baron, M.D., Barrett, T., Rima, B.K., 2005. 'Rescue' of mini-genomic constructs and viruses by combinations of morbillivirus N, P and L proteins. J. Gen. Virol. 86, 1077–1081.

Chew, T., Noyce, R., Collins, S.E., Hancock, M.H., Mossman, K.L., 2009. Characterization of the interferon regulatory factor 3-mediated antiviral response in a cell line deficient for IFN production. Molecular immunology 46, 393–399.

Devaux, P., von Messling, V., Songsungthong, W., Springfeld, C., Cattaneo, R., 2007. Tyrosine 110 in the measles virus phosphoprotein is required to block STAT1 phosphorylation. Virology 360, 72–83.

Dong, J.B., Saito, A., Mine, Y., Sakuraba, Y., Nibe, K., Goto, Y., Komase, K., Nakayama, T., Miyata, H., Iwata, H., Haga, T., 2009. Adaptation of wild-type measles virus to cotton rat lung cells: E89K mutation in matrix protein contributes to its fitness. Virus Genes 39, 330–334.

Emeny, J.M., Morgan, M.J., 1979. Regulation of the interferon system: evidence that Vero cells have a genetic defect in interferon production. J. Gen. Virol. 43, 247–252.

Giraudon, P., Wild, T.F., 1981. Differentiation of measles virus strains and a strain of canine distemper virus by monoclonal antibodies. J. Gen. Virol. 57, 179–183.

Hashimoto, K., Ono, N., Tatsuo, H., Minagawa, H., Takeda, M., Takeuchi, K., Yanagi, Y., 2002. SLAM (CD150)-independent measles virus entry as revealed by recombinant virus expressing green fluorescent protein. J. Virol. 76, 6743–6749.

Hirama, K., Goto, Y., Uema, M., Endo, Y., Miura, R., Kai, C., 2004. Phylogenetic analysis of the hemagglutinin (H) gene of canine distemper viruses isolated from wild masked palm civets (Paguma larvata). J. Vet. Med. Sci. /Jpn. Soc. Vet. Sci. 66, 1575–1578.

Hur, K., Bae, J.S., Choi, J.H., Kim, J.H., Kwon, S.W., Lee, K.W., Kim, D.Y., 1999. Canine distemper virus infection in binturongs (Arctictis binturong). J. Comp. Pathol. 121, 295–299.

Ikegame, S., Takeda, M., Ohno, S., Nakatsu, Y., Nakanishi, Y., Yanagi, Y., 2010. Both RIG-I and MDA5 RNA helicases contribute to the induction of alpha/beta interferon in measles virus-infected human cells. J. Virol. 84, 372–379.

Iwasaki, M., Yanagi, Y., 2011. Expression of the Sendai (murine parainfluenza) virus C protein alleviates restriction of measles virus growth in mouse cells. Proc. Nat. Acad. Sci. U.S.A. 108, 15384–15389.

Kotani, T., Jyo, M., Odagiri, Y., Sakakibara, Y., Horiuchi, T., 1989. Canine distemper virus infection in lesser pandas (Ailurus fulgens). Nihon juigaku zasshi. Jpn. J. Vet. Sci. 51, 1263–1266.

Lan, N.T., Yamaguchi, R., Furuya, Y., Inomata, A., Ngamkala, S., Naganobu, K., Kai, K., Mochizuki, M., Kobayashi, Y., Uchida, K., Tateyama, S., 2005a. Pathogenesis and phylogenetic analyses of canine distemper virus strain 007Lm, a new isolate in dogs. Vet. Microbiol. 110, 197–207.

Lan, N.T., Yamaguchi, R., Hirai, T., Kai, K., Morishita, K., 2009. Relationship between growth behavior in vero cells and the molecular characteristics of recent isolated classified in the Asia 1 and 2 groups of canine distemper virus. J. Vet. Med. Sci./Jpn. Soc. Vet. Sci. 71, 457–461.

Lan, N.T., Yamaguchi, R., Inomata, A., Furuya, Y., Uchida, K., Sugano, S., Tateyama, S., 2006. Comparative analyses of canine distemper viral isolates from clinical cases of canine distemper in vaccinated dogs. Vet. Microbiol. 115, 32–42.

Lan, N.T., Yamaguchi, R., Uchida, K., Sugano, S., Tateyama, S., 2005b. Growth profiles of recent canine distemper isolates on Vero cells expressing canine signalling lymphocyte activation molecule (SLAM). J. Comp. Pathol. 133, 77–81.

Li, H., Durbin, R., 2010. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics 26, 589–595.

Miyajima, N., Takeda, M., Tashiro, M., Hashimoto, K., Yanagi, Y., Nagata, K., Takeuchi, K., 2004. Cell tropism of wild-type measles virus is affected by amino acid substitutions in the P, V and M proteins, or by a truncation in the C protein. J. Gen. Virol. 85, 3001–3006.

Muhlebach, M.D., Mateo, M., Sinn, P.L., Prufer, S., Uhlig, K.M., Leonard, V.H., Navaratnarajah, C.K., Frenzke, M., Wong, X.X., Sawatsky, B., Ramachandran, S., McCray Jr., P.B., Cichutek, K., von Messling, V., Lopez, M., Cattaneo, R., 2011. Adherens junction protein nectin-4 is the epithelial receptor for measles virus. Nature 480, 530–533.

Nakatsu, Y., Takeda, M., Ohno, S., Shirogane, Y., Iwasaki, M., Yanagi, Y., 2008. Measles virus circumvents the host interferon response by different actions of the C and V proteins. J. Virol. 82, 8296–8306.

Norrby, E., Appel, M.J., 1980. Humoral immunity to canine distemper after immunization of dogs with inactivated and live measles virus. Arch. Virol. 66, 169–177.

Noyce, R.S., Bondre, D.G., Ha, M.N., Lin, L.T., Sisson, G., Tsao, M.S., Richardson, C.D., 2011. Tumor cell marker PVRL4 (nectin 4) is an epithelial cell receptor for measles virus. PLoS Pathog. 7, e1002240.

Ono, N., Tatsuo, H., Hidaka, Y., Aoki, T., Minagawa, H., Yanagi, Y., 2001. Measles viruses on throat swabs from measles patients use signaling lymphocytic activation molecule (CDw150) but not CD46 as a cellular receptor. J. Virol. 75, 4399–4401.

Orvell, C., Norrby, E., 1980. Immunological relationships between homologous structural polypeptides of measles and canine distemper virus. J. Gen. Virol. 50, 231–245.

Osterhaus, A.D., Groen, J., De Vries, P., UytdeHaag, F.G., Klingeborn, B., Zarnke, R., 1988. Canine distemper virus in seals. Nature 335, 403–404.

Perpinan, D., Ramis, A., Tomas, A., Carpintero, E., Bargallo, F., 2008. Outbreak of canine distemper in domestic ferrets (Mustela putorius furo). Vet. Rec. 163, 246–250.

Pratakpiriya, W., Seki, F., Otsuki, N., Sakai, K., Fukuhara, H., Katamoto, H., Hirai, T., Maenaka, K., Techangamsuwan, S., Lan, N.T., Takeda, M., Yamaguchi, R., 2012. Nectin4 is an epithelial cell receptor for canine distemper virus and involved in the neurovirulence. J. Virol..

Qiu, W., Zheng, Y., Zhang, S., Fan, Q., Liu, H., Zhang, F., Wang, W., Liao, G., Hu, R., 2011. Canine distemper outbreak in rhesus monkeys, China. Emerg Infect Diseases 17, 1541–1543.

Roelke-Parker, M.E., Munson, L., Packer, C., Kock, R., Cleaveland, S., Carpenter, M., O'Brien, S.J., Pospischil, A., Hofmann-Lehmann, R., Lutz, H., Mwamengele, G.L., Mgasa, M.N., Machange, G.A., Summers, B.A., Appel, M.J., 1996. A canine distemper virus epidemic in Serengeti lions (Panthera leo). Nature 379, 441–445.

Roscoe, D.E., 1993. Epizootiology of canine distemper in New Jersey raccoons. J. Wildl. Dis. 29, 390–395.

Rothlisberger, A., Wiener, D., Schweizer, M., Peterhans, E., Zurbriggen, A., Plattet, P., 2010. Two domains of the V protein of virulent canine distemper virus selectively inhibit STAT1 and STAT2 nuclear import. J. Virol. 84, 6328–6343.

Sawatsky, B., Delpeut, S., von Messling, V., 2011. Canine distemper virus. In: Samal, S.K. (Ed.), The Biology of Paramyxoviruses. Caister Academic Press, Norfolk, pp. 275–291.

Seki, F., Ono, N., Yamaguchi, R., Yanagi, Y., 2003. Efficient isolation of wild strains of canine distemper virus in Vero cells expressing canine SLAM (CD150) and their adaptability to marmoset B95a cells. J. Virol. 77, 9943–9950.

Seki, F., Yamada, K., Nakatsu, Y., Okamura, K., Yanagi, Y., Nakayama, T., Komase, K., Takeda, M., 2011. 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. J. Virol. 85, 11871–11882.

Shirogane, Y., Takeda, M., Tahara, M., Ikegame, S., Nakamura, T., Yanagi, Y., 2010. Epithelial-mesenchymal transition abolishes the susceptibility of polarized epithelial cell lines to measles virus. J. Biol. Chem. 285, 20882–20890.

Stephenson, J.R., ter Meulen, V., 1979. Antigenic relationships between measles and canine distemper viruses: comparison of immune response in animals and humans to individual virus-specific polypeptides. Proc. Nat. Acad. Sci. U.S.A. 76, 6601–6605.

Sun, Z., Li, A., Ye, H., Shi, Y., Hu, Z., Zeng, L., 2010. Natural infection with canine distemper virus in hand-feeding Rhesus monkeys in China. Veterinary microbiology 141, 374–378.

Tahara, M., Takeda, M., Yanagi, Y., 2005. Contributions of matrix and large protein genes of the measles virus Edmonston strain to growth in cultured cells as revealed by recombinant viruses. J. Virol. 79, 15218–15225.

Tahara, M., Takeda, M., Yanagi, Y., 2007. Altered interaction of the matrix protein with the cytoplasmic tail of hemagglutinin modulates measles virus growth by affecting virus assembly and cell-cell fusion. J. Virol., 81.

Takeda, M., Kato, A., Kobune, F., Sakata, H., Li, Y., Shioda, T., Sakai, Y., Asakawa, M., Nagai, Y., 1998. Measles virus attenuation associated with transcriptional impediment and a few amino acid changes in the polymerase and accessory proteins. J. Virol. 72, 8690–8696.

Takeda, M., Tahara, M., Hashiguchi, T., Sato, T.A., Jinnouchi, F., Ueki, S., Ohno, S., Yanagi, Y., 2007. A human lung carcinoma cell line supports efficient measles virus growth and syncytium formation via a SLAM- and CD46-independent mechanism. J. Virol. 81, 12091–12096.

Takeda, M., Tahara, M., Nagata, N., Seki, F., 2011. Wild-type measles virus is intrinsically dual-tropic. Front. Microbiol. 2, 279.

Takeuchi, K., Miyajima, N., Kobune, F., Tashiro, M., 2000. Comparative nucleotide sequence analysis of the entire genomes of B95a cell-isolated and Vero cell-isolated measles viruses from the same patient. Virus Genes 20, 253–257.

Tatsuo, H., Ono, N., Tanaka, K., Yanagi, Y., 2000. SLAM (CDw150) is a cellular receptor for measles virus. Nature 406, 893–897.

Tatsuo, H., Ono, N., Yanagi, Y., 2001. Morbilliviruses use signaling lymphocyte activation molecules (CD150) as cellular receptors. J. Virol. 75, 5842–5850.

Taylor, J., Pincus, S., Tartaglia, J., Richardson, C., Alkhatib, G., Briedis, D., Appel, M., Norton, E., Paoletti, E., 1991. Vaccinia virus recombinants expressing either the measles virus fusion or hemagglutinin glycoprotein protect dogs against canine distemper virus challenge. J. Virol. 65, 4263–4274.

Wang, L.-F., Collins, P.L., Fouchier, R.A.M., Kurath, G., Lamb, R.A., Randall, R.E., Rima, B.K., 2012. Family paramyxoviridae. In: King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.), Virus Taxonomy, 9th ed. Elsevier Academic Press, London, pp. 672–685.

von Messling, V., Svitek, N., Cattaneo, R., 2006. Receptor (SLAM [CD150]) recognition and the V protein sustain swift lymphocyte-based invasion of mucosal tissue and lymphatic organs by a morbillivirus. J. Virol. 80, 6084–6092.

Note

Manuscript type: Notes:

Manuscript ID MAI-2012-191 revision II

Title: Simple method to differentiate measles vaccine from wild-type strains using loop-mediated isothermal amplification (LAMP)

Tetsuo Nakayama [1)], Akihito Sawada [1)], Hideyuki Kubo [2)], Atsushi Kaida [2)], Toshimitsu Tanaka [3)], Naoki Shigemoto [4)], Katsuhiro Komase [5)], Makoto Takeda [5)]

1) Kitasato Institute for Life Sciences, Laboratory of Virus Infection.
2) Osaka City Institute of Public Health and Environmental Sciences.
3) Chiba City Institute of Public Health and Environmental Sciences.
4) Center for Public Health and Environment, Hiroshima Prefectural Technology Research Institute.
5) National Institute for Infectious Diseases, Department Virology III

Running title: Identification of measles vaccine strain

Corresponding author: T. Nakayama.
Kitasato Institute for Life Sciences, Laboratory of Virus Infection, Shirokane 5-9-1, Minato-ku, Tokyo 108-8641, Japan.
TEL: 81-3-5791-6269, FAX: 81-3-5791-6130
E-mail: tetsuo-n@lisci.kitasato-u.ac.jp

Accepted Article

Abstract

Through increasing measles vaccine coverage, proportion of patients with modified measles has been increasing. They demonstrate low-grade fever with very mild eruptions similar to vaccine-related adverse events. Differentiation between the two pathogenic conditions is required to improve the quality of laboratory-based measles surveillance, In this report, vaccine-specific and wild-type specific primer sets were designed for the loop-mediated isothermal amplification (LAMP) in the N gene, and vaccine strains, C1, D3, D4, D5, D8, D9, G3, and H1 wild strains were examined. Three vaccine strains were efficiently amplified using a vaccine-specific primer set with an approximately 10-times higher sensitivity than wild-type primer.

Modified measles was differentiated from vaccine-associated cases using this system, but with some limitations for the other genotypes.

Key words: measles vaccine, LAMP, modified measles

Abbreviations

BIP (backward inner primer)

FIP (forward inner primer)

LAMP (loop-mediated isothermal amplification)

RFLP (restriction fragment length polymorphisms)

RT-LAMP (reverse transcription loop-mediated isothermal amplification)

vac-specific (vaccine-specific)

wt-specific (wild type-specific)

The number of measles-related deaths decreased from 873,000 in 1999 to 164,000 in 2008, and the goal of 2010 to reduce measles deaths by 90% before 2000 was not realized (1). Global measles vaccination coverage increased from 72% in 2000 to 82% for the first dose in 2007, and the two-dose immunization strategy was recommended for countries with a high coverage of the first-dose measles vaccine, at more than 95%. Most countries (88%) now implement the two-dose strategy (1). But, measles transmission has increased, and outbreaks have become widespread since late 2009 in EU region because of failure to vaccinate to susceptible populations (2). The World Health Assembly updated the goal of measles elimination of 95% reduction of measles mortality by 2015, compared to 2000 (3). In Japan, measles outbreaks occurred in 2007-2008, and they showed different characteristics whereby most patients were young adults or adolescents attending high schools and universities at an early stage of the outbreak (4, 5). A relatively large proportion of those with adult measles had a previous history of single dose of measles vaccine and, thus, typical measles symptoms were not observed, only mild grade fever and rash (5). These patients with modified measles exhibited symptoms similar to the adverse events of vaccines with low-grade fever in 10-20% of vaccine recipients (6). These modified measles patients were confirmed by virus isolation, detection of virus genome, or serological responses of significant increase in IgG antibodies and/or presence of IgM antibodies (7), but it is difficult to diagnose a modified measles by serology for IgM positivity because of the past history of vaccination. Virus isolation is not always successful because of the timing of sample collection, transport, a low virus load, and short period of virus excretion in modified measles. Measles virus genome was directly detected in clinical materials, and should be verified whether it is related to the adverse effects of vaccines or modified measles by sequencing of the PCR products for

molecular genotyping, targeting the N gene. This technique is time consuming and clinical samples do not contain sufficient amounts of viral RNA to perform sequencing analysis.

Circulating wild-type genotypes of measles virus classified into 24 subclades and, among them, B1, C1, D1, E, and F were considered inactive (8). In Japan, three measles vaccine strains have been used; the AIK-C and Schwarz FF8 strains were further attenuated from the Edmonston strain and CAM from the domestic wild-type, genotype A (9). It is critical to make an accurate diagnosis with high sensitivity and specificity using a simple method. Recent advances in molecular technology have improved the sensitivity and simplicity of genome amplification. A rapid diagnostic procedure for the detection of the measles genome was reported using loop-mediated isothermal amplification (RT-LAMP) (10, 11). In this report, it was modified for the discrimination of measles vaccine strain from wild circulating strains.

In Japan, C1, D3, D5 Palau-type, H1, D5 Bangkok-type, and D9 have circulated since 1984 (5,12). Representative strains were used in this study: MVi/Tokyo.JPN/84-K [C1], MVi/Tokyo.JPN/37.99(Y) [D3], MVi/Tokyo.JPN/21.00 (O) [D5 Palau-type], MVi/Tokyo.JPN/20.00(S) [H1], MVi/Tokyo.JPN/17.07 [D5 Bangkok-type], and MVi/Aichi.JPN/44.06 [D9]. In 2011, D4, D8, D9, and G3 strains were imported from outsides [National Institute of Infectious Diseases, Infectious Disease Surveillance Center; http://idsc.nih.go.jp/disease/measles/2011]. MVi/Chiba C.JPN/08.11 [G3], MVi/Hiroshima.JPN/09.11 [D8], and MVi/Osaka C.JPN/09.11 [D4] were also investigated. During the measles outbreak in 2007-08, 18 clinical samples were obtained from measles patients and those with non-typical measles demonstrating mild febrile illness and eruptions (5). Among them, 13 stocked samples were available for this study.

Five vaccine-associated cases after 2008 were investigated.

Total RNA was extracted from 200 µl of culture medium or clinical samples using magnetic beads RNA purification kit (TOYOBO Co. Ltd., JPN), and re-suspended in 30 µl. cDNA was synthesized from 5 µl of RNA, using One Step PrimeScript® RT-PCR Kit (TaKaRa Bio, Otsu, Japan) with poly T primer and random hexamer. Five µl of cDNA was used for the amplification of measles genome by LAMP. The LAMP method is characterized by auto-cycling DNA synthesis using *Bst* DNA polymerase with strand displacement (NEW ENGLAND BioLabs, U.S.) and a specially designed set of primers, as is shown in Fig. 1. Six LAMP primers were synthesized, recognizing eight different positions: F3, B3, F Loop, and BIP were the same as previously reported (11). In this report, vaccine- and wild-type-specific FIPs and B Loop primers were used: vaccine-specific FIP (FIP-*Vac*: 5'-TTGTCCTCAGTAGTATGCATTGCAGGTATCACTGCCGAGGATG), wild-specific FIP (FIP-*Wt*: 5'-CTGTCCTCAGTAGTATGCATTGCAGGTATCACTGCCGAGGATG), vaccine-specific B Loop (B Loop-*VAC*: 5'-TGAGAATGAGCTACCGA), and wild-specific B Loop (B Loop-*Wt* : 5'-TGAGAATGAGCTACCAG) (Fig. 1). The bold letters at the 5' end of the FIP and the 3' of the B Loop were specific for the vaccine and wild-type sequences. The reaction mixture and procedure were already reported and the differentiation of vaccine or wild-type strain was realized based on which primer set reached the threshold of LAMP amplification faster (11).

N gene ORF of the AIK-C and MVi/Tokyo.JPN/37.99(Y) [D3] were amplifed and inserted into the multi-cloning sites of pBluescript SK II. pAIK-N and pWt D3-N were constructed. Serial dilutions of pAIK-N and pWt D3-N were subjected to vac-specific and wt-type specific LAMP, and the results are shown in Fig. 2. Detection limit of the

Accepted Article

measles AIK-C vaccine genome was 1-10 copies by vac-specific LAMP but 10-100 copies with lower efficiency by wt-specific LAMP. Wild-type pWt D3-N was detected with a detection limit of 10-100 copies by wt-specific LAMP, more than 10 min faster than vac-specific LAMP. It showed the similar detection limit of 30-100 copied by the original RT-LAMP (11). The measles vaccine strain genomes from Schwarz FF-8 and CAM (10^4 TCID50/ml) were amplified using vac-specific LAMP more than ten min faster than wt-specific LAMP. The time difference in amplification between vac-specific and wt-specific LAMP increased with further dilutions.

Wild-type measles genotypes C1, D3, D4, D5 Palau, D5 Bangkok, D8, D9, G3, and H1 were also investigated. These isolates contained approximately 10^4 TCID 50/ml and and serial 10-fold dilutions of cDNA were subjected to or vac- and wt-specific LAMP. The results of recent isolates of D4, D5, D8, D9, and G3 are shown in Fig. 3. The measles genome was efficiently amplified by wt-specific LAMP, more than 10 min earlier than by vac-specific LAMP in D4, D5 Palau, D5 Bangkok, D8, and D9 genotypes. As for the G3, 1:100 dilution of G3 was amplified by wt-LAMP three min faster and approximately 10 min faster for 1:1,000 dilutions than by vac-LAMP. Amplification profile of H1 was similar to that of G3. But C1 was amplified within 1-2 min differences by both vac-specific and wt-specific LAMP (data not shown).

Clinical samples obtained from the patients with low-grade fever and rash were examined and the results are shown in Fig. 2. One demonstrated febrile illness 10 days after immunization and another during measles outbreak, as previously reported (5). In patient suspected of vaccine adverse event, the measles genome was amplified only by the vac-specific LAMP, and the wt-specific LAMP amplified the wild-type genome more efficiently in another patient with modified measles during measles outbreak. These two

cases were confirmed by sequence analysis. Among 13 stocked samples in 2007-08 outbreak, all were efficiently amplified by wt-specific LAMP as did by the original RT-LAMP. Among five vaccine-associated cases after 2008, four were identified as vaccine strain. The remaining one case was negative for LAMP and also negative for RT-PCR. Conventional nested PCR and real-time PCR were done but all were negative. We supposed that sample taking or transporting condition was not appropriate.

The WHO in Western Pacific Region set a goal to eliminate measles by 2012, and a two dose schedule with a catch-up campaign for forthcoming five years was implemented in Japan in 2008 (4). Thereafter, the number of reported cases of measles decreased from 11,015 in 2008 to 457 in 2010. Among 457 cases in 2010, approximately two thirds were laboratory- confirmed cases, being estimated as 3.58 per million. Some imported cases were reported with D4, D8, D9, and H1 genotypes (13). After Tsunami disaster in 2011, D4, D8, D9, and G3 strains were imported from outsides [National Institute of Infectious Diseases, Infectious Disease Surveillance Center; http://idsc.nih.go.jp/disease/measles/2011pdf]. In the control phase, identification of the index case was difficult especially for modified measles, making it hard to correctly diagnose measles infection, excluding rubella, Parvovirus B19 infection, and other virus infection, because of cross-reactivity with IgM measles antibody (14). The proportion of modified measles cases is increasing, with low-grade febrile illness and mild rash, similar to vaccine-associated illness. The restriction fragment length polymorphisms (RFLP) was developed to determine whether they were vaccine-associated adverse events or wild measles infection (15). The measles genome was amplified by RT-nested PCR, PCR products were electrophoresed after treatment with restriction enzymes, and it took several hours to obtain the results.

LAMP is characterized by high sensitivity and specificity to amplify target DNA (10, 11). RT-LAMP systems were reported to detect several virus genomes with higher sensitivity than conventional nested PCR. Mumps vaccine has the problem of aseptic meningitis after vaccination, and a differentiation method was reported whereby LAMP products were digested with specific restriction enzyme. Mumps Hoshino strain has *Sca* I site in the LAMP target region, and the LAMP-specific ladder pattern disappeared after digestion with *Sca* I but not for the wild-type strains (16). This detection system required additional procedures: opening the LAMP tube, treatment with restriction enzyme, and electrophoresis. It is an undesirable operative step to open the lid of the LAMP tube as it may lead to DNA cross-contamination. Single-step operation is ideal to obtain results. As RSV has two distinctly different subgroups, A and B, sequence-specific LAMP primers were designed specifically for those subgroups in the same target region, and the subgroup-specific primer sets amplified the respective genomes (17).

The key reaction for LAMP is dumb-bell loop formation to react with FIP or BIP primers, and inner sequences of F1 and B1 are critical for loop construction (10). The FIP primer consisted of 5' F1 complement connected to the F2 sequence, and the 5' end of FIP or BIP was critical to bind to the 3' portion of F1 or B1. In a previous report, LAMP primers were designed in the conserved regions to detect the measles genome with a high sensitivity. Sequence results between the F1 and B1 of vaccine and wild-type strains showed that all vaccine strains had A at genome position 1321 but G in all wild-types (11). In this study, vaccine-specific and wild-type-specific FIP primers were designed to have one nucleotide shift to the inner region in comparison with the previous original LAMP FIP primer sequence, adding T at the 5' end of FIP for vaccine-specific and C for wild-type-specific FIP. The addition of loop primers resulted in shorter reaction time to

work for displacement of stem loop region and most genotypes had GG or AG (genome position 1397 and 1398) at the 3' end of B Loop primer. All wild-types except C1 strain were amplified more efficiently by wt-specific LAMP than vac-specific LAMP, but C1 was amplified within 1-2 min differences in comparison with vac-specific and wt-specific LAMP. Discrimination difficulty would be be similar to B2, B3 and C2 genotypes, having the same sequence of the 5'end of FIP and the 3'end of B loop. C1 genotype was inactive but, at present, B2, B3, and C2 genotypes are not introduced into Japan, but still active (8, 12, 13). There would be a possibility of invasion. When definite amplification delay was not observed, sequence analysis should be performed. As for the G3 and H1, the time difference in genome amplification by wt-specific and vac-specific LAMP became apparent in samples with lower genome concentration.

Five clinical samples are obtained and four were vaccine-associated adverse events, demonstrating the amplification by vac-specific primer set. In Japan, C1, D3, D5 Palau-type, H1, D5 Bangkok-type, and D9 have circulated since 1984 (5, 12, 13), and D4, D8, D9, and G3 strains were imported from outsides in 2011 [National Institute of Infectious Diseases, Infectious Disease Surveillance Center; http://idsc.nih.go.jp/disease/measles/2011]. Clinical samples of D4, D8, D9, and G3 were not obtained but the differentiation method using vac- and wt-specific LAMPs would work for surveillance study.

Disclosure

All authors had no conflict of interest regarding this study.

References

1. WHO. (2009) Measles vaccines: WHO position paper. *Wkly Epidemiol Rec* **84**: 349-60.
2. CDC. (2011) Increased transmission and outbreaks of measles – European region, 2011. *MMWR* **60**:1605-10.
3. Okwo-Bele J.M., Cherian T. (2011) The expanded programme on immunization: a lasting legacy of smallpox eradication. *Vaccine* **29S**: D74-D79
4. CDC. (2008) Progress toward measles elimination - Japan, 1999-2008. *MMWR* **57**: 1049-52.
5. Nagai M., Ji Y-X., Yoshida N., Miyata A., Fujino M., Ihara T., Yoshikawa T., Asano Y., Nakayama T. (2009) Modified adult measles in outbreaks in Japan, 2007-2008. *J Med Virol* **81**: 1094-101.
6. Makino S. (1983) Development and characteristics of live AIK-C measles virus vaccine: a brief report. *Rev Infect Dis* **5**: 504-5.
7. WHO. (2005) Global measles and rubella laboratory network - update. *Wkly Epidemiol Rec* **80**: 384-8.
8. WHO. (2012) Measles virus nomenclature update: 2012. *Wkly Epidemiol Rec* **87**: 73-81.
9. Hirayama M. (1983) Measles vaccines used in Japan. *Rev Infect Dis* **5**: 495-503.
10. Notomi T., Okayama H., Masubuchi H., Yonekawa T., Watanabe K., Amino N., Hase T. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic. Acids Res.* **28**: e63.
11. Fujino M., Yoshida N., Yamaguchi S., Hosaka N., Ota Y., Notomi T., Nakayama T. (2005) A simple method for the detection of measles virus genome by loop-mediated

isothermal amplification (LAMP). *J Med Virol* 76: 406-13.

12. Nakayama T., Fujino M., Yoshida N. (2004) Molecular epidemiology of measles virus in Japan. *Pediatr Int* 46: 214-21.
13. National Institute of Infectious Diseases of Japan, Infectious Diseases Surveillance Center. (2011) Measles in 2010. *IASR* 32: 31-2.
14. Thomas H.I.J., Barrett E., Hesketh L.M., Wynne A., Morgan-Carpner P. (1999) Simultaneous IgM reactivity by EIA against more than one virus in measles, parvovirus B19 and rubella infection. *J Clin Virol* 14: 107-18.
15. Mori T. (1994) A simple method for genetic differentiation of the AIK-C vaccine strain from wild strains of measles virus. *Biologiclals* 22: 179-85.
16. Yoshida N., Fujino M., Ota Y., Notomi T., Nakayama T. (2007) Simple differentiation method of mumps Hoshino vaccine strain from wild strains by reverse transcription loop-mediated isothermal amplification (RT-LAMP). *Vaccine* 25: 1281-6.
17. Ushio M., Yui I., Yoshida N., Fujino M., Yonekawa T., Ota Y., Notomi T., Nakayama T. (2005) Detection of respiratory syncytial virus genome by subgroups-A, B specific reverse transcription loop-mediated isothermal amplification (RT-LAMP). *J Med Virol* 77: 121-7.

Figure legends

Fig. 1. Primer design for vaccine-specific and wild-type specific LAMP primers.

The upper panel shows the schematic location of LAMP primers. Sequence alignments of vaccine and wild strains are shown between F1C and B2 primer positions in the middle panel. Different nucleotides are depicted in comparison with the Edmonston and vaccine strains (AIK-C, Schwarz FF8, CAM70, and S191). The underlined sequence was the original measles LAMP primers. The lower panel shows vac-specific and wt-specific LAMP primer sequences. The first bold letter at the 5'end of FIP-*Vac* and FIP-*Wt* and two bold letters at the 3'end of B Loop-*Vac* and B Loop-*Wt* are specific to the vaccine or wild-type sequence.

Fig. 2. LAMP reaction of the vaccine strains and comparison of the sensitivity.

N gene expressing plasmids were constructed from AIK-C vaccine (pAIK-N) and D3 wild strain (pWt D3-N) and plasmid concentration was adjusted to 1,000 copies/one reaction and serial 10-fold dilutions pAIK-N and pWt D3-N were subjected to LAMP specific to the vaccine- and wild-type primer sets. The reaction times were compared to reach to the threshold of 0.1 in turbidity. Dotted lines show amplification responses using wild-type specific (Wt) primer set and solid lines by vaccine-specific (Vac) primer set. Two clinical samples are shown in the lower panels obtained from the patients suspected as vaccine associated or modified measles cases.

Fig. 3. LAMP reaction of wild types, D4, D5, D8, D9 and G3.

RNA of virus isolates was converted to cDNA and serial 10-fold dilutions were subjected to LAMP reaction. The reaction times were compared to reach to the threshold of 0.1 in turbidity. Dotted lines show amplification responses using wild-type specific (LAMP-Wt) primer set and solid lines by vaccine-specific (LAMP-Vac) primer set.

Accepted Article

Figure 1.

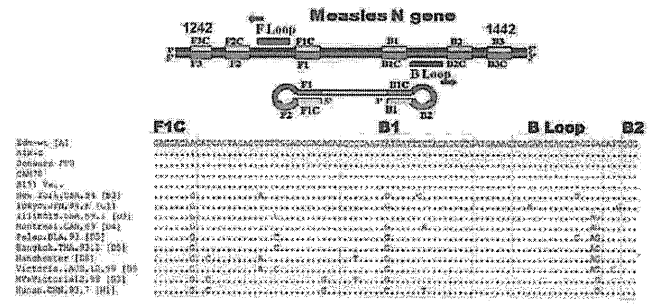
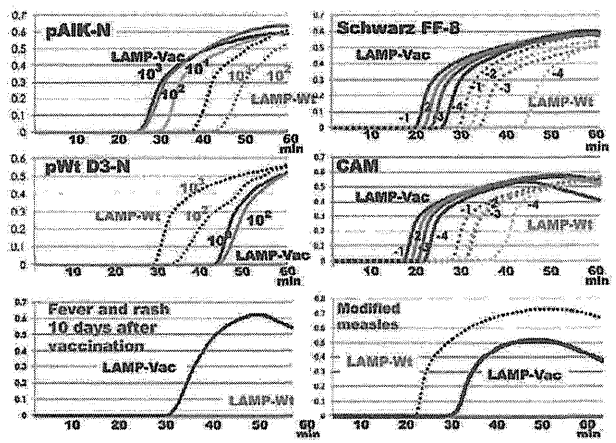
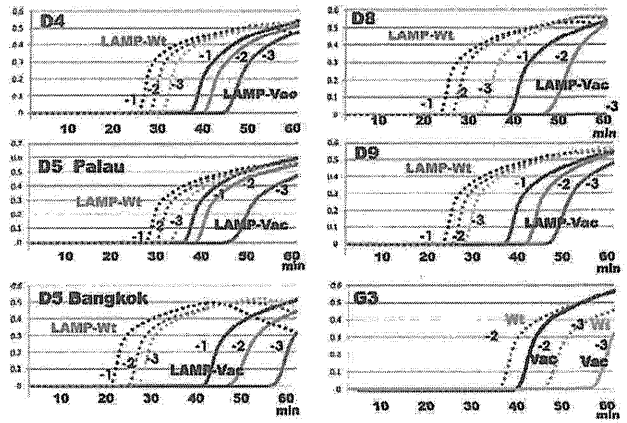


Figure 2.



Accepted Article

Figure 3.



Laboratory and Epidemiology Communications

Progress toward Measles Elimination between 2008 and 2010 in the Hokkaido District, Japan

Hideki Nagano*, Masaru Jinushi, Rika Komagome, Masahiro Miyoshi, Masayuki Kikuchi[1], Emi Muratsubaki[1], Harumi Ito[1], Maki Inoue, and Motohiko Okano

Hokkaido Institute of Public Health, Sapporo 060-0819; and
[1]Sapporo City Institute of Public Health, Sapporo 003-8505, Japan

Communicated by Makoto Takeda

(Accepted August 2, 2011)

Measles is an acute and highly contagious disease mainly characterized by high fever, cough, and a maculopapular rash (1). Since the occurrence in Japan of a nation-wide measles epidemic in 2001 (2), measles control efforts have intensified across the county, resulting in a dramatic decrease in total cases. However, a small and local epidemic of measles did occur in 2006 in the Kanto area and expanded to impact the entire country. In the Hokkaido district, the northern islands of Japan, we experienced several local epidemics of measles between 2007 and 2008 (3). A feature of these local epidemics was that the infection mainly targeted both vaccinated and unvaccinated teenagers. This measles epidemic led to a change in the surveillance system used in Japan, requiring mandatory reporting instead of sentinel reporting as of January 1, 2008 (2). Herein, we report the status of measles infections and surveillance systems between 2008 and 2010 in the Hokkaido district.

In 2008, 1,462 measles cases were reported in the Hokkaido district, of which 951 (65%) were diagnosed clinically, 408 (28%) were confirmed by laboratory diagnoses, and 103 (7%) were deemed as "modified" measles (Table 1). Regarding age distribution, the highest peak occurred in patients aged 16 years, and patients aged less than 20 years accounted for approximately 70% of all cases. Vaccinated patients numbered 419 (29%), 664 (45%) were unvaccinated, and the vaccination status was unknown for 379 (26%) patients. Fever, rash, and cough were observed in greater than 80% of the patients. Throat swab specimens were obtained from 28 suspected cases, and viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Germantown, Md., USA). A one-step RT-PCR Kit (Qiagen) was used to amplify the measles virus nucleoprotein (N) gene as previously described (3). Measles virus RNA was detected in 20 specimens, and further isolation was conducted on 5 specimens using Vero/hSLAM cells (4). These 20 specimens containing measles virus RNA all belonged to the genotype D5, identical to the virus isolated in other areas of Japan.

A drastic decline in reported cases (17 total) was observed in 2009, and the portion of clinical diagnoses also decreased to 6 cases (35%; Table 1). The measles IgM enzyme immunoassay (EIA) was performed at commercial laboratories on specimens from the remaining 11 cases. However, the EIA values were not reported to the regional Public Health Centers. We did not have the opportunity to perform RT-PCR on any clinical specimens from these reported cases. Only 2 throat swabs were obtained from the other suspected measles cases, and both were negative by RT-PCR.

Table 1. Measles cases by the surveillance system between 2008 and 2010 in the Hokkaido district, Japan

Year	Reported measles cases	% only by clinical diagnosis	Positive no. of RT-PCR/ cases tested[1)]
2008	1,462	65	20/28
2009	17	35	0/2
2010	5	20	2/15

[1)]; "Cases tested" indicates number of clinical specimens obtained as a suspected case at Prefectural and Municipal Public Health Institutes in the Hokkaido district.

The number of reported measles cases further decreased to 5 in 2010 (Table 1). One case was a clinically diagnosed 2-year-old boy, who presented with fever, cough, runny nose, and rash, did not have laboratory confirmation, and had not been vaccinated. Another case was "modified" measles with clinical symptoms that included fever, rash, and positive IgM for measles by EIA performed in a commercial laboratory; however, the IgM EIA results were not reported to the regional Public Health Center. The clinical specimens for these 2 cases were not submitted to Prefectural and Municipal Public Health Institutes for the confirmation of infection. The other 2 were imported measles cases: one (Case no. 2 shown in Table 2) was a Chinese woman aged 28 years with unknown vaccination status visiting Sapporo on May 1, and the other (Case no. 13) was a Japanese woman aged 35 years with a history of no measles vaccination who had traveled to India from November 2 to 10. Clinical specimens were obtained 7 and 11 days after fever onset from the Chinese (throat swab, urine, and whole blood) and Japanese (whole blood and urine) patients, respectively. Measles IgM EIA (Denka Seiken Co., Tokyo, Japan) was performed according to the manufacturer's instructions and EIA index values were highly positive (14.03 and 25.87, respectively). The RT-PCR results for specimens from both patients were all positive. Phylogenetic analyses were performed by direct sequencing of the amplified products of the N gene (450 nucleotides [nt]) as previously described (3). The 2 strains detected (Mvi/Sapporo. JPN/19.10/1 and Mvi/Sapporo. JPN/48.10/1) were identified as H1 (the Chinese patient, GenBank accession no. AB569977) (the Infectious Agents Surveillance Report [IASR] online at http://idsc.nih.go.jp/iasr/rapid/pr3652.html in Japanese) and D4 (the Japanese patient, GenBank accession no. AB605257) (IASR online at http://idsc.nih.go.jp/iasr/rapid/pr3722.html in Japanese), respectively (Fig. 1). Measles virus was successfully isolated from the specimens of the Chinese patient alone using Vero/hSLAM cells. Case

Table 2. Laboratory test results of suspected measles cases performed at Prefectural and Municipal Public Health Institutes in the Hokkaido district, 2010

Case no.	Day after the onset of fever	Age (y)	Sex	Specimen	IgM index value	RT-PCR	Vaccination status
1	1	1	Male	Ts	ND	—	Vaccinated
2	7	28	Female	Ts, U, WB	14.03	H1	Unknown
3	4	1	Male	Ts, U, WB	1.14	—	Vaccinated
4	6	1	Female	Ts, WB	0.8	—	Vaccinated
5	2	32	Female	Ts, WB	0.02	—	Unknown
6	15	51	Male	Ts, WB	4.73	—	Unknown
7	5	4	Male	Ts, WB	0.86	—	Vaccinated
8	4	1	Male	Ts, U, WB	0.05	—	Vaccinated
9	14	0	Male	Ts, WB	0.46	—	Unvaccinated
10	6	28	Female	Ts, U, WB	2.32	—	Vaccinated
11	12	2	Male	S	1.66	—	Vaccinated
12	11	1	Male	Ts, WB	0.06	—	Unvaccinated
13	11	35	Female	U, WB	25.87	D4	Unvaccinated
14	?	?	Female	U	ND	—	Unknown
15	7	30	Female	U	ND	—	Vaccinated

?, unspecified; Ts, throat swab; U, urine; WB, whole blood; S, serum; ND, not done; —, negative; H1, measles virus genotype H1; D4, measles virus genotype D4.

*Corresponding author: Mailing address: Hokkaido Institute of Public Health, North 19 West 12, Kita-ku, Sapporo 060-0819, Japan. Tel: +81-11-747-2722, Fax: +81-11-747-2757, E-mail: nagano@iph.pref.hokkaido.jp

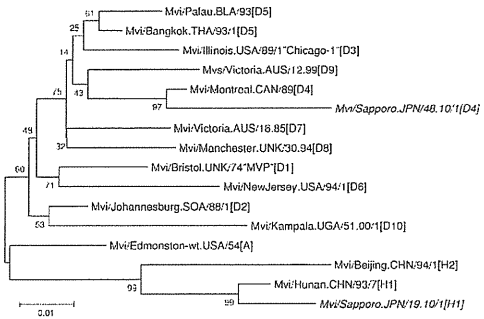


Fig. 1. Phylogenetic tree of 450 nucleotides of N gene of measles virus. Numerals at each branch indicate the bootstrap values of the clusters in percent. The present strains are represented in italics.

no. 10 was a female aged 28 years with "modified" measles and a history of one measles vaccination, who presented with fever and rash and whose blood specimen indicated a measles IgM index value of 2.32 (weakly positive) but was negative for RT-PCR test on measles RNA and negative by IgM and PCR for parvovirus B19, which commonly causes an illness with rash (5). This case was not able to be confirmed by intensive laboratory tests; however, the possibility of true measles could not be ruled out. Furthermore, all other suspected cases were not confirmed by the RT-PCR test. Thus, as described above, only 2 cases could have been classified as laboratory-confirmed ones.

Since the measles epidemic in 2008, the number of reported cases has dramatically declined each year. We had learned of only 5 reported measles cases in the Hokkaido district in 2010, 2 of which were confirmed by laboratory tests and were imported. Therefore, judging from the criterion of measles elimination that indicates an incidence of <1 case of measles confirmed by laboratory or epidemiologic linkage per 1,000,000 population is necessary, while excluding imported cases (6), endemic measles transmission in the Hokkaido district (approximately 5,500,000 population) may be considered as eliminated. With the low number of measles cases occurring due to high vaccination coverage, some laboratory tests, including molecular tests and those that approach virus isolation in ways other than serologic assays, e.g., IgM EIA, may be useful for final diagnosis in suspected measles cases that have low values for IgM EIA, possibly due to cross-reactivity to other viral infections such as parvovirus B19 and human herpes virus type 6 (5). Thus, the use of molecular-based tests, in addition to serologic assays, to confirm measles infections is required. Maintaining high vaccination coverage and using an improved surveillance system that includes viral detection are important for the elimination of measles.

Acknowledgments We thank the medical practitioners and staff members of public health officers in Hokkaido for collecting samples and their epidemiological surveillance.

This work was financially supported in part by the Hokkaido Government and Grant-in-Aid from the Ministry of Health, Labour and Welfare, Japan.

Conflict of interest None to declare.

REFERENCES

1. Perry, R.T. and Halsey, N. (2004): The clinical significance of measles: a review. J. Infect. Dis., 189, S4–16.
2. Sunagawa, T., Shimada, T., Ueno-Yamamoto, K., et al. (2008): Progress toward measles elimination—Japan, 1999–2008. Morbid. Mortal. Wkly. Rep., 57, 1049–1052.
3. Nagano, H., Jinushi, M., Tanabe, H., et al. (2009): Epidemiological and molecular studies of measles at different clusters in Hokkaido district, Japan, 2007. Jpn. J. Infect. Dis., 62, 209–211.
4. Ono, N., Tatsuo, H., Hidaka, Y., et al. (2001): Measles viruses on throat swabs from measles patients use signaling lymphocytic activation molecule (CDw150) but not CD46 as a cellular receptor. J. Virol., 75, 4399–4401.
5. Thomas, H.I., Barnet, E., Hesketh, I.M., et al. (1999): Simultaneous IgM reactivity by EIA against more than one virus in measles, parvovirus B19 and rubella infection. J. Clin. Virol., 14, 107–118.
6. World Health Organization (2010): Monitoring progress towards measles elimination. Wkly. Epidemiol. Rec., 49, 490–495.

JVI
Journals.ASM.org

Mutant Fusion Proteins with Enhanced Fusion Activity Promote Measles Virus Spread in Human Neuronal Cells and Brains of Suckling Hamsters

Shumpei Watanabe,[a] Yuta Shirogane,[a] Satoshi O. Suzuki,[b] Satoshi Ikegame,[a] Ritsuko Koga,[a] Yusuke Yanagi[a]

Department of Virology[a] and Department of Neuropathology,[b] Faculty of Medicine, Kyushu University, Fukuoka, Japan[a]

Subacute sclerosing panencephalitis (SSPE) is a fatal degenerative disease caused by persistent measles virus (MV) infection in the central nervous system (CNS). From the genetic study of MV isolates obtained from SSPE patients, it is thought that defects of the matrix (M) protein play a crucial role in MV pathogenicity in the CNS. In this study, we report several notable mutations in the extracellular domain of the MV fusion (F) protein, including those found in multiple SSPE strains. The F proteins with these mutations induced syncytium formation in cells lacking SLAM and nectin 4 (receptors used by wild-type MV), including human neuronal cell lines, when expressed together with the attachment protein hemagglutinin. Moreover, recombinant viruses with these mutations exhibited neurovirulence in suckling hamsters, unlike the parental wild-type MV, and the mortality correlated with their fusion activity. In contrast, the recombinant MV lacking the M protein did not induce syncytia in cells lacking SLAM and nectin 4, although it formed larger syncytia in cells with either of the receptors. Since human neuronal cells are mainly SLAM and nectin 4 negative, fusion-enhancing mutations in the extracellular domain of the F protein may greatly contribute to MV spread via cell-to-cell fusion in the CNS, regardless of defects of the M protein.

Measles virus (MV) causes measles, a common acute infectious disease characterized by high fever and a maculopapular rash (1). Despite the availability of effective vaccines, measles still remains epidemic in developing countries. MV sometimes invades the central nervous system (CNS), causing a fatal degenerative disease, subacute sclerosing panencephalitis (SSPE), several years after acute measles (1–4). SSPE has been treated with broad-spectrum antiviral drugs, including ribavirin, interferons, and isoprinosine (4). Although these treatments may be beneficial, causing temporal stabilization of disease progression and prolonged survival, complete remission is not achieved. Therefore, establishment of effective therapy for SSPE is highly desirable, in addition to vaccination against measles to reduce SSPE occurrence.

MV, a member of the family *Paramyxoviridae*, is an enveloped virus with a nonsegmented, negative-strand RNA genome. The MV genome has six genes that encode the nucleocapsid (N), phospho- (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins. The genomic RNA is encapsidated with the N protein and, together with RNA-dependent RNA polymerase composed of the L and P proteins, forms a ribonucleoprotein (RNP) complex (3). There are two envelope glycoproteins, the H and F proteins, which are responsible for receptor binding and membrane fusion, respectively (3). The M protein plays a role in the assembly of virus particles by interacting with the RNP and the cytoplasmic tails of the H and F proteins. MV enters host cells by pH-independent membrane fusion at the cell surface. Binding of the H protein to a cellular receptor is thought to trigger the conformational changes of the F-protein trimer (5–7), thereby inducing the formation of the six-helix bundle (6-HB) structure involved in membrane fusion (8–11). The interaction between two heptad repeat (HR) domains, HR-A and HR-B, in individual F-protein monomers is responsible for the formation of 6-HB. While the F protein, upon activation, induces virus-to-cell fusion, it also causes cell-to-cell fusion in infected cells, producing syncytia (3).

Wild-type MV strains infect immune cells using signaling lymphocyte activation molecule (SLAM) (also called CD150) as a receptor (12), and the tissue distribution of SLAM is consistent with the lymphotropism and immunosuppressive nature of MV (13, 14). CD46, a complement-regulatory molecule expressed on all human cells except red blood cells, functions as a receptor for vaccine and laboratory-adapted strains of MV (15–17), but not for wild-type strains, as specific amino acid changes in the H protein are needed for MV to use CD46 (18, 19). Wild-type MV strains have also been shown to infect SLAM-negative cells, including polarized epithelial cells (20–22) and neuronal cells (23, 24). Recently, nectin 4 was identified as an epithelial cell receptor (25, 26).

A number of studies have reported successful virus isolation from brain tissues of SSPE cases (27–30). Sequencing analyses have revealed characteristic mutations in these viruses (SSPE strains) compared with wild-type MV isolates. In many SSPE strains, A-to-G-biased hypermutations occur in the genome, especially in the M gene (31–33). In some strains, a single deletion or mutation in the P gene causes a transcriptional error, leading to an increase in the level of dicistronic P-M mRNA compared with that of M mRNA. In yet other strains, mutations cause the elongation

Received 26 September 2012 **Accepted** 12 December 2012
Published ahead of print 19 December 2012
Address correspondence to Shumpei Watanabe, wshunpei@virology.med.kyushu-u.ac.jp, or Yusuke Yanagi, yyanagi@virology.med.kyushu-u.ac.jp.
S.W. and Y.S. contributed equally to this article.

doi:10.1128/JVI.02632-12

or shortening of the cytoplasmic tail of the F protein, affecting its interaction with the M protein (34–36). All these mutations in SSPE strains abrogate the expression or function of the M protein, thereby precluding the production of virus particles. Whereas standard MVs are efficiently eliminated by host defense mechanisms, assembly-defective MVs may persist in the body by evading immune responses (37). In addition, it was shown that the deletion of the M protein or the cytoplasmic domain of the F protein enhanced cell-cell fusion in SLAM- or CD46-dependent infection (38, 39). In short, defects of the M protein have been thought to play a crucial role in MV pathogenicity in the CNS.

In this study, we incidentally identified several substitutions in the extracellular domain of the F protein that enhance its fusion activity. We also noticed that most SSPE strains possess substitutions in the extracellular domain of the F protein that are likely to affect its fusion activity, judging from their positions within the protein. Therefore, we examined whether these substitutions in the F protein play a role in MV pathogenicity. Our results revealed that fusion-enhancing mutations in the extracellular domain of the F protein indeed facilitate MV spread in SLAM- and nectin 4-negative cells, as well as neuropathogenicity in suckling hamsters, independently of defects of the M protein.

MATERIALS AND METHODS

Cells. Vero cells expressing human SLAM (Vero/hSLAM cells) (14), Vero cells, and IMR-32 cells were maintained in Dulbecco's minimum essential medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS). SYM-1 cells were maintained in 1:1 DMEM/RPMI medium supplemented with 10% FBS. Vero/hSLAM cells constitutively expressing the T7 RNA polymerase (Vero/hSLAM-T7 cells) were established using a retrovirus vector as follows. To generate the retrovirus expressing the T7 RNA polymerase, the cDNA encoding the T7 RNA polymerase was cloned into pMX-IRES-Puro (a gift from M. Shimojima and T. Kitamura) (40–42), producing pMX-T7pol-IRES-Puro. PLAT-gp cells (Invivogen, San Diego, CA) cultured in 6-well cluster plates were transfected with 7.5 μg of pMX-T7pol-IRES-Puro and 0.5 μg of pCVSV-G using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and the retrovirus was recovered. Vero/hSLAM cells in 24-well plates were infected with this retrovirus, and at 72 h after transduction, the culture medium was replaced with complete DMEM containing 2 μg/ml puromycin (Invivogen). A single clone was isolated after several passages with puromycin and used for experiments. Vero/hSLAM-T7 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 2 μg/ml puromycin.

Plasmid constructions. The F genes of mutant viruses were amplified by PCR using the primer pair 5′-TTGAATTCGCCACCATGGGTCTCA AGGTGAACGT-3′ and 5′-TTGCGGCCGCTCAGAGCGACCTTACAT AGG-3′. After digestion with EcoRI and NotI, the PCR products were cloned into pCA7, the eukaryotic expression vector (43), producing pCA7-ICF(I87T), pCA7-ICF(M94V), pCA7-ICF(S262R), pCA7-ICF(L354M), and pCA7-ICF(N462K). pCA7-ICH and pCA7-ICF, which encode the H and F proteins of the IC-B strain of MV, respectively, have been described previously (19). Six different sets of mutations were introduced independently into pCA7-ICF by site-directed mutagenesis using complementary primer pairs, producing pCA7-ICF(T461I), pCA7-ICF(S103I), pCA7-ICF(N462S), pCA7-ICF(N465S), pCA7-ICF(N462S/N465S), and pCA7-ICF(S103I/N462S/N465S). All full-length-genome plasmids were derived from pHHRz(+)MV323-EGFP (44, 45), which carries the antigenomic full-length cDNA of the wild-type IC-B strain together with enhanced green fluorescent protein (EGFP). The F gene of pHHRz(+)MV323-EGFP was replaced with those of pCA7-ICF-derived plasmids with different mutations, producing pHHRz(+)MV323-F(S262R)-EGFP, pHHRz(+)MV323-F(L354M)-EGFP, pHHRz(+)MV323-F(N462K)-EGFP, pHHRz(+)MV323-F(T461I)-EGFP, and pHHRz(+)MV323-F(S103I/N462S/N465S)-EGFP. To construct the plasmid pHHRz(+)MV323-ΔM-EGFP, the gene encoding the 5′ and 3′ untranslated regions (UTR) of the M protein but lacking the coding sequence of the M protein was amplified by the overlapping-PCR method with the primer set 5′-ATCCGCGGTCAG GGATCTGG-3′, 5′-CTGGGCACTGCGGTTGTGGAACTTAGGAGGC AATC-3′, 5′-CTCCTAAGTTCCACAACCGCAGTGCCCAGCAATAC C-3′, and 5′-TGCCGCGGGCCGGGGTCTG-3′ and ligated to the plasmid pHHRz(+)MV323-EGFP after digestion with SacII. The plasmids pCITE-RL and pTKminusT7-FL used in the quantitative fusion assay were constructed as follows. To construct the plasmid pCITE-RL, the gene encoding *Renilla* luciferase was amplified by PCR from p18MGKLuc01 (46) and ligated to the plasmid pCITE-2a(+) (Novagen, Madison, WI). To construct the plasmid pTKminusT7-FL, the gene encoding firefly luciferase was amplified by PCR from p18MGFLuc01 (46) and ligated to the plasmid pTK-RL (Promega, Madison, WI), from which the T7 promoter and *Renilla* luciferase sequence had been removed.

Viruses. Recombinant MVs were generated as reported previously (45). Briefly, BHK/T7-9 cells, which constitutively express T7 RNA polymerase, 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. At 2 days after transfection, the cells were cocultured with Vero/hSLAM cells. The recombinant viruses IC323-F(S262R)-EGFP, IC323-F(L354M)-EGFP, IC323-F(N462K)-EGFP, IC323-F(T461I)-EGFP, IC323-F(S103I/N462S/N465S)-EGFP, and IC323-ΔM-EGFP were generated from pHHRz(+)MV323-F(S262R)-EGFP, pHHRz(+)MV323-F(L354M)-EGFP, pHHRz(+)MV323-F(N462K)-EGFP, pHHRz(+)MV323-F(T461I)-EGFP, pHHRz(+)MV323-F(S103I/N462S/N465S)-EGFP, and pHHRz(+)MV323-ΔM-EGFP, respectively. The generated MVs were propagated in Vero/hSLAM cells. The number of PFU of each recombinant virus was determined in Vero/hSLAM cells.

Quantitative fusion assay. 293T cells cultured in a 12-well plate were transfected with pCA7-ICH (0.4 μg), an F-protein-encoding plasmid (pCA7-ICF or pCA7 encoding each mutant F protein; 0.1 μg) and pCITE-RL encoding *Renilla* luciferase driven by T7 polymerase (1.2 μg), using Polyethylenimine (PEI) "Max" high-potency linear PEI (Polysciences, Warrington, PA). These transfectants were used as effector cells. As an internal control, pTKminusT7-FL encoding firefly luciferase driven by the herpes simplex virus (HSV) thymidine kinase (TK) promoter was cotransfected into the effector cells. Vero/hSLAM-T7 cells were cultured in a 12-well plate and used as target cells. At 6 h posttransfection, effector cells were washed with PBS 3 times, detached with 0.05% EDTA in PBS, and centrifuged (400 × *g*; 5 min; 4°C). The cell pellet was resuspended in 500 μl of growth medium, and 100 μl of cell suspension was overlaid onto the target cells. After 18 h of incubation, cell-cell fusion was quantified by measuring the luciferase activity. Firefly and *Renilla* luciferase activities were independently assayed by using the dual-luciferase reporter assay system (Promega) with a Mithras LB940 plate reader (Berthold Technologies, Pforzheim, Germany). The relative fusion activity (*Renilla* luciferase activity divided by firefly luciferase activity) was calculated. The value obtained with pCA7-ICH and pCA7-ICF was set to 100%.

Plasmid-mediated fusion assay in Vero/hSLAM cells. Vero/hSLAM cells cultured in a 12-well cluster plate were transfected with pCA7-ICH (0.2 μg) plus pCA7 encoding the wild-type or each mutant F protein (1.5 μg), using Lipofectamine 2000 (Invitrogen). One day after transfection, the cells were observed under a light microscope after Giemsa staining.

Plasmid-mediated fusion assay in Vero cells. Vero cells cultured in a 24-well cluster plate were transfected with pCA7-ICH (0.75 μg) plus pCA7 encoding each mutant F protein (0.75 μg). Plasmid DNAs were incubated with 1.5 μl of Lipofectamine LTX Plus reagent (Invitrogen) for 5 min at room temperature and incubated with 4.5 μl of Polyethylenimine "Max" high-potency linear PEI for 30 min at room temperature, followed by dropping the mixture onto Vero cells. At 48 h posttransfection, the cells were observed under a light microscope after Giemsa staining. Then, the

number of nuclei in syncytia per visual field was determined with a 10× objective lens.

Overlay fusion assay. Recombinant viruses were rescued in BHK-T7/9 cells as described above. The transfected BHK-T7/9 cells were maintained in growth medium containing fusion block peptide (Z-D-Phe-Phe-Gly; Peptide Institute Inc., Osaka, Japan) to prevent cell-to-cell fusion. At 24 h posttransfection, the transfected BHK-T7/9 cells were washed 3 times with PBS and detached by trypsin digestion. The preparation, containing 200 EGFP-positive cells, was overlaid onto a confluent monolayer of Vero or IMR-32 cells cultured in a 10-cm dish. At 1 or 3 days after overlay, EGFP fluorescence was observed under a fluorescence microscope.

Surface biotinylation. Vero/hSLAM cells were transfected with 4 μg of plasmid DNA encoding MV F variants as indicated. After washing with PBS three times, cells were incubated in PBS with 0.5 mg of NHS-SS-Biotin (Thermo Scientific, Rockford, IL)/ml for 20 min at 4°C, followed by washing and quenching for 5 min at 4°C in DMEM. The cells were scraped in 1 ml immunoprecipitation buffer (10 mM HEPES [pH 7.4], 50 mM sodium pyrophosphate, 50 mM sodium fluoride, 50 mM sodium chloride, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100) containing protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan), and the lysates were cleared by centrifugation for 20 min at 20,000 × *g* and 4°C. The biotinylated proteins were adsorbed to Sepharose-coupled streptavidin (GE Healthcare, Waukesha, WI) for 90 min at 4°C, washed once in immunoprecipitation buffer, buffer 1 (100 mM Tris [pH 7.6], 500 mM lithium chloride, 0.1% Triton X-100) and buffer 2 (20 mM HEPES [pH 7.2], 2 mM EGTA, 10 mM magnesium chloride, 0.1% Triton X-100). Finally the proteins were incubated in urea buffer for 25 min at 50°C and subjected to Western blot analysis using antibodies specific for the MV-F tail. As a loading control, 10 μl of cell lysates was mixed with urea buffer and subjected to Western blot analysis using antibodies specific for beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA). For densitometric quantification of F proteins, blots were analyzed using a VersaDoc digital imaging system (Bio-Rad, Richmond, CA), and the signals were quantified with QuantityOne software (Bio-Rad).

Virus challenge and histopathological examination. Ten-day-old Syrian golden hamsters (SLC-Japan, Shizuoka, Japan) were anesthetized with sevoflurane. Then, 25 μl of diluted viruses was inoculated into the right or left hemisphere of the brains of hamsters. After the inoculation, clinical symptoms were observed every day, and moribund hamsters were euthanized. The brains were collected from dead and moribund hamsters. Three hamsters inoculated with wild-type MV or IC323-F(L354M)-EGFP were euthanized 6 days postinoculation, and the brains were also collected. EGFP autofluorescence of the MV-infected cells in each brain was observed under a fluorescence stereomicroscope. 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, Fukuoka, Japan. For histopathological analysis, the collected brain was fixed in 10% buffered formalin and processed into paraffin sections. Sections were stained with hematoxylin and eosin (HE). Immunohistochemical analysis was performed, using the following antibodies: mouse anti-NeuN (1:100; Chemicon, Temecula, CA), mouse anti-glial fibrillary acidic protein (GFAP) (1:1,000; Dako A/S, Glostrup, Denmark), rabbit anti-green fluorescent protein (GFP) (1:500; Invitrogen), and anti-MV-N (1:100; Novus Biological, Littleton, CO). Fluorescence images were captured with a confocal microscope (A1 Confocal Laser Microscope; Nikon Corporation, Tokyo, Japan).

RESULTS

Substitutions in the F protein that enhance its fusion activity. In two different experiments, we identified substitutions in the F protein of IC323-EGFP (the EGFP-expressing recombinant MV based on the wild-type IC-B strain) (44) that enhanced its fusion activity. First, in our attempt to modify IC323-EGFP so that it possessed the H protein with an epitope tag at its C terminus, we found that the virus did not induce syncytia in Vero/hSLAM cells (14). This presumably occurred because the added tag adversely affected the interaction of the H protein with the receptor SLAM and/or the F protein. However, after a few passages in Vero/hSLAM cells, several mutant viruses emerged, which regained the ability to induce syncytia. These mutant viruses were plaque purified, and their H, F, and M genes, encoding the proteins involved in membrane fusion, were subjected to sequencing analysis. Three mutant viruses had single-amino-acid substitutions in the F protein but no substitutions in the H and M proteins (Table 1). Second, Vero cells (SLAM and nectin 4 negative) were infected with IC323-EGFP. As expected, no syncytia were detected at the beginning, but after a few passages, we found two syncytium-producing viruses that had single-amino-acid substitutions in the F protein but none in the H and M proteins (Table 1).

TABLE 1 Single-amino-acid substitutions found in the F genes of mutant viruses with enhanced fusion activity

Substitution	Virus used for identification	Cells used for identification
I87T	Epitope tagged	Vero/hSLAM
S262R	Epitope tagged	Vero/hSLAM
N462K	Epitope tagged	Vero/hSLAM
M94V	Wild type	Vero
L354M	Wild type	Vero

To determine whether these single amino acid substitutions indeed endow the F protein with enhanced fusion activity, Vero/hSLAM cells were cotransfected with the expression plasmid encoding the F protein with each substitution and the plasmid encoding the H protein (without the epitope tag). Compared with the wild-type F protein, all mutant F proteins produced larger syncytia in Vero/hSLAM cells (Fig. 1A). To further evaluate the fusion activities of these F-protein mutants, a quantitative plasmid-mediated fusion assay was established. The S262R, L354M, and N462K substitutions allowed the F protein to exhibit more than 2-times-higher fusion activity than the wild-type F protein, while the M94V substitution slightly enhanced its fusion activity (Fig. 1B).

The fusion activities of these F-protein mutants were also examined in Vero cells by expressing them together with the wild-type H protein. While the wild-type F protein did not induce syncytium formation in Vero cells, all mutant proteins did so (Fig. 1C). Since the sizes of syncytia observed in Vero cells were much smaller than those in Vero/hSLAM cells, the quantitative fusion assay did not give large values so as to reliably evaluate fusion activity. Thus, fusion activity in Vero cells was evaluated by counting the nuclei in syncytia (Fig. 1D). The S262R and N462K substitutions conferred on the F protein more enhanced fusion activity than the other substitutions. When these mutant F proteins were expressed alone (without the H protein), they did not induce syncytia in Vero or Vero/hSLAM cells (data not shown).

Figure 2 shows the locations of these 5 substitutions within the full-length F-protein structure. The substitutions I87T, M94V, and S262R are located in the "microdomain," the region near the HR-A domain that is involved in the initiation of fusion (47–49). The L354M substitution is located in the cysteine-rich region, which is thought to interact with the H protein (50). Mutant MVs resistant to a fusion-inhibitory peptide contain amino acid substi-