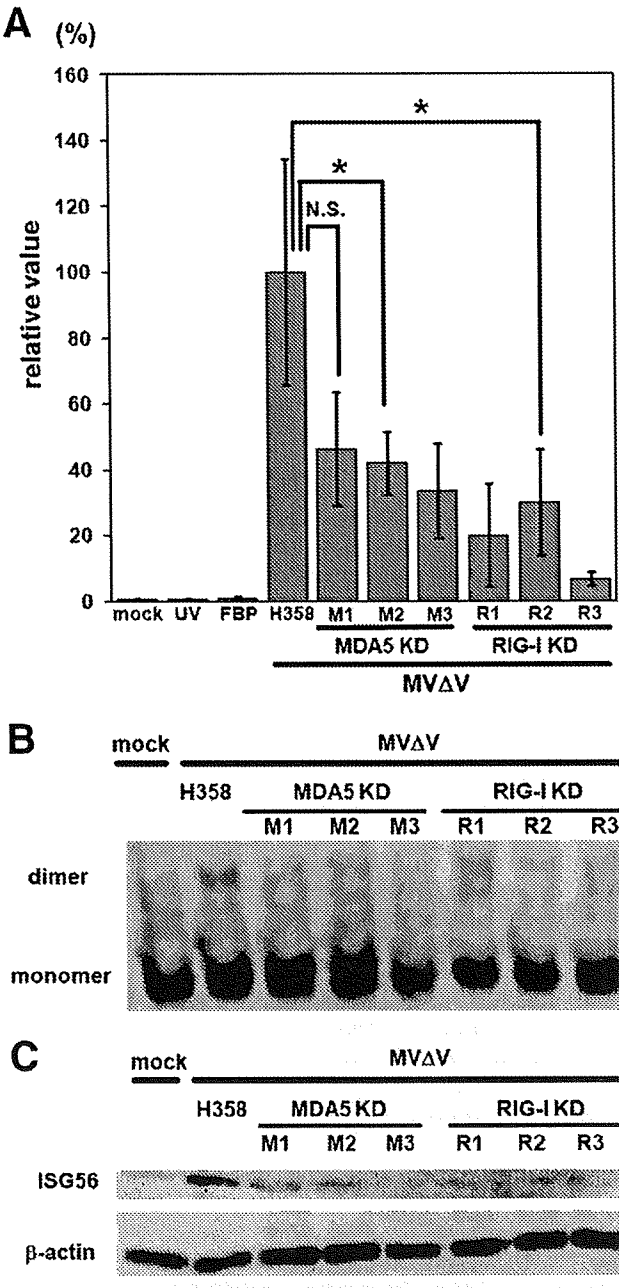

FIG. 4. Inductions of IFN-β mRNA and ISG56 and dimerization of IRF3 in MVΔV-infected cells. (A) The parental H358 cells, MDA5 knockdown clones (MDA5 KD: M1, M2, and M3) and RIG-I knockdown clones (RIG-I KD: R1, R2, and R3) were infected with MVΔV at an MOI of 0.5. H358 cells were also infected with UV-inactivated MVΔV (UV), treated with an FBP before MVΔV infection (FBP), or mock infected (mock). Total RNAs were extracted from the cells at 24 h after infection, and the IFN-β mRNA levels were quantified by RT-qPCR. The data were normalized by the β-actin mRNA levels, and the mean value in MVΔV-infected H358 cells was set to 100%. *, $P < 0.05$ (significant difference based on a t test). N.S., not significant. (B and C) H358 cells and MDA5 and RIG-I knockdown clones were infected with MVΔV at an MOI of 0.5. H358 cells were also mock infected. At 24 h after infection, the monomeric and dimeric forms of IRF3 in the cells were examined by native PAGE and Western blot analysis (B), and the ISG56 levels in the cells were detected by SDS-PAGE and Western blot analysis (C). β-Actin was examined as an internal control.

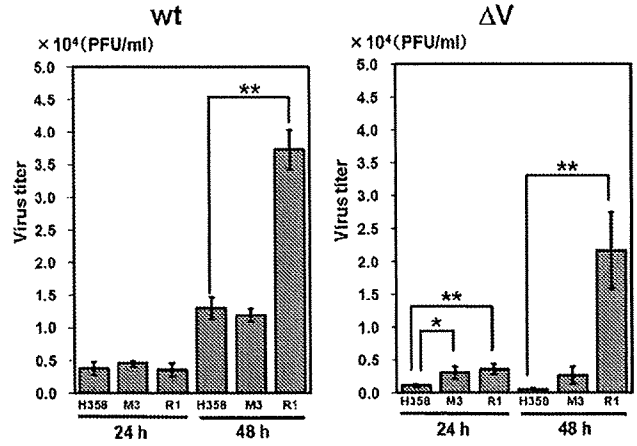

FIG. 5. Growth kinetics of wt MV and MVΔV in H358 cells and knockdown clones. The parental H358 cells, an MDA5 knockdown clone (M3) and an RIG-I knockdown clone (R1) were infected with wt MV or MVΔV at an MOI of 0.5. At 24 and 48 h after infection, the cells were harvested, together with the culture media, and the virus titers were determined by plaque assays. The data represent the means ± the standard deviations of triplicate samples. *, $P < 0.05$; **, $P < 0.01$ (significant differences based on a t test).

IFN-β mRNA (Fig. 4A), the dimerization of IRF3 (Fig. 4B), and the production of ISG56 (Fig. 4C). As expected, these three parameters were also suppressed in RIG-I knockdown clones (Fig. 4A to C). In control experiments, all of the knockdown clones were found to exhibit IRF3 dimer formation and express ISG56 after transfection with Cardif/VISA/MAVS/IPS-1, the common adaptor molecule downstream of MDA5 and RIG-I (42; data not shown). These findings indicate that MDA5 does indeed contribute to the detection of MV and resulting induction of IFN-β and that MDA5 and RIG-I are both required for H358 cells to fully produce IRF3-activated IFN-β and ISG56 after MVΔV infection. It was noted that significant levels of IFN-β mRNA were produced in MVΔV-infected RIG-I knockdown clones (Fig. 4A), unlike the case for RIG-I knockdown clones infected with wt MV, in which the production of IFN-β mRNA was negligible compared to wt MV-infected parental H358 cells (Fig. 2A). These observations may further support the role of MDA5 in IFN induction after MV infection, which is only revealed in the absence of the V protein.

Growth of wt MV and MVΔV in knockdown clones. The growth kinetics of wt MV and MVΔV were examined in H358 cells, an MDA5 knockdown clone (M3), and a RIG-I knockdown clone (R1) (Fig. 5). At 24 h after infection when the productions of IFN-β mRNA were still very limited (Fig. 3C), there was little difference in the growth of wt MV among H358, M3, and R1 cells. At 48 h after infection, the growth of wt MV was accelerated in R1 cells, but not in M3 cells, compared to H358 cells. Virus titers may be determined by the balance between the speed of virus replication and antiviral action of IFN. Thus, the reduction of IFN-β mRNA found in M3 cells may not have been sufficient to enhance wt MV replication at 48 h after infection, unlike that in R1 cells. On the other hand, the growth of MVΔV was strongly suppressed in H358 cells. Growth retardation of MVΔV may be expected to be compen-

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sated for in MDA5 knockdown cells, and MVΔV did indeed grow better in M3 cells than in H358 cells. However, the growth of MVΔV in M3 cells was still less efficient than that of wt MV in H358 cells. Furthermore, MVΔV growth was greatly enhanced in R1 cells compared to H358 and M3 cells at 48 h after infection, reconfirming the primary importance of RIG-I in the detection of MV and subsequent IFN production.

DISCUSSION

In the present study, we have shown that RIG-I and MDA5 are both involved in the detection of MV in human cells and that the V protein plays an important role in MV growth by inhibiting the MDA5-mediated induction of the host IFN responses.

Previous studies using embryonic fibroblasts from MDA5$^{-/-}$ or RIG-I$^{-/-}$ knockout mice in vitro, as well as these knockout mice in vivo, have shown that these RNA helicases have differential roles in the recognition of different viruses. Specifically, RIG-I is essential for the detection of many RNA viruses including paramyxoviruses, orthomyxoviruses, rhabdoviruses and flaviviruses, while MDA5 is critical for the detection of picornaviruses (15, 23, 28). Interestingly, dengue virus type 2, a flavivirus, and reoviruses were shown to induce innate immune responses in embryonic fibroblasts obtained from either MDA5$^{-/-}$ or RIG-I$^{-/-}$ mice, indicating that they trigger both RIG-I- and MDA5-dependent responses (28).

Although the above generalization may be made, the data are more complicated for individual viruses. In studies with knockout mice, IFN production was significantly diminished in RIG-I$^{-/-}$ cells, but not in MDA5$^{-/-}$ cells, after infection with paramyxoviruses such as Sendai virus (SeV), including that lacking the V protein, Newcastle disease virus (NDV), and respiratory syncytial virus (23, 28). On the other hand, Yoneyama et al. (58) demonstrated that expression of IFN mRNA after NDV infection was inhibited in mouse fibroblast L929 cells by siRNA-mediated knockdown of either RIG-I or MDA5. Studies with the monoclonal antibody J2, which specifically recognizes dsRNAs of more than 40 bp in length (55), detected dsRNAs in NDV-infected cells (52) but not in SeV-infected cells (52, 55).

In the case of MV, infection was found to trigger IFN responses in human Huh7 cells but not in their derivative Huh7.5 cells with nonfunctional RIG-I, although the MDA5 pathway was intact in both cell lines (38). Another study suggested that MDA5 is involved in MV-induced expression of IFN-β since forced expression of MDA5, but not RIG-I or TLR3, led to enhanced IFN-β promoter activity in MV-infected human A549 cells (6). Shingai et al. (46) reported that vaccine strains of MV contain defective interfering (DI) particles, which are responsible for IFN-β induction. These authors also showed that both RIG-I and MDA5 detect the stem-loop structure of DI RNAs, whereas only RIG-I senses DI RNAs with 5′-triphosphate. Therefore, differences in the cell types (knockout mouse-derived cells versus cultured cells) and assay systems used may have led to various conclusions regarding the relative roles of RIG-I and MDA5 in the detection of different paramyxoviruses. Furthermore, although all paramyxoviruses have a common life cycle including genome replication and transcription (25), host cells may recognize different paramyxoviruses in different manners.

To determine the individual roles of MDA5 and RIG-I in IFN induction after MV infection, we established human epithelial H358 cells constitutively expressing siRNAs targeting the MDA5 and RIG-I mRNAs. Even in the RIG-I knockdown clones that still expressed considerable levels of RIG-I (clones R2 and R3), induction of IFN-β mRNA after MV infection was almost completely blocked, indicating that RIG-I plays a major role in the recognition of MV. The results are consistent with those obtained using Huh7 and Huh7.5 cells (38), as well as with the general notion regarding the importance of RIG-I in the detection of paramyxoviruses. However, our data further showed that MDA5 is also involved in the detection of MV, since IFN-β mRNA production in MDA5 knockdown clones was reduced compared to that in the parental H358 cells. This observation appears to contradict the finding that RIG-I knockdown clones (expressing normal levels of MDA5) hardly produced IFN-β mRNA after MV infection. Without RIG-I, MDA5-mediated signaling probably does not function sufficiently to induce IFN in the presence of the antagonizing V protein.

To evaluate the contribution of MDA5 to IFN induction in the absence of the V protein, a V protein-deficient MV (MVΔV) was generated. IFN-β mRNA and ISG56 were induced at higher levels in MVΔV-infected cells than in wt MV-infected cells at 24 h after infection. Similar enhancement of IFN production has also been reported for the paramyxovirus simian virus 5 with a defective V protein (18). Activation of IRF3 and production of IFN-β mRNA and ISG56 in response to MVΔV infection were reduced in MDA5 knockdown cells compared to the parental H358 cells. Importantly, significant levels of IFN-β mRNA were produced in RIG-I knockdown cells infected with MVΔV, unlike the case for those infected with wt MV. Furthermore, the growth of MVΔV, but not that of wt MV, was enhanced in MDA5 knockdown cells. All of these results indicate that MDA5 is indeed involved in the recognition of MV and that the V protein is required for MV to inhibit the MDA5-mediated IFN induction.

MDA5 and RIG-I appear to recognize different RNA structures (19, 22, 37, 44). Leader RNA, a 5′-triphosphate ended single-stranded RNA, is produced during the transcription of MV and other paramyxoviruses (9, 26), and in vitro-synthesized MV leader RNA was found to induce IFN through RIG-I (38). Therefore, RIG-I probably detects leader RNA in MV-infected cells and induces IFN production. Using the dsRNA-specific antibody J2 (55), we tried to detect intracellular dsRNA, which is the ligand for MDA5 (22). Although dsRNA was not found in wt MV- or MVΔV-infected Vero/hSLAM cells, it was clearly detected in MVΔC-infected cells (data not shown). A recent study also reported that dsRNA exists in cells infected with a C protein-deficient SeV, but not in SeV-infected cells (52). Therefore, the C proteins of both SeV and MV may act to limit the generation of intracellular dsRNA. It is likely that dsRNA is generated at a low level in wt MV- or MVΔV-infected cells, although it cannot be detected by the J2 antibody in the presence of the C protein. Alternatively, unknown structural motifs may exist in MV RNA that can activate MDA5.

The V protein interferes with multiple steps of host antiviral

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responses such as IFN-α/β induction (36, 39) and IFN-α/β-mediated signal transduction (32, 34, 51, 57). Since the MV P protein can inhibit IFN signaling like the V protein (13, 32), the raison d'être for the MV V protein may largely reside in its blockade of MDA5-mediated IFN induction. However, the significance of this V protein function may vary among different cell types, because MV lacking the V protein grows comparably to the parental virus in B95a cells (present study) but not in H358 cells (present study) and rhesus monkeys (12). In the present study, knockdown of MDA5 weakened IFN induction in H358 cells after infection with wt MV or MVΔV. On the other hand, the growth defect of MVΔV was not completely compensated for in MDA5 knockdown H358 cells, indicating that the V protein may contribute to efficient MV growth partly by inhibiting MDA5-mediated IFN induction and partly by other mechanisms.

Given the importance of RIG-I in the recognition of paramyxoviruses, including MV, inhibition of RIG-I, rather than MDA5, may be a better strategy for MV to achieve efficient growth. Indeed, some paramyxoviruses block RIG-I-mediated IFN-α/β induction (4, 27). Strong suppression of innate immunity through blockade of RIG-I may increase MV growth but possibly kill the host too quickly for the virus to spread to other hosts. Blockade of MDA5 may be more advantageous for MV survival, by allowing both sufficient growth within a host and efficient spread to different hosts.

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Reduced ability of hemagglutinin of the CAM-70 measles virus vaccine strain to use receptors CD46 and SLAM

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ABSTRACT

The CAM-70 measles virus (MV) vaccine strain is currently used for vaccination against measles. We examined the fusion-inducing ability of the CAM-70 hemagglutinin (H) protein and found that it was impaired in both CD46- and signaling lymphocyte activation molecule (SLAM)-expressing cells. We also generated recombinant MVs possessing H genes derived from the CAM-70 strain. The CAM-70 H protein impaired viral growth in both CD46- and SLAM-expressing cells. In peripheral blood lymphocytes (PBL) and monocyte-derived dendritic cells (Mo-DC), the CAM-70 strain did not grow efficiently. Infection with recombinant MVs revealed that impaired growth of the CAM-70 strain was attributed to the H gene only partly in PBL and largely in Mo-DC. Thus, impaired fusion-inducing ability of the H protein may be one of the underlying molecular mechanisms resulting in the attenuation of the CAM-70 strain.
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1. Introduction

Measles is an extremely contagious disease and is a major cause of both childhood morbidity and mortality. However, the availability of effective live attenuated measles vaccines has resulted in the control of measles in many industrialized countries. Moreover, mortality due to measles was reduced from 873,000 deaths to 345,000 deaths worldwide between 1999 and 2005 following mass vaccination in developing countries [1]. In Japan, measles vaccines were introduced in 1978 as part of routine immunization schedules and resulted in a dramatic decrease in measles cases, although relatively large outbreaks still occur [2,3].

One of the attenuated live vaccines for measles virus (MV), the CAM-70 strain, was originally developed in Japan from the Tanabe strain by serial propagation in amniotic and chorioallantoic membranes of chicken embryos and then subsequently in chicken embryonic fibroblasts [4,5]. This strategy is similar to those utilized for the development of other live attenuated measles vaccine strains. The live attenuated CAM-70 vaccine was licensed for vaccination in Japan in 1971 and its safety and effectiveness have been well established [6,7]. However, the mechanism of its attenuation and that for other measles vaccine strains remains unknown.

MV, an enveloped virus with a non-segmented negative-strand RNA genome, belongs to the genus *Morbilivirus* in the family *Paramyxoviridae*. MV has two surface glycoproteins, hemagglutinin (H) and fusion (F). The H protein recognizes the cellular receptor and its binding to this receptor leads to activation of the F protein, resulting in fusion between the viral envelope and the cellular membrane and entry of the nucleocapsid into the cytoplasm [8]. The cellular receptors for MV include CD46 and signaling lymphocyte activation molecule (SLAM, also called CD150) [9–13]. CD46, a member of the regulators of complement activation family, is expressed on all nucleated human cells [14] and is recognized by both vaccine and laboratory-adapted strains, but generally not by wild-type MV strains [15]. On the other hand, SLAM, a member of the immunoglobulin superfamily, is expressed on activated B and T cells, dendritic cells, and macrophages [16–18], and is now well established as the receptor for all MV strains including wild-type strains [15]. In addition to CD46 and SLAM, an unidentified receptor for MV has recently been suggested to be located on the basolateral side of the epithelium [19–21].

Numerous studies have identified several amino acid residues on the MV H protein that play an important role in its interaction with CD46 and SLAM. The MV H residue Y481 is known to be essential for interaction with CD46 [22–26] and amino acids 473–477 region is reported to be another site required for CD46 interaction [27]. In addition, studies have found that S546G substitution increases CD46 binding [28,29], while residues S548 and

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F549 appear to reduce CD46 binding [30]. Moreover, a recent study clarified that, in addition to Y481, the amino acids I390, D416, S446, N484, and G492, are necessary for efficient use of CD46 [31]. On the other hand, it was reported that the MV H residues D505, D507, Y529, D530, T531, R533, F552, Y553, and P554 are important in the interaction with SLAM [32,33] and that these residues form a putative SLAM-binding site in the crystal structure of the MV H protein [34]. A more recent surface plasmon resonance assay has shown that I194 is essential for binding to SLAM and that Y529, D530, R533, and Y553 are not involved in binding to SLAM but contribute to events after the receptor binding [35].

In the current study, we compared CD46-dependent and SLAM-dependent fusion-inducing ability of the CAM-70 H protein with those of the other MV strains. We found that both the CD46-dependent and SLAM-dependent fusion-inducing abilities of the CAM-70 H protein were lower than those of the other strains examined, although impairment of the SLAM-dependent fusion was not as prominent as that of the CD46-dependent fusion. To evaluate the effects of the impaired fusion-inducing ability of the CAM-70 H protein on virus growth, we next generated recombinant MVs possessing H genes derived from the CAM-70 and other MV strains. The CAM-70 H protein impaired viral growth significantly in CD46-expressing cells, but only slightly in SLAM-expressing cells. Given that lymphocytes and dendritic cells are thought to be two of the major targets of MV *in vivo* [36], infection of peripheral blood lymphocytes (PBL) and monocyte-derived dendritic cells (Mo-DC) with the CAM-70 and the recombinant MVs was also examined. We observed that the CAM-70 strain did not grow efficiently in PBL and Mo-DC. Infection with recombinant MVs revealed that the impaired growth of the CAM-70 strain could be attributed to the H gene in PBL in part, while the H gene largely accounted for the impaired growth in Mo-DC. Thus, impaired fusion-inducing ability of the H protein may represent one of the molecular mechanisms underlying the attenuation of the CAM-70 strain. Lastly, we identified amino acid substitutions that are responsible for the low fusion-inducing ability of the CAM-70 H protein.

2. Materials and methods

2.1. Cells and viruses

The adherent marmoset B-cell line B95a [37] was incubated in RPMI 1640 medium (RPMI; Nissui pharmaceutical, Tokyo, Japan) containing 5% fetal bovine serum (FBS) (RPMI-5% FBS). The human epithelial cell line derived from cervical cancer (HeLa) and the epithelial cell line derived from the African green monkey kidney (Vero) were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui pharmaceutical) containing 5% (DMEM-5% FBS) and 10% FBS (DMEM-10% FBS) respectively. Chinese hamster ovary (CHO) cells expressing human CD46 (CHO/CD46) [38] were cultured in DMEM-10% FBS supplemented with 700 μg of hygromycin (Nacalai Tesque, Tokyo, Japan) per milliliter. CHO cells expressing human SLAM (CHO/SLAM) [13] were cultured in DMEM-10% FBS supplemented with 500 μg of G418 (Geneticin; Nacalai Tesque) per milliliter. Normal human dermal fibroblasts (NHDF) were purchased from PromoCell (Heidelberg, Germany) and were cultured according to the manufacturer's instruction. The vaccine strain CAM-70 derived from a commercially available vaccine vial (The Research Foundation for Microbial Diseases of Osaka University, Osaka, Japan) was propagated in B95a cells once or twice at 37 °C to prepare virus stocks. The MV Tanabe strain (a gift from Y. Gomi and S. Ueda, The Research Foundation for Microbial Diseases of Osaka University), which had been passaged in monkey kidney cells twice and African green monkey kidney cells once after its isolation, was propagated in B95a cells once at 37 °C. Recombinant MVs were generated from cDNAs using CHO/SLAM cells and the vaccinia virus carrying T7 RNA polymerase, vTF7-3, following the procedure reported previously [39]. Infectivity titers of virus stocks were determined by measuring the 50% tissue culture infectious dose ($TCID_{50}$) in B95a cells.

2.2. Preparation of peripheral blood lymphocytes and the generation of monocyte-derived dendritic cells

PBL and Mo-DC were prepared following a previously reported procedure [40]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from the peripheral blood of normal healthy volunteers by Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation and the non-adherent fraction of PBMC used as PBL. Monocytes were isolated from PBMC using the MACS monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The recovered monocytes were >90% pure according to flow cytometry with a fluorescein isothiocyanate (FITC)-conjugated anti-CD14 monoclonal antibody (MAb) (BD Biosciences, San Diego, CA, USA). To generate Mo-DC, monocytes were cultured in RPMI containing 10% FBS (RPMI-10% FBS) supplemented with 50 ng of GM-CSF and 30 ng of IL-4 (PeproTech, London, UK) per milliliter for seven days with fresh cytokines being added on day 4. Surface markers of the prepared Mo-DC were $HLA\text{-}DR^+CD80^+CD86^{+/-}CD40^+CD1a^+CD83^-$, which are typical of immature Mo-DC.

2.3. Flow cytometry analysis

PBL were cultured in RPMI-10% FBS supplemented with 2.5 μg PHA-L (SIGMA, St. Louis, MO, USA) per milliliter for two days before analysis. Mo-DC and PHA-stimulated PBL were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD46 MAb (BD Biosciences, San Jose, CA, USA). For detection of SLAM, the cells were treated with Fc receptor-blocking reagent (MBL, Nagoya, Japan) and stained with anti-SLAM MAb IPO-3 (Kamiya, Seattle, WA, USA) and FITC-conjugated goat anti-mouse IgG $F(ab')_2$ fragment (ICA Pharmaceuticals, Aurora, OH, USA). Stained cells were analyzed on a FACS Calibur (BD Biosciences, San Jose, CA, USA).

2.4. Cloning and sequencing of the MV Tanabe and CAM-70 H genes

B95a cells cultured in 75-cm^2 flasks were infected with MVs at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$/cell and the infected cells harvested at two days post-infection (p.i.). Total RNA was then isolated from the infected cells using an RNeasy mini kit (Qiagen, Hilden, Germany) and was reverse transcribed into cDNA using the specific sense primer, $5'\text{-}^{6949}$ATCCTGATTGCAGTGTGTCT$^{6968}\text{-}3'$ and the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). The cDNA was amplified following 30 PCR cycles under the following conditions: 30 s at 94 °C, 30 s at 55 °C and 120 s at 68 °C using Platinum Pfx DNA polymerase (Invitrogen) with the MV specific sense primer, $5'\text{-}^{6949}$ATCCTGATTGCAGTGTGTCT$^{6968}\text{-}3'$ and the antisense primer $5'\text{-}^{9186}$TTTCACACTAGTGGGTATGCCTG$^{9164}\text{-}3'$. The amplified PCR product was cloned into pCR-Blunt-II-TOPO vector (Invitrogen) and three clones of the cDNA were sequenced using the Big Dye terminator cycle sequencing kit and an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The consensus sequence of the H gene of the CAM-70 strain is the same as the reported sequence (GenBank accession number DQ345723). The nucleotide sequence of the H gene for the MV Tanabe strain has been deposited in DDBJ/EMBL/GenBank with the accession number AB467283.

2.5. Plasmid construction

All plasmids encoding mutant MV genome were based on p(+)MV323 which encodes the antigenomic full-length cDNA of the wild-type IC-B strain of MV [41]. Using the restriction enzymes PacI and SpeI, the region between nucleotide positions 7242 and 9175 of p(+)MV323 was replaced with the corresponding regions of the CAM-70, Tanabe, and AIK-C H genes, generating full-length genome plasmids termed p(+)MV323-CAM H, p(+)MV323-Tanabe H, and p(+)MV323-AIK H respectively. The cDNA of the AIK-C H gene was obtained from the full-length genome plasmid of the AIK-C strain, pAIK-C [42].

Individual H genes were also cloned into the mammalian expression vector pME18S, generating pME-CAM H, pME-Tanabe H, and pME-AIK H. The cDNA of the CAM-70 F gene was generated following the same protocol for the cloning of the H-gene cDNA described above, using the MV-specific sense primer 5′-^{4592}CAGCACAGAACAGCCCTGACACAA^{4615}-3′ and antisense primer 5′-^{7273}CATTGTGGATGATCTTGCACCCTA^{7250}-3′. The PCR fragments were then cloned into pME18S, generating pME-CAM F. The generation of the plasmids expressing the H and F proteins of the IC-B strains, designated pME-IC H and pME-IC F, have been described previously [43].

Plasmids expressing chimeric MV H proteins between CAM-70 and AIK-C strains were also generated. In order to achieve this, the region between nucleotides 7242 and 8082 of the CAM-70 H cDNA was replaced with the corresponding region of the AIK-C H gene using the restriction enzymes PacI and NdeI, generating CAM-AIK H I cDNA. Similarly, the region between nucleotides 8082 and 9175 of the CAM H cDNA was replaced with the corresponding region of the AIK-C H gene using NdeI and SpeI, generating CAM-AIK H II cDNA. The chimeric CAM-AIK H I and II cDNA were cloned into the pME18S vector, generating pME-CAM-AIK H I and pME-CAM-AIK H II. Point mutations were introduced into the cDNA of the CAM-70 H gene, using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and mutagenic primers to generate the cDNAs CAM-70 H N455T, G505D, and E603G, encoding mutant CAM-70 H proteins possessing amino acid substitutions N455T, G505D, and E603G respectively. cDNAs encoding mutant CAM-70 H proteins possessing amino acid substitutions in the combinations N455T + G505D and N455T + E603G, were generated by replacing the region between nucleotides 8751 and 9175 of the CAM-70 H N455T cDNA with the corresponding region of the cDNAs CAM-70 H G505D and CAM-70 H E603G, respectively. The cDNAs encoding mutant CAM-70 H proteins were also cloned into pME18S, generating pME-CAM H N455T, G505D, E603G, N455T + G505D, and N455T + E603G.

2.6. Fusion assays

CHO/CD46 and CHO/SLAM cells were seeded into four-well Lab-tek II chamber slides (Nalgen Nunc International, Roskilde, Denmark). The cells were transfected with 0.5 μg of each plasmid expressing the various MV H proteins plus 0.5 μg of pME-IC F or pME-CAM F using the FuGENE HD transfection reagent (Roche Diagnostics, Indianapolis, IN, USA). After various times post-transfection, cells were air-dried and fixed with 95% ethanol for 15 min at −20 °C. The fixed cells were incubated with MAb against MV H protein (MN-6/cl.32) [43] for 1 h, extensively washed with phosphate-buffered saline (PBS), and incubated with FITC-conjugated goat anti-mouse IgG antibody (MBL, Nagoya, Japan) for 1 h. Finally, the cells were washed three times with PBS and mounted in fluorescent mounting medium (Vector Laboratories, Burlingame, CA, USA). CHO/CD46 and CHO/SLAM cells were also seeded into 24-well plates and transfected as described above. After various times post-transfection, the transfected cells were stained with Giemsa's solution and the number of nuclei within syncytia as a proportion of a total of 1000 nuclei in several microscopic fields was counted. The fusion index (100 × the total number of nuclei within syncytia per 1000 nuclei) was calculated by the formula described previously [44].

2.7. Growth kinetics of MV

Cells were incubated with MV at an MOI of 0.01 or 0.05 $TCID_{50}$/cell for 1 h at 37 °C. After two washes with DMEM or RPMI, the cells were seeded at a density of 2×10^5 cells per well in 500 μl of medium and incubated at 37 °C. B95a cells and HeLa cells were incubated in RPMI-5% FBS and DMEM-5% FBS respectively. NHDF was incubated in Fibroblast Growth Medium (PromoCell). PBL was incubated in RPMI-10% FBS supplemented with 2.5 μg of PHA-L per milliliter and Mo-DC in RPMI-10%FBS supplemented with GM-CSF and IL-4. Infected culture medium and cells were harvested together at various times post-infection. After freezing and thawing, infectivity titers were determined by measuring $TCID_{50}$ in B95a cells.

2.8. Inhibition of MV infection by anti-CD46 monoclonal antibody

Vero cells were incubated in the presence of 10 μg/ml of anti-CD46 MAb (M177) (MONOSAN, Uden, the Netherlands) or mouse IgG_1 isotype control antibody (R&D Systems, Minneapolis, MN, USA) for 1 h at 37 °C and then infected with the MV CAM-70 strain at an MOI of 0.1 $TCID_{50}$/cell in the presence of the antibody. At 65 h post-infection, infected cells were harvested and homogenized in lysis buffer (150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 50 mM Tris buffer, pH 8.0) containing 1 mM phenylmethylsulfonylfluoride. The lysates were resolved by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (PVDF, Millipore, Bedford, MA, USA). Non-specific binding was blocked with dried skim milk powder in PBS containing 0.1% Tween 20 and the membrane incubated with anti-MV M MAb (Chemicon, Temecula, CA, USA). The membranes were then washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (MBL), followed by the ECL (enhanced chemiluminescence) Western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK).

3. Results

3.1. Cloning and sequencing of the MV Tanabe strain H gene

Given that the genome sequence of the MV Tanabe strain from which the vaccine CAM-70 strain was derived has not been reported to date, we cloned the cDNA of the Tanabe H gene, determined its nucleotide sequence and compared the predicted amino acid sequence with those of the CAM-70, Edmonston-derived vaccine AIK-C, and wild-type IC-B strains (Table 1). The Tanabe H protein contained the Y481 and G546 amino acid residues, which have been shown to be important for binding to CD46 [22,23,25,26,28,29]. We identified eight amino acid differences between the Tanabe and CAM-70 strains, suggesting that these substitutions occurred during the serial passaging in the chicken embryonic cells. Among these substitutions, seven changes were unique to the CAM-70 strain when compared to the four strains analyzed in our study (Table 1), and also when compared to those of other Edmonston-lineage vaccine strains [45] including the D505G substitution, which has been suggested to reduce interaction with SLAM [32]. Therefore, we decided to examine CD46- and SLAM-dependent fusion-inducing ability of the CAM-70 H protein.

Table 1
Amino acid differences in the H protein among MV strains.

Amino acid position	Strain			
	IC-B[a]	AIK-C[a]	CAM-70[a]	Tanabe
93	T	T	I	T
157	V	V	A	V
174	A	T	T	T
175	R	R	K	R
176	A	T	T	T
211	S	G	G	G
235	G	E	E	E
243	G	R	R	R
252	H	Y	Y	Y
276	F	L	L	L
284	F	L	L	L
296	F	L	L	L
302	R	R	G	G
334	R	Q	Q	Q
338	P	T	S	P
390	N	I	I	I
416	N	D	D	D
446	T	S	S	S
455	T	T	N	T
481	N	Y	Y	Y
484	T	N	T	T
505	D	D	G	D
546	S	S	S	G
575	K	Q	Q	Q
592	G	G	E	E
603	G	G	E	G

[a] Amino acid sequences were deduced from the reported nucleotide sequences. Accession numbers of nucleotide sequences of the IC-B, AIK-C, and CAM-70 strains are NC_001498, AF266286, and DQ345723, respectively.

3.2. Fusion-inducing ability of the CAM-70, AIK-C, Tanabe, and IC-B H proteins in CD46- and SLAM-expressing cells

To examine fusion-inducing ability of the CAM-70 H protein, a plasmid expressing the CAM-70 H protein was generated and transfected into CHO cells expressing CD46 (CHO/CD46 cells) and SLAM (CHO/SLAM cells) together with an expression plasmid of the wild-type IC-B F protein. For comparison, plasmids expressing the AIK-C, Tanabe, and IC-B H proteins were also included. Transfected cells were fixed at 24, 48, and 65 h post-transfection (p.t.) and immunostained for the MV H protein (Fig. 1). When CHO/CD46 cells were used as target cells, no syncytium was observed in cells expressing the CAM-70 H protein up to 48 h p.t. and only a small number of syncytia were observed at 65 h p.t. In contrast, obvious syncytia were observed in cells expressing the AIK-C and Tanabe H proteins at 24 h p.t. (Fig. 1A). The IC-B H protein did not induce syncytium formation in CHO/CD46 cells over the course of the experiment, consistent with previous findings that reported that IC-B H protein dose not interact with CD46 [46,47] (Fig. 1A). In CHO/SLAM cells, the AIK-C, Tanabe, and IC-B H proteins, but not the CAM-70 H protein, induced prominent syncytia at 24 h p.t. (Fig. 1B). After 48 h p.t., syncytia were observed in CHO/SLAM cells expressing the CAM-70 H protein (Fig. 1B). Given the possibility that CAM-70 H protein was unable to induce efficient syncytium formation in co-operation with the IC-B F protein, the same experiments together with the CAM-70 F protein were also undertaken and the same results as those with the IC-B F protein were obtained (data not shown). These results indicated that both the CD46- and SLAM-dependent fusion-inducing abilities of the CAM-70 H protein were reduced in comparison to those of the H proteins of the other MV strains. Moreover, CD46-dependent fusion-inducing ability of the CAM-70 H protein was more reduced in comparison to the SLAM-dependent one, as indicated by the observed slow progression of syncytium formation by the CAM-70 H protein, an effect that was more prominent in CHO/CD46 cells than in CHO/SLAM cells. Cell surface expression of the H proteins was checked by transfection of CHO cells and flow cytometry, and the expression levels were found to be comparable among the H proteins examined in this study (data not shown).

Given that the fusion-inducing ability of the CAM-70 H protein was very low in CHO/CD46 cells, we next examined whether CD46 serves as the receptor for the CAM-70 H protein. In order to achieve this, Vero cells that had been pre-treated with an anti-CD46 MAb M177 for 1 h were infected with the CAM-70 strain and cultured in the presence of anti-CD46 MAb. The anti-CD46 MAb was found to inhibit syncytium formation and accumulation of the M protein in virus-infected cells (Fig. 1C), indicating that the CAM-70 H protein utilizes CD46 as its receptor, albeit in an inefficient manner.

3.3. Growth of recombinant MVs possessing the CAM-70, AIK-C, and Tanabe H genes in cultured cells

We next examined whether the low fusion-inducing ability of the CAM-70 H protein affects virus growth in cultured cells. For this experiment, we used a cloned MV IC323 that can be rescued from the full-length genomic cDNA of the wild-type IC-B strain [41]. The H gene of IC323 genomic cDNA was replaced with that of the CAM-70, AIK-C, or Tanabe strains and IC323s possessing the CAM-70, AIK-C, and Tanabe H genes instead of the IC-B H gene were rescued. These rescued recombinant MVs were designated IC-CAM H, IC-AIK-H, and IC-Tanabe H respectively (Fig. 2A). The growth kinetics of these recombinant MVs were then compared in various cell types (Fig. 2B). In HeLa cells and primary cultures of normal human dermal fibroblasts (NHDF), which express CD46 but not SLAM, IC-AIK H and IC-Tanabe H grew efficiently. IC-CAM H, however, exhibited slower and less efficient growth than both IC-AIK H and IC-Tanabe H (Fig. 2B). IC-AIK H and IC-Tanabe H induced syncytia in both HeLa cells (data not shown) and NHDF as outlined in Fig. 2C. However, the induction of syncytia by IC-CAM H was much less extensive in HeLa cells (data not shown) and not observed in NHDF (Fig. 2C). IC323 did not grow or induce syncytium formation in the two cell types (Fig. 2B and C), which is consistent with the result of the cell-fusion assay in CHO/CD46 cells expressing IC-B H protein. In marmoset B-lymphoid B95a cells, where SLAM is available for MV but not CD46 [48,49], all of the recombinant MVs examined grew efficiently; however, the growth kinetics of IC-CAM H was always slightly slower than those of the others (Fig. 2B). Although syncytia formation was observed in all recombinant MV-infected B95a cells, the spreading of syncytium was always slightly slower in the IC-CAM H-infected cells when compared to that in the cells infected with other recombinant MVs (data not shown). These findings indicate that the lower CD46- and SLAM-dependent fusion-inducing ability of the CAM-70 H protein resulted in slower growth of the recombinant MV. In addition, growth of IC-CAM H was more reduced in CD46-expressing cells compared to SLAM-expressing cells, a result consistent with those described above, indicating that the ability of the CAM-70 H protein to utilize CD46 as a receptor is more impaired in comparison to its use of SLAM.

3.4. Growth of the CAM-70 and Tanabe strains in cultured cells

To assess how the impairment in fusion-inducing ability of the CAM-70 H protein affects the character of the CAM-70 strain, we examined the infection of cultured cells with the CAM-70 strain and compared this infection with that of the parental Tanabe strain and IC323. In HeLa cells and NHDF, both the CAM-70 and Tanabe strains grew efficiently, although the growth rate of the CAM-70 strain was slightly slower than that of the Tanabe strain (Fig. 3A). This was in contrast to the impaired growth of IC-CAM H in these

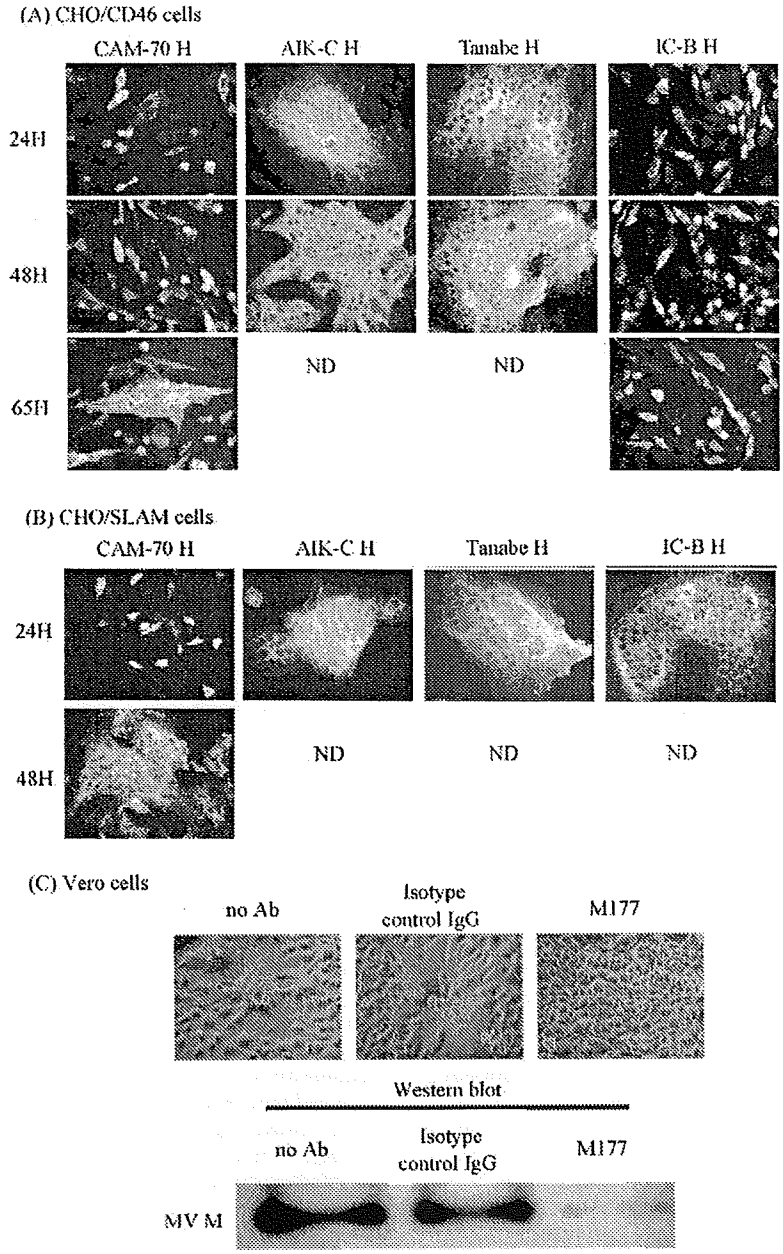

Fig. 1. Fusion-inducing ability of MV H proteins. CHO/CD46 cells (A) and CHO/SLAM cells (B) were transfected with plasmids expressing MV H protein of the indicated strain together with a plasmid expressing the F protein of the IC-B strain. At various time points post-transfection (24–65 h), cells were fixed, stained with anti-MV H protein MAb and FITC-conjugated goat anti-mouse IgG polyclonal serum and observed under a fluorescence microscope. In case cells were damaged severely because of extensive syncytium formation, photographs were not taken (ND). (C) Inhibition of MV infection by anti-CD46 MAb. Vero cells were pre-treated with anti-CD46 MAb (M177), IgG_1 isotype control antibody, or without antibody (no Ab) for 1 h. The cells were then infected with the MV CAM-70 strain with or without the presence of the antibody. Cells were observed under a phase-contrast microscope at 65 h post-infection. Infected cells were also harvested and cell lysates resolved by SDS-polyacrylamide gel electrophoresis under reducing conditions, transferred to PVDF membrane and immunoblotted for the MV M protein.

cells, suggesting that additional factors other than the H gene play important roles in the growth of the CAM-70 strain in the epithelial cells and fibroblasts. The formation of syncytium was less extensive in HeLa cells infected with the CAM-70 strain when compared to that in the cells infected with the Tanabe strain (data not shown), and was not observed in the CAM-70 strain-infected NHDF. Syncytium formation was observed in the Tanabe strain-infected NHDF (Fig. 3B). This result is consistent with the low fusion-inducing ability of the CAM-70 H protein in cells expressing CD46.

In B95a cells, both the CAM-70 and Tanabe strains grew, however the growth kinetics of the CAM-70 was slightly slower than those of the Tanabe strain and IC323 (Fig. 3A). The spread of syncytium was also slightly slower in B95a cells infected with the CAM-70 strain when compared to that in the cells infected with the Tanabe strain and IC323 (data not shown). These observations were consistent with those for the infection with IC-CAM H (Fig. 2B) and indicated that low fusion-inducing ability of the CAM-70 H protein was likely to slow growth kinetics of the parental CAM-70 strain in SLAM-expressing lymphoid cells.

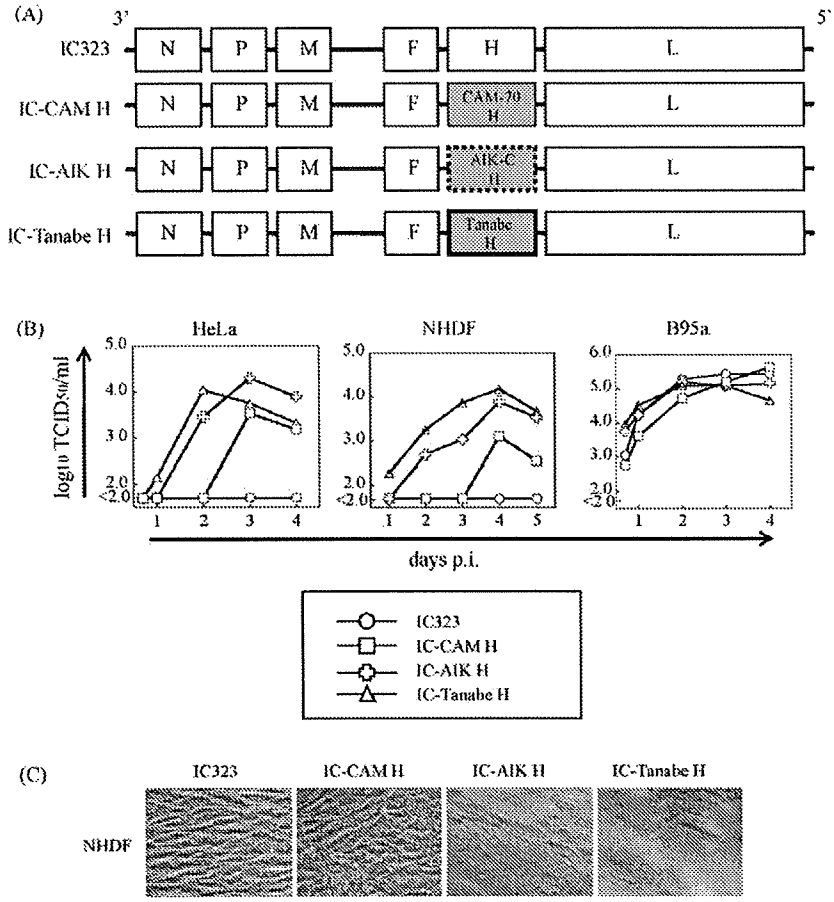

Fig. 2. Characterization of recombinant MVs containing H genes of various strains. (A) Schematic diagram of the recombinant MVs analyzed. Boxes indicate open reading frames of the MV nucleocapsid (N), phospho- (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins. The H gene of IC323 was replaced with that of the CAM-70, AIK-C, and Tanabe strains, generating IC-CAM H, IC-AIK H, and IC-Tanabe H respectively. (B) Growth kinetics of recombinant MVs in HeLa, NHDF, and B95a cells. Cells were infected with recombinant MVs at an MOI of 0.01 $TCID_{50}$/cell for HeLa and B95a cells, or at an MOI of 0.05 $TCID_{50}$/cell for NHDF. Infected cultures (both culture medium and cells) were harvested at the indicated time points. After freezing and thawing, infectivity titers were determined by measuring $TCID_{50}$ in B95a cells. Representative results from more than two independent experiments are shown. (C) Cytopathic effects of the recombinant MVs in NHDF. NHDF were infected with IC323, IC-CAM H, IC-AIK H, and IC-Tanabe H at an MOI of 0.05 $TCID_{50}$/cell. Cells were observed using a phase-contrast microscope at 65 h post-infection. In the photographs of the cells infected with IC-AIK H and IC-Tanabe H, the whole field is occupied by a syncytium.

3.5. The growth of the CAM-70 strain and recombinant MV expressing the CAM-70 H gene in peripheral blood lymphocytes and monocyte-derived dendritic cells

In MV-infected patients, it is thought that lymphocytes and dendritic cells serve as two of the major target cells for the virus [36]. Therefore, we next examined the infection of PBL and Mo-DC with the CAM-70 strain and compared this infection with that of the parental Tanabe strain and wild-type IC323. In addition, we also assessed infection with recombinant MVs, IC-CAM H and IC-Tanabe H. We found that PHA-stimulated PBL and Mo-DC expressed both CD46 and SLAM, however the levels of their expression were lower in Mo-DC than PHA-stimulated PBL (Fig. 4A). This result is consistent with those reported previously [50,51]. In PHA-stimulated PBL, the Tanabe strain grew efficiently and induced syncytia, a result also observed for IC323 (Fig. 4B and C). IC-Tanabe H also grew efficiently and induced syncytia, to an extent similar to that observed for IC323. However, the CAM-70 strain grew much less efficiently and induced syncytium formation to a lesser degree than both IC323 and the Tanabe strain (Fig. 4B and C). Although IC-CAM H grew efficiently, its growth kinetics was slightly slower than those of IC323 and IC-Tanabe H and its fusion induction was much less extensive (Fig. 4B and C). These results suggest that the impaired growth of the CAM-70 strain in PHA-stimulated PBL could be attributed to both the low fusion-inducing ability of the CAM-70 H protein and to other factors in addition to the H gene.

In Mo-DC, the CAM-70 strain did not grow efficiently and was unable to induce syncytium formation (Fig. 4B and C). Similarly, IC-CAM H did not grow efficiently and syncytium formation was not observed (Fig. 4B and C). In addition, immunoblot analysis revealed that viral protein accumulation was below the limits of detection in the Mo-DC infected with the CAM-70 strain and IC-CAM H (data not shown). These results indicated that the impaired growth of the CAM-70 strain in Mo-DC could be largely attributed to the low fusion-inducing ability of the CAM H protein and that the entry of the CAM-70 strain into Mo-DC was likely to be restricted. Although the Tanabe strain induced extensive syncytium formation in Mo-DC (Fig. 4C), infectious virus production was almost always under detection limits (Fig. 4B). This result is similar to our previous observations in Mo-DC, where we have shown that the Edmonston-derived vaccine strains AIK-C and FF-8 enter cells, replicate efficiently, and induce syncytia extensively, however, that infectious virus production is poor [40]. In contrast to this, IC-Tanabe H were shown to grow efficiently in Mo-DC and to induce extensive syncytium formation (Fig. 4B and C), indicating that the inefficient infectious virus production

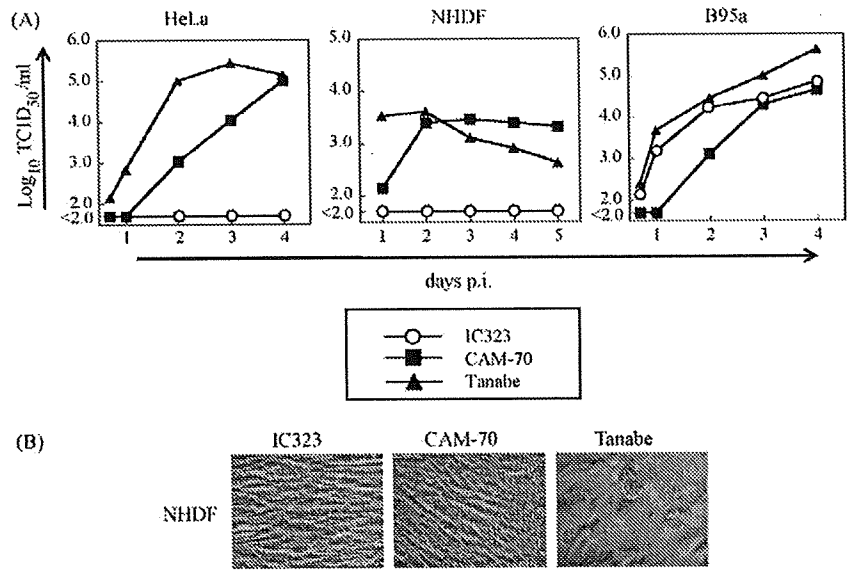

Fig. 3. Characterization of the MV CAM-70 and Tanabe strains. (A) Growth kinetics of MVs in HeLa, NHDF, and B95a cells. The growth kinetics of the CAM-70 and Tanabe strains were examined as described in Fig. 2B. The growth kinetics of IC323 was also examined for comparison. Representative results from more than two independent experiments are shown. (B) Cytopathic effects of MVs in NHDF. NHDF infected with IC323, the CAM-70, and Tanabe strains were observed as described in Fig. 2C. In the photograph of the cells infected with the Tanabe strain, the whole field is occupied by a syncytium.

of the Tanabe strain could be attributed to factors other than the H gene.

3.6. Identification of the amino acids responsible for the low CD46- and SLAM-dependent fusion-inducing ability of the CAM-70 H protein

To identify the amino acid regions responsible for the low fusion-inducing ability of the CAM-70 H protein, we generated plasmids expressing two chimeric H proteins between the CAM-70 and AIK-C strains using convenient restriction enzyme sites. This process resulted in the dividing of the H protein into two regions. Region I encompassed the endoplasmic, transmembrane, stalk domains and a small part of the head region, while region II encompassed a large part of the head region including the predicted CD46- and SLAM-binding sites (Fig. 5A). These plasmids were transfected into CHO/CD46 and CHO/SLAM cells together with a plasmid expressing IC-B F protein. The transfected cells were strained with Giemsa's solution at 24 h p.t., and then fusion indices were determined as described in Section 2 (Fig. 5B and C). In CHO/CD46 cells, CAM-AIK H II, in which region II was derived from the AIK-C H protein, induced syncytium formation, although the extent was less than that induced by the AIK-C H protein (Fig. 5B). CAM-AIK H I, in which region II originated from the CAM-70 H protein, was unable to induce any syncytia at this time point (Fig. 5B). In CHO/SLAM cells, CAM-AIK H II induced syncytia to a similar extent as the AIK-C H protein at 24 h p.t. (Fig. 5C). CAM-AIK H I did not produce any syncytia at this time point (Fig. 5C). These results indicated that lower fusion-inducing ability of the CAM-70 H protein could be attributed to region II, the area encompassing a large part of the head domain. Region I was also thought to play a role in the low CD46-dependent fusion-inducing ability of the CAM-70 H protein, given that the fusion-inducing ability of CAM-AIK H II in CHO/CD46 cells was observed to be less than that of the AIK-C H protein.

By comparing the amino acid sequences of region II in the CAM-70, Tanabe, and AIK-C H proteins, we found that the amino acid composition differed only in the CAM-70 H protein at positions 455, 505, and 603 (Table 1 and Fig. 5A). As a result, we focused our study on the amino acids located at these three positions. We replaced these residues in the CAM-70 H protein with those of the AIK-C and Tanabe H proteins, generating the mutants CAM H N455T, G505D, and E603G (Fig. 5A). We then transfected plasmids expressing these mutants into CHO/CD46 and CHO/SLAM cells. In CHO/CD46 cells, the mutants failed to induce any obvious syncytia at 24 h p.t. (data not shown). Of these three mutants, only the CAM H N455T mutant was able to induce a few small-sized syncytia at 48 h p.t. (Fig. 5B). The remaining two mutants, CAM H G505D and E603G, did not induce any syncytia at this time point (Fig. 5B). Given that the fusion-inducing ability of the CAM H N455T mutant was still lower than that of CAM-AIK H II, it was expected that either G505 or E603 in addition to N455 could result in the lower CD46-dependent fusion-inducing ability of the CAM-70 H protein. Therefore, we generated the combination mutants CAM H N455T + G505D and N455T + E603G (Fig. 5A). None of them induced syncytia at 24 h p.t. (data not shown). CAM H N455T + E603G induced more extensive syncytia than the single mutant CAM H N455T at 48 h p.t. (Fig. 5B). However, fusion-inducing ability of the CAM H N455T + E603G combination mutant was still lower than that of CAM-AIK H II (Fig. 5B). There was no obvious enhancement in fusion induction observed in CHO/CD46 cells expressing CAM H N455T + G505D compared with the cells expressing CAM H N455T (Fig. 5B). Inversely, we also replaced amino acid residues at positions 455, 505, and 603 in the AIK-C H protein with those of the CAM-70 H protein, generating the mutants AIK H T455N, D505G, and G603E. Consistent with the results described above, the CD46-dependent fusion-inducing ability of AIK H T455N was less than that of the AIK-C H protein (data not shown). However, neither D505G nor G603E reduced fusion induction in CD46-expressing cells (data not shown). These results indicated that the amino acid substitution T455N resulted in the low CD46-dependent fusion-inducing ability of the CAM-70 H protein and that the substitution G603E may also play a role in this process.

In CHO/SLAM cells, CAM H G505D induced syncytium formation equally as well as CAM-AIK H II and the AIK-C H protein at 24 h p.t. (Fig. 5C). CAM H N455T also induced syncytium formation at 24 h p.t., although it was less extensive than that induced by CAM-AIK H II and the AIK-C H protein. In contrast, CAM H E603G

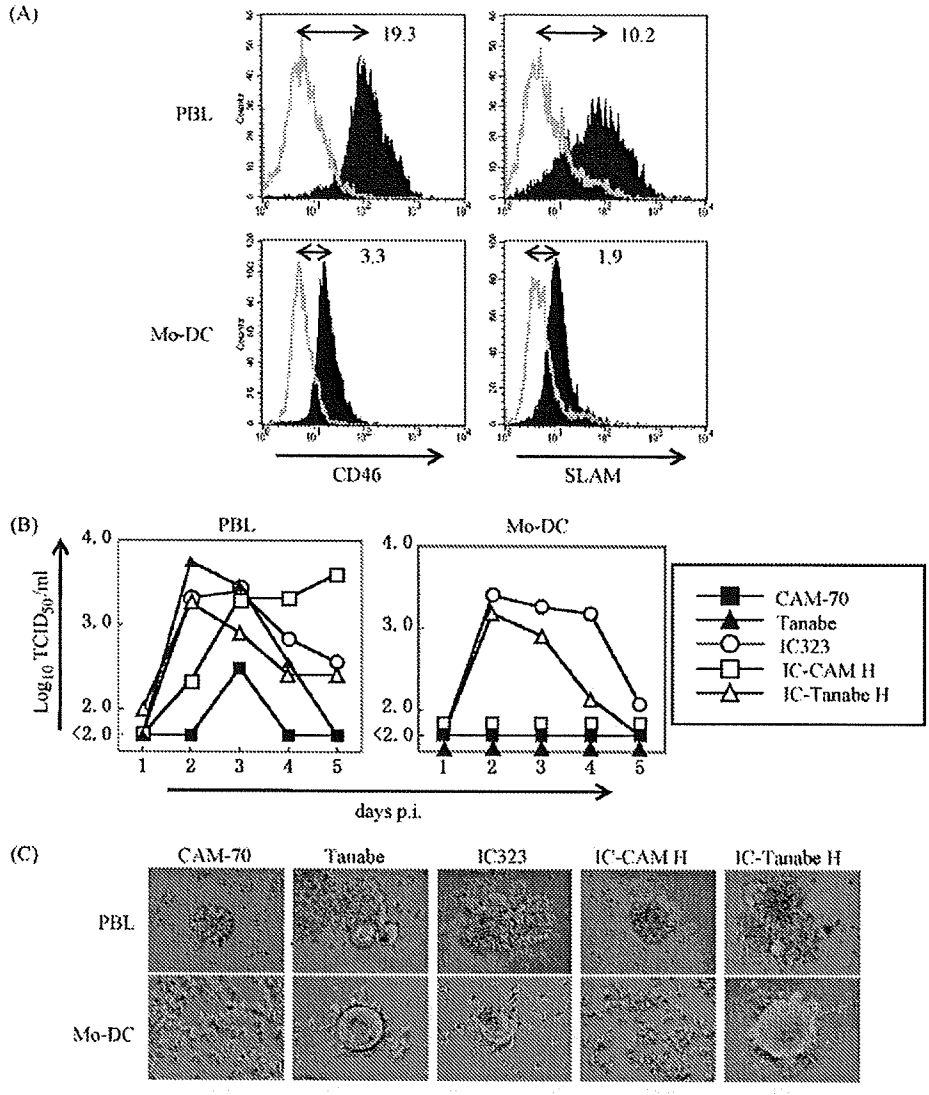

Fig. 4. Infection of PBL and Mo-DC with MVs. (A) Expression of CD46 and SLAM on PBL and Mo-DC. PBL stimulated with PHA (2.5 μg/ml) for two days and immature Mo-DC were stained with FITC-conjugated anti-CD46 MAb (filled profile) or mouse IgG_1 isotype control antibody (gray line) for detection of CD46. To detect SLAM, the cells were incubated with anti-SLAM MAb (IPO-3) (filled profile) or mouse IgG_1 isotype control antibody (gray line), followed by incubation with FITC-conjugated goat anti-mouse IgG $F(ab')_2$ fragment. Fluorescent cells were analyzed with a flow cytometer. The ratio of mean fluorescence intensity of the filled profile: the gray line is shown and indicates expression levels on the cells. (B) Growth kinetics of MVs in PBL and Mo-DC. PBL and Mo-DC were infected with MVs at an MOI of 0.05 $TCID_{50}$/cell. PBL was incubated in RPMI-10% FBS supplemented with 2.5 μg of PHA-L per milliliter and Mo-DC in RPMI-10%FBS supplemented with GM-CSF and IL-4. Infected culture medium and cells were harvested together every day up to five days post-infection. After freezing and thawing, infectivity titers were determined by measuring $TCID_{50}$ in B95a cells. Representative results from more than two independent experiments are shown. (C) Cytopathic effects of MVs in PBL and Mo-DC. PBL and Mo-DC were infected with MVs and cultured as described above. Cells were observed using a phase-contrast microscope at two days post-infection.

did not produce any syncytia at this time point (Fig. 5C). Moreover, there was no enhancement in fusion induction observed in CHO/SLAM cells expressing CAM H N455T + E603G compared with the cells expressing CAM H N455T (Fig. 5C). Inversely, in the experiments with mutant AIK-C H proteins, the substitutions T455N and D505G reduced the SLAM-dependent fusion-inducing ability but not G603E (data not shown). These results suggested that the amino acid substitutions T455N and D505G were responsible for the low SLAM-dependent fusion-inducing ability of the CAM-70 H protein.

4. Discussion

In the current study, we demonstrated that the ability of the MV vaccine strain CAM-70 H protein to use the CD46 and SLAM MV receptors is low. In addition, we showed that the ability of the CAM-70 H protein to use CD46 is far more impaired in comparison to that of SLAM. Accordingly, growth of IC-CAM H was significantly reduced in CD46-expressing HeLa cells and NHDF, and was slightly slower than that of IC323, IC-AIK H, and IC-Tanabe H in SLAM-expressing B95a cells. The CAM-70 strain grew efficiently in HeLa cells and NHDF, although the growth kinetics was slightly slower than that of the parental Tanabe strain. This indicated to us that factors other than the H gene of the CAM-70 strain may play a role in its efficient growth in both epithelial cells and fibroblasts. It has been reported previously that the H, M, and L genes contributed to the adaptation of the Edmonston B vaccine strain in African green monkey kidney epithelial Vero cells [52]. Thus, we generated a recombinant MV based on IC323, that contained the M gene of the CAM-70 strain in place of that of IC323. This recombinant MV was able to grow in Vero cells, although IC323 was not (S. Kato and S. Ohgimoto, unpublished data), suggesting that the M gene of the CAM-70 strain is likely to

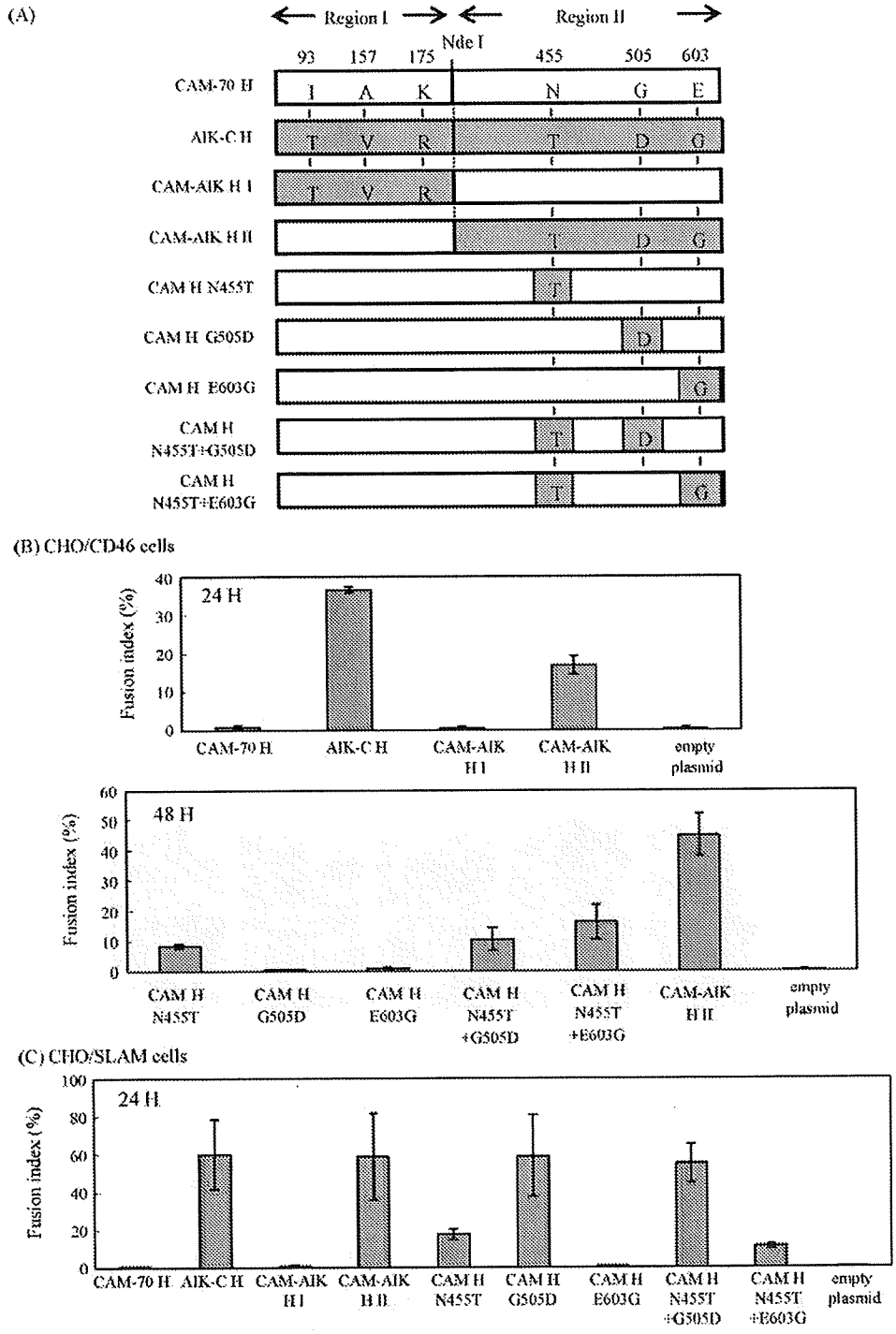

Fig. 5. Fusion-inducing ability of mutant MV H proteins. (A) Schematic diagram of chimeric H proteins between the CAM-70 and AIK-C strains and mutant H proteins of the CAM-70 strain. Plasmids expressing two chimeric H proteins between the CAM-70 and AIK-C strains were generated using the restriction enzyme NdeI and resulted in the division of the H protein into two regions termed region I and region II. Amino acid sites differing only in the CAM-70 H protein when compared to the amino acid sequences of CAM-70, Tanabe, and AIK-C H proteins are indicated. Regions and amino acids derived from the AIK-C H protein are shaded and those from the CAM-70 H protein are in white. (B and C) Fusion induction by chimeric and mutant H proteins. CHO/CD46 (B) and CHO/SLAM (C) cells were transfected with plasmids expressing chimeric H proteins between the CAM-70 and AIK-C strains and mutant CAM-70 H proteins together with the plasmid expressing the F protein of the IC-B strain. At the time points post-transfection indicated (24–48 h), cells were stained with Giemsa's solution and fusion indices were determined as described in Section 2. The bars indicate the mean ± standard deviations for triplicate counts.

be responsible for the efficient growth of the CAM-70 strain in both epithelial and fibroblastic cells. We are now investigating whether other genes of the CAM-70 contribute to efficient growth in Vero cells.

IC-CAM H was found to grow in PHA-stimulated PBL to a similar degree as the other recombinant MVs studied, although the growth kinetics was slightly slower. IC-CAM H did not grow efficiently in Mo-DC, even though both PHA-stimulated PBL and Mo-DC

express CD46 and SLAM [50,51,53]. This may be due to the observation that the expression levels of CD46 and SLAM on the surface of Mo-DC are lower than those observed on PHA-stimulated PBL (Fig. 4C) [50,51]. As described above, the ability of the CAM-70 H protein to utilize CD46 was vastly impaired, while its ability to use SLAM was only slightly reduced. Thus in PHA-stimulated PBL, the CD46-dependent entry of IC-CAM H is likely to be much less efficient but the SLAM-dependent entry likely to be only slightly impaired. Similarly, CD46-dependent entry of IC-CAM H into Mo-DC also seems to be significantly less efficient. In addition, the CAM-70 H protein may not be able to efficiently use the low levels of SLAM expressed on the surface of Mo-DC. In support of this hypothesis, it has been reported that there are expression level thresholds for MV receptors that are required to induce cell fusion. The MV H protein cannot induce cell fusion in cells expressing MV receptors at levels lower than the threshold [54,55]. Thus, the expression of SLAM on Mo-DC may be lower than the threshold level for the CAM-70 H protein but be higher than those for the Tanabe and IC323 H proteins, of which the ability to use SLAM is higher than that of the CAM-70 H protein.

In PBL and Mo-DC, the CAM-70 strain did not grow efficiently or induce syncytium formation. As lymphocytes and dendritic cells are thought to be two of the major target cells for the MV in infected patients [36], the impaired growth of the CAM-70 strain in these cell types may be one of the underlying mechanisms of its attenuation. In the current study, the impaired growth of the CAM-70 strain was found to be attributed to the H gene only in part in PBL and largely in Mo-DC. Therefore, the H gene may be one of the factors responsible for the attenuation of the CAM-70 strain. Future investigation in our laboratory will seek to identify additional factors on the CAM-70 strain which may cause impaired growth in these target cells.

The amino acid substitutions T455N and G603E were shown to be partly responsible for the lower CD46-dependent fusion-inducing ability of the CAM-70 H protein, while T455N and D505G were found to be largely responsible for the lower SLAM-dependent process. The crystal structure of the MV H protein has recently been reported and demonstrates that the MV H protein forms a disulfide-linked homodimer and exhibits a six-bladed β-propeller fold with β1–β6 sheets [34]. Putative binding sites for CD46 and SLAM have been shown to be situated independently on the surface of the molecule [22–26,28,29,31–33]. T455 is located in the β4 sheet where several amino acid residues involved in CD46 binding have been identified [22–26,31,33]. T455 is, however, thought to be located internally and outside the putative CD46 and SLAM binding sites on the surface of the MV H protein. This suggests that T455N may change the conformation of the H protein, thus reducing the affinity of the molecule to the receptors or its ability to trigger membrane fusion. G603 is located within the β6 sheet and outside the receptor binding sites. Therefore, G603E may also change the conformation of the H protein. This may result in the impairment of CD46-dependent fusion induction, although the effect of this change appears to be subtle. D505 is located at the putative SLAM-binding site where it forms an "acidic patch" with other negatively charged residues including E503, D507, D530, and E535 [32–34]. Substitution D505G reduces the negative charge at the SLAM-binding site and thus, most likely impairs interactions with SLAM. In addition to D505G, T455N was also found to reduce the SLAM-dependent fusion-inducing ability of the CAM-70 H protein, a result likely caused by a change in the conformation of the molecule.

The CAM-70 strain was derived from the Tanabe strain following serial propagation in chicken embryonic cells. As shown in this study, the H protein of the Tanabe strain was able to use both CD46 and SLAM receptors efficiently. Therefore, the serial propagation undertaken in the cells appears to have weakened the ability of the H protein to utilize the receptors. Given that CD46 and SLAM are not expressed on chicken embryonic cells, the CAM-70 strain should enter the cells via another pathway. It has been reported that adaptation at the entry step is necessary for MV to grow in chicken embryonic fibroblasts [56]. There have been eight amino acid differences demonstrated between the Tanabe and CAM-70 H proteins (Table 1), including differences at positions 455, 505, and 603 which have been reported in this study to be related to the reduced ability of the CAM-70 H protein to use CD46 and SLAM efficiently. Some of these changes may have resulted in the H protein efficiently using the entry pathway on chicken embryonic cells, and as such these changes may have weakened their ability to use CD46 and SLAM. We are now investigating whether these amino acid substitutions are related to adaptation of the CAM-70 strain to chicken embryonic cells.

Numerous mechanisms have been hypothesized for the attenuation of MV strains. The temperature sensitive nature due to the P gene is thought to be responsible for the attenuation of the AIK-C vaccine strain [57,58]. Reduced gene expression in cultured cells has also been suggested as a mechanism for the attenuation of Edmonston vaccine strain [59]. Interferons are thought to be induced more readily in cells infected with attenuated MV strains than in cells infected with wild-type MV strains [60,61] and resistance against interferons is impaired in the attenuated MV strain Edmonston-tag [62]. An impaired infectious virus production of MV vaccine strains in Mo-DC may also be related to their attenuation [40]. Moreover, infection experiments in human tonsillar tissues have suggested that vaccine attenuation may be the result of alterations in cell tropism [63]. In conclusion, we have shown in the current study that the ability of the H protein of the CAM-70 strain to utilize the CD46 and SLAM MV receptors is low. This result outlines one of the possible mechanisms for attenuation of the CAM-70 strain.

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Key words
ムンプス
ムンプスウイルス
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血清抗体
ウイルス分離

特集 ワクチンで予防可能な疾患の病原診断とその読み方 3

ムンプス

庵原 俊昭*

要旨

ムンプス流行時の急性耳下腺腫脹例の多くはムンプスであり，非流行時の急性耳下腺腫脹例の多くはムンプス以外が原因である．ムンプス確定診断の基本は，病巣からのウイルス分離，ウイルス遺伝子の検出であるが，これらの検査を一般医療機関で行うのは困難なため，多くの医療機関ではムンプスの診断に血清抗体測定を用いている．ムンプスに対する免疫の確認には血清 EIA-IgG 抗体を測定する．急性耳下腺腫脹の既往歴やムンプスワクチン歴がある急性耳下腺腫脹例の診断には，血清 EIA-IgM 抗体と EIA-IgG 抗体を同時に測定する．また，ムンプス流行中の無菌性髄膜炎例や難聴例では血清抗体を測定し，ムンプスとの関連を検討することが大切である．

はじめに

ムンプスは流行性耳下腺炎，おたふくかぜともよばれ，パラミクソウイルス科モルビリウイルス属に属するムンプスウイルスによる全身性ウイルス感染症である．潜伏期間は通常 16～18 日である．急性耳下腺腫脹が特徴的な臨床症状であるが，反復性耳下腺炎，化膿性耳下腺炎など，ムンプス以外にも急性耳下腺腫脹を示す疾患があり，臨床症状だけではムンプスの診断が困難な場合がある[1]．また，ムンプス流行時には急性耳下腺腫脹を伴わないムンプスウイルスによる無菌性髄膜炎例や難聴例を経験することがある[2]．本稿では，ウイルス感染症診断の基本および種々のムンプスの病態における病原診断方法とそのデータの読み方について解説する．

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I ウイルス感染症の確定診断方法（表 1）

ウイルスがホストに感染すると，ウイルスによる直接の侵襲とそれに伴うホストの免疫反応により臨床症状が出現する．臨床症状を伴わない場合はウイルス感染であり，軽症の臨床経過を示す場合は修飾感染である．ワクチン後の免疫減衰により発症する軽症の麻疹，水痘，ムンプスは，修飾麻疹，修飾水痘，修飾ムンプスとよばれている．

ウイルス感染症診断の基本は，病巣にウイルスの存在を証明することである[3]．なかでも病巣からのウイルス分離は，病巣に生きたウイルスの存在の証明であり，診断の gold standard である．分離が困難なときは，polymerase chain reaction（PCR）法や loop-mediated isothermal amplification（LAMP）法にてウイルス遺伝子を検出する．検出ウイルス量が多いほど発症に強く関連している．また生検などにより組織が得られたときは，免疫染色などの方法

表1　ウイルス感染症の確定診断方法

1．直接的な診断方法
1）病巣からのウイルス分離
2）病巣からのウイルス抗原検出（迅速診断法）
3）病巣からのウイルス遺伝子の検出（PCR，LAMP など）
4）病巣におけるウイルス蛋白の証明
2．間接的な診断方法
1）血清 IgM 抗体の検出
2）血清抗体の有意上昇
3）病巣以外からのウイルス分離
4）病巣以外からのウイルス遺伝子の検出（PCR，LAMP など）

PCR：polymerase chain reaction，LAMP：loop-mediated isothermal amplification

表2　ウイルス分離用サンプルの適切な採取方法と取り扱い方

・急性期できるだけ早期にサンプルを採取する
・原則病巣，または病巣の分泌液からサンプルを採取する
・採取したサンプルは生理食塩水またはトランスポートメディウムに入れ，乾燥させないようにする
・髄液や尿はそのまま使用する
・サンプルは－60℃以下で保存しても壊れない滅菌された保存容器に保存する
・サンプル採取後できるだけ早期に目的とするウイルスに応じた培養細胞にサンプルを接種する
・ウイルス分離を行うまでの期間が短い場合は 4℃で，長い場合は－60℃以下でサンプルを保存する

で病巣にウイルス蛋白の存在を証明する．

病巣からのサンプル採取が困難な場合や，病巣からのウイルス分離，ウイルス遺伝子の検出，ウイルス蛋白の証明が困難な場合は，ウイルス感染の傍証として，血清抗体測定や病巣以外の部位からのウイルス分離，ウイルス遺伝子の検出などが行われている．急性期血清での IgM 抗体の検出や血清抗体の有意上昇は，ウイルス感染症診断に有用な診断方法である．IgM 抗体の検出には，多くは酵素免疫（EIA）法が用いられている．血清抗体の有意上昇とは，2 週間以上あけて測定した血清抗体価が，測定誤差以上の上昇を示すことであり，中和（NT）法や赤血球凝集抑制（HI）法では 2 管（4 倍）以上の，EIA 法では 2 倍以上の上昇である．

エンテロウイルスやポリオウイルス感染では便や咽頭からのウイルス分離や，血清のウイルス遺伝子検出は有用な補助診断検査である．

ウイルス感染症診断の gold standard であるウイルス分離用サンプルの取り扱い方法を表2に示した．ウイルス分離はサンプル採取後早急に行うべきである[4]．細胞に接種するまでの期間が短い場合は 4℃で，接種までの期間が長い場合は－60℃以下でサンプルを保存する．エンベロープをもつウイルスでは融解時にウイルスが壊れやすくなるため，分離用のサンプルは－20℃では保存しない．PCR や LAMP 用のサンプルは－20℃での保存は可能である．血清は－20℃で保存する．

表3 ムンプスの診断

【疫学】ムンプス患者との接触がある，地域でムンプスの流行がある
【臨床】・2日間以上持続する，片側ないし両側の急性耳下腺腫脹
・他に耳下腺腫脹の原因がないこと
【検査室確定診断】
1．ワクチン歴なし（PVFを含む）
・急性期血清EIA-IgM抗体陽性
・血清EIA-IgG抗体の有意上昇（2倍以上の上昇）
・唾液からのムンプスウイルス分離陽性，唾液のPCRまたはLAMP陽性
2．ワクチン歴あり（再感染を含む）
・急性期血清EIA-IgM抗体弱陽性
・急性期血清EIA-IgG抗体高値（25.9EIA単位以上）
・唾液からのムンプスウイルス分離陽性，唾液のPCRまたはLAMP陽性

PVF：primary vaccine failure，PCR：polymerase chain reaction，LAMP：loop-mediated isothermal amplification

Ⅱ ムンプスの診断方法

臨床上のムンプスの診断基準は，ムンプス流行中の48時間以上持続する急性耳下腺腫脹である（表3）．ワクチン後のムンプス罹患例や乳児では，ときに耳下腺腫脹期間が短い例を経験する．一方，ムンプスが流行していないときの急性耳下腺腫脹の原因はムンプス以外である[5]．反復性耳下腺炎例の多くは片側の耳下腺腫脹であるが，ときに両側耳下腺の腫脹を認める例がある．

ムンプスのウイルス学的診断方法として，ウイルス分離，ウイルス遺伝子検出および血清抗体測定がある．ムンプスにおいてウイルス学的診断が必要な場合は，①ムンプス流行時期以外に急性耳下腺腫脹を認めたとき，②ムンプス流行中にワクチン接種を受け，その後に耳下腺腫脹や無菌性髄膜炎を発症したとき，③ムンプス流行中に無菌性髄膜炎や難聴を認めたとき，④ワクチン後1カ月以内に耳下腺腫脹や無菌性髄膜炎を発症したとき，⑤ワクチン後1カ月以上経過して流行中に耳下腺腫脹を認めたとき，⑥ムンプスの診断を受けていた人がムンプス流行中に再度耳下腺腫脹を認めたとき，などである．耳下腺腫脹の原因や，髄膜炎を発症させた株の同定が必要である．

1．ムンプスウイルス分離の方法

急性耳下腺腫脹を認めたときは唾液からウイルス分離を行い，無菌性髄膜炎を合併したときは髄液からウイルス分離を行う．唾液の採取は綿棒を用いて行う．唾液を十分に染み込ませた綿棒を生理食塩水の中でよく撹拌し，綿球を絞った後，木製の棒はウイルスを不活化させる危険性があるため綿棒を取り出しておく．髄液からウイルス分離を行うときは腰椎穿刺で採取した髄液をそのまま培養細胞に接種する．ムンプスウイルスの分離には，アフリカミドリザル腎臓由来の培養細胞であるVero細胞を用いる．以前ムンプスでは尿からのウイルス分離も推奨されていたが，唾液と比較し分離率が低いため，近年は尿からのウイルス分離は推奨されなくなっている．

2．血清抗体の測定

ムンプスにおいてもっとも感度の高い血清抗体測定方法はEIA法であり，HI法はEIA法に比べると感度は50%程度の低率である[6]．ムンプス既往の診断やワクチン後の抗体保有状況を確認するためには血清EIA-IgG抗体を測定する．一方，ムンプス急性期の診断には血清EIA-IgM抗体を測定する．ワクチン後の自然ムンプス罹患の病態を検討するためには，EIA

表4　急性耳下腺腫脹例におけるムンプス抗体の特徴と診断

既往歴	ワクチン歴	IgM 抗体	IgG 抗体		診　断*
			抗体価	avidity	
なし	なし	＋～＋＋	＋	弱い	ムンプス初感染*
なし	なし	－～＋	＋＋＋†	強い	ムンプス再感染*
なし	なし	－	－or＋†	強い	ムンプス以外の原因
なし	あり	＋～＋＋	＋	弱い	PVF*
なし	あり	－～＋	＋＋＋	強い	SVF*
なし	あり	－	＋	強い	ムンプス以外の原因
あり	なし	－	＋	強い	ムンプス以外の原因
あり	なし	－～＋	＋＋＋	強い	ムンプス再感染†

（滝原俊昭，2007[3]より一部改変）

PVF：1次性ワクチン不全（primary vaccine failure），SVF：2次性ワクチン不全（secondary vaccine failure）

*：唾液からのウイルス分離は診断の参考になる．

†：ムンプスの顕性感染率は70%．

法で血清IgM抗体とIgG抗体を同時に測定するほうが望ましい．

ムンプスではウイルス血症により親和性臓器に到達したウイルスがそれぞれの臓器で増殖してから症状が出現するため，ウイルス血症の時期からムンプスの主要症状出現までの時期にタイムラグがある[7]．多くの全身性ウイルス感染症では，ウイルス血症のピーク時頃から血清IgM抗体が検出され始めるが，ムンプスの初感染例では耳下腺腫脹時にIgM抗体だけではなくIgG抗体も検出される[8]．

Ⅲ 種々の病態におけるムンプスの診断方法

疫学的関連性のある急性耳下腺腫脹例の診断は比較的容易であるが，いろいろな状況から診断に苦慮する例や由来株の同定が必要な例を紹介する．

1．ムンプス非流行時の急性耳下腺腫脹例

ムンプス非流行時ではほとんどの急性耳下腺腫脹例の唾液からウイルスは分離されず，血清EIA-IgM抗体は陰性である．EIA-IgG抗体は，発症者の免疫状態（既往歴やワクチン歴）により異なっている（表4）．非流行時の急性耳下腺腫脹の原因の多くは，反復性耳下腺炎か化膿性耳下腺炎である．耳下腺の超音波検査を行うと，反復性耳下腺炎では耳下腺内の導管がアップルツリー状に拡張している像が認められる．なお，ムンプスにおける超音波所見はびまん性の耳下腺腫脹である．

2．ムンプス流行中にムンプスワクチンを受けた後の急性耳下腺腫脹例と無菌性髄膜炎例

ムンプスが流行するとムンプスワクチンの接種希望者が増加する．ムンプス非流行時のムンプスワクチン接種後の耳下腺腫脹率は2%であるのに対し，流行中にムンプスワクチンの接種を受けると，接種後の急性耳下腺腫脹率は5%に増加する[9]．増加した3%は自然感染によるものである（表5）．

ワクチン接種後15日以内に認める耳下腺腫脹は自然感染によるものであり，19日以降に認める耳下腺腫脹は，多くはワクチン株によるものである．16～18日の間に認める耳下腺腫脹は，自然感染例とワクチン後の臨床反応例が混在している[10]．ワクチン後1カ月以内に耳下腺腫脹を認めた場合は，唾液からウイルス分離を行ってから由来株の同定を行うか，唾液から直接PCR法またはLAMP法で由来株を同定す

表5 流行時期による耳下腺腫脹例のウイルス分離結果

	非流行時	流行時	p value
接種例	1,136	949	
耳下腺腫脹例	24	47	0.0004
	2.11%	4.95%	
分離陽性 星野株*	14	14	0.6281
	1.23%	1.48%	
野生株	1	25	<0.0001
	0.09%	2.63%	
分離陰性	9	8	0.8950
	0.79%	0.84%	

（落合 仁ほか，2008[9]より一部改変）

*：ムンプスワクチン株

ることが大切である．PCR法またはLAMP法にて増幅された部位の制限酵素切断パターンで由来株を同定する[10][12]．

由来株の同定を行うときは，ウイルス量が多い病初期にサンプルを採取後，接種したワクチンのメーカーに相談する．現在日本で市販されているムンプスワクチン株は，星野株と鳥居株の2株である．なお，急性期血清抗体パターンは，自然感染初感染時の抗体パターンと同様のIgM抗体陽性，IgG抗体弱陽性であり，抗体反応パターンから由来株の推定は不可能である．

ムンプスワクチン後の臨床反応である無菌性髄膜炎はワクチン接種後20日頃に発症する．無菌性髄膜炎を発症した場合も由来株の同定が大切である．

3．ムンプス流行中の無菌性髄膜炎例と難聴例

ムンプスでは感染したウイルスが上気道で増殖後ウイルス血症により，耳下腺を含む唾液腺，膵臓，中枢神経系，内耳，腎臓，精巣や卵巣などの親和性臓器に散布され，それぞれの臓器で増殖して症状が出現する．このような病態のため，ムンプス流行中にときに耳下腺腫脹を認めずにムンプスウイルスによる無菌性髄膜炎やムンプス難聴を発症することがある[2]．

ムンプスウイルスによる無菌性髄膜炎と確定診断するためには，髄液からムンプスウイルスを分離するか，ムンプスウイルス遺伝子を検出することが必須である．血清抗体の測定は診断の参考になる．無菌性髄膜炎急性期の抗体パターンは，IgM抗体陽性，IgG抗体弱陽性の初感染パターンである．

ムンプス難聴は400～1,000人に1人の割合で発症する予後不良の合併症である[13][14]．多くは片側の難聴であり，補聴器を用いても聴力の向上は困難である．蝸牛膜迷路にムンプスウイルスが感染して発症する[14]．ムンプス流行中に耳下腺腫脹を認めずに難聴を発症した症例を経験した場合は，ムンプス血清抗体価を測定する．血清抗体パターンはムンプス初感染パターンと同じである．

4．ムンプスワクチンを受けた後1カ月以内の急性耳下腺腫脹例と無菌性髄膜炎例

ムンプスが流行していないときにワクチン接種後16～21日に認める急性耳下腺腫脹は，ムンプスワクチンによる臨床反応である．確定診断するためには唾液を採取し，ウイルス分離後に由来株を同定するか，直接PCR法などで由来株を同定する．

ムンプスワクチン接種後20日頃に無菌性髄膜炎を発症することがある．わが国のワクチン株による無菌性髄膜炎の発症頻度は1/2,000接種である[15]．採取した髄液からウイルス分離後に由来株を同定するか，直接PCR法などで由来株を同定する．ムンプスワクチン接種により