Leucine at position 278 of the AIK-C measles virus vaccine strain fusion protein is responsible for reduced syncytium formation

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The live measles virus (MV) vaccine strain AIK-C was attenuated from the wild-type strain Edmonston by plaque purification at 33°C. Strain AIK-C grew well at 33°C with a mixture of small- and medium-sized plaques in Vero cells, but did not grow well at 40°C. To investigate fusion inducibility, expression plasmids for the fusion (F) and haemagglutinin (H) protein regions of MV strains AIK-C (pAIK-F01 and pAIK-H) and Edmonston (pEdm-F and pEdm-H) were constructed. pEdm-F induced extensive cell fusion in B95a and Vero cells under the control of T7 RNA polymerase, whereas a sharp reduction in syncytium formation was observed when pAIK-F01 was used. Six amino acid differences were determined between pAIK-F01 and pEdm-F. Direct sequencing showed that the seed strain AIK-C contained either Leu or Phe at position 278 of the F protein. Experiments using recombinant F protein plasmids demonstrated that those with Leu at position 278 induced poor syncytium formation, while those with Phe at position 278 (Edmonston type) induced extensive cell fusion. Replacement of Phe with Leu at position 278 of pEdm-F reduced fusion-inducing capability. A full-length infectious clone of AIK-C with Leu at position 278 was constructed. The rescued virus produced small plaques in Vero cells. However, the same rescued virus with Phe at position 278 produced large plaques. It was concluded that Leu at position 278 of the F protein of the MV vaccine strain AIK-C is responsible for the formation of small plaques.

Introduction

Measles virus (MV) is a member of the family Paramyxoviridae, genus Morbillivirus. Acute infection with MV begins in the upper respiratory tract and, through subsequent secondary viraemia, the virus is disseminated to organs and tissues causing a variety of clinical symptoms. MV infects immunocompetent cells, lymphocytes and macrophages and causes a transient immunosuppressive state. Encephalitis is the most serious illness in measles patients and occurs at a rate of one case in 1000 to 2000. Subacute sclerosing panencephalitis, which develops several years after primary acute MV infection, occurs at a rate of less than one case in 100,000. Although an ordinary MV infection is debilitating, the majority of patients recover completely without serious complications (Griffin et al., 1995; Redd et al., 1999). The number of measles cases has decreased due to the widespread use of a live MV vaccine in developed countries (Rota et al., 1996). It is now thought that throughout the world every year, approximately 30 million individuals become infected with MV and, according to the Expanded Programme on Immunization (EPI) newsletter (Pan American Health Organization, 2000), 0–88 million children die of measles or measles-related complications. The EPI wants to eradicate MV, but has been unable to achieve this goal for various reasons, including the unexpected high infectivity of MV, the poor immunogenicity of MV vaccines for infants under 9 months of age and problems with vaccine distribution. The EPI recommends vaccination against MV at 9 months of age, but one-third of measles patients are infants under this age (Whittle et al., 1988). When high-dose MV vaccines of the Schwarz or Edmonston–Zagreb strains were given to 6-month-old infants in a 3 year follow-up study, the mortality
rate was high (Garenne et al., 1991). Therefore, the World Health Organization decided to discontinue the use of these high-dose MV vaccines (Weiss, 1991; WHO, 1992). To eradicate MV, a vaccine that can provide high immunogenicity in young infants is needed to overcome conferred maternal immunity.

The MV vaccine strain AIK-C, attenuated from the Edmonston strain (Sasaki, 1974; Makino, 1983), was developed in Japan in 1976 by plaque cloning and passage in sheep kidney and chicken embryonic cells at 33 °C. It shows optimal growth at 33 °C and extremely poor or no growth at 40 °C, i.e. temperature sensitivity (ts), with small plaques in Vero cells (Sasaki, 1974). To date, over 15 million doses of this vaccine have been used, mainly in Japan, and low reactogenicity has been reported. The AIK-C strain induced a higher seroconversion rate in infants of 4 to 6 months of age than the Schwarz strain in infants of 9 months of age (Tidjani et al., 1994; Rota et al., 1998). Most current MV vaccines were developed from the Edmonston strain by passage in chicken embryonic cells (Hirayama, 1983). We have reported that the genome sequence of the AIK-C strain has 56 nucleotide changes and 31 amino acid substitutions compared with the sequence of the Edmonston strain (Mori et al., 1993). The sequences of the fusion (F), haemagglutinin (H) and nucleocapsid (N) protein regions of current vaccine strains have been determined (Bellini et al., 1994; Rota et al., 1994). There is, however, no information about mutations that relate to the characteristics of vaccine strains or to the process of attenuation.

In this study, we have constructed expression plasmids for the F and H regions of the MV vaccine seed strain AIK-C and its parental wild-type strain Edmonston. We have examined the fusion inducibility of these plasmids in B95a, HeLa and Vero cells infected with recombinant vaccinia virus expressing T7 RNA polymerase, at an m.o.i. of 1 for B95a cells or 0.25 for HeLa cells. Cells were fixed at 37 °C for 1 h in 24-well plates. Inoculated virus was removed and cells were washed with Opti-MEM (GIBCO BRL). Vero cell monolayers in 24-well plates were infected with replication-deficient vaccinia virus expressing T7 RNA polymerase (MVAT7pol) at an m.o.i. of 10, kindly supplied by G. Sutter (Sutter et al., 1995). The F and H expression plasmids were transfected (0.2 µg per well) using SuperFect transfection reagent (Qiagen) for B95a and Vero cells or DMRIE-C reagent (GIBCO BRL) for HeLa cells. After overnight incubation, cells were fixed with 0.5% glutaraldehyde and stained with Giemsa’s staining solution (Merck).

**Methods**

**Measles virus strains.** The AIK-C seed strain for vaccine production was used. Strain AIK-C was attenuated from the wild-type MV strain Edmonston, kindly provided by the late John F. Enders (Makino, 1983; Sasaki, 1974).

**Construction of expression plasmids.** Total RNA was extracted from tissue culture fluid and the F and H protein-coding regions were amplified by RT–PCR using the primers F-ATG (5′ CATGAATTCATGGGTCTCAAGGTGAAACGT), F-TCGA (5′ GAGTCCTACGGTATTTGTCCA), F-TGA (5′ TTAGCCGCGCGCTCAGGCCGACCTTACATA), H-ATG (5′ GTGGAATTCATGTCCACCAACCGAGACCCGAAG) and H-TAG (5′ AATGCGCGCCGCTCTATGCGATTGGTTCCA). The linker restriction enzyme sites EcoRI and NotI are underlined. The F and H protein-coding regions were amplified by nested RT–PCR as reported previously (Nakayama et al., 1995). Expression plasmids were constructed by inserting the full coding region sequences of the F and H proteins into pbLueScript II SK(−) using the EcoRI and NotI multicloning sites located downstream of the T7 promoter. The F and H expression plasmids constructed from the AIK-C strain were designated pAIK-F01 and pAIK-H, and from the Edmonston strain, pEdm-F and pEdm-H. Constructs were sequenced by the dye terminator method using an ABI 377 sequencer (ABI PRISM, Perkin-Elmer).

**Site-directed mutagenesis.** To introduce a point mutation at position 278 of the F protein, the primers 278R+ (5′ GAGTCCTACGGTATTTGTCCA), 278R− (5′ GAGGACAATAAGGTAGGACCT), 278Y+ (5′ GAGTCCTACATATTTGTCCA) and 278Y− (5′ GAGGACAATAAGGTAGGACCT) were synthesized. Mutated amino acids, Arg and Tyr, are underlined. Mutated DNA fragments were amplified by PCR and, using the appropriate restriction enzymes, were inserted into pEdm-F. The two mutated clones were designated pEdm-F278Arg and pEdm-F278Tyr.

**Fusion analysis of cells co-transfected with expression plasmids.** Plasmid constructs were expressed using the vaccinia virus T7 expression system, kindly supplied by B. Moss (Fuerst et al., 1986). Monolayers of confluent B95a or HeLa cells were infected with recombinant vaccinia virus vTF7-3, which expresses bacteriophage T7 RNA polymerase, at an m.o.i. of 1 for B95a cells or 0.25 for HeLa cells. Cells were incubated at 37 °C for 1 h in 24-well plates. Inoculated virus was removed and cells were washed with Opti-MEM (GIBCO BRL). Vero cell monolayers in 24-well plates were infected with replication-deficient vaccinia virus expressing T7 RNA polymerase (MVAT7pol) at an m.o.i. of 10, kindly supplied by G. Sutter (Sutter et al., 1995). The F and H expression plasmids were transfected (0.2 µg per well) using SuperFect transfection reagent (Qiagen) for B95a and Vero cells or DMRIE-C reagent (GIBCO BRL) for HeLa cells. After overnight incubation, cells were fixed with 0.5% glutaraldehyde and stained with Giemsa’s staining solution (Merck).

**Immunofluorescence assay.** HeLa cells were cultured in a LabTek 8-well chamber slide (Nunc) and transfected with the F and/or H expression plasmids. Cells were fixed with cold acetone and an indirect immunofluorescence assay was performed using an anti-MV hyperimmune mouse antiserum followed by a FITC-conjugated monoclonal antibody against mouse IgG (Sigma).

**Construction of full-length AIK-C cDNA and recovery of recombinant virus.** We constructed a full-length cDNA clone of the vaccine seed strain AIK-C. A fragment of the recombinant F protein plasmid was inserted into the cDNA clone using appropriate restriction sites. A diagram of the cloning strategy is shown in Fig. 1. We constructed plasmids in two parts. The first half of pNEB193M-278Leu contains the leader sequence of the AIK-C genome to the trailer sequence. To change the amino acid at position 278 from Leu to Phe, a fragment of the recombinant plasmid pMV-F278Phe (as shown in Fig. 3) digested with AseI (positions 172 and 1211 of the F gene in F-ATG) was introduced into the pNEB193M-278Leu fragment, also digested with AseI. The resultant plasmid, designated pNEB193M-278Phe was digested with NotI/PacI and ligated into the second half of the plasmid, also digested with NotI/PacI. This construct was designated pIC-MVAIK-F278Phe. The original AIK-C cDNA was designated pIC-MVAIK-F278Phe. For the recovery of recombinant MV, B95a cells were infected with MVAT7pol, a replication-deficient vaccinia virus expressing T7 RNA polymerase, and then transfected with pAIK-N, pAIK-F, pAIK-L (pCIAL001, pCIAL001 and pCIAL001) and pIC-MVAIK-F278Leu (original of AIK-C) or pIC-MVAIK-F278Phe (mutant).
Leu278 critical for fusion of MV strain AIK-C

Fig. 1. Construction of a full-length cDNA clone of the MV strain AIK-C and substitution of Leu at position 278 of the F protein to Phe. pMV-F278Phe (Fig. 3) was used to introduce mutations.

Fig. 2. Differences in the deduced amino acid sequences of pAIK-F01, pEdm-F, pAIK-H and pEdm-H. Fusion inducibility is also shown. Different combinations of the F and H expression plasmids were co-transfected into vTF7.3-infected B95a cells. pBluescript II SK(--) was used as a control plasmid.

as reported previously (Schneider et al., 1997). B95a cells were cultured at 32.5 °C in 5% CO₂ until syncytia were apparent.

[Plaque and infectivity assays. Vero cell monolayers were inoculated with viruses and overlaid with 0.5% agar in Eagle’s minimal essential medium supplemented with antibiotics and 5% foetal calf serum. After 7 days of incubation in 5% CO₂ at 37 °C, cells were fixed with 0.25% glutaraldehyde. Anti-MV hyperimmune rabbit antibodies were added to the plate and after vigorous washing, biotinylated anti-rabbit IgG goat antibodies were added. Infected cells were stained with an Elite ABC Vectastain kit (Vector Laboratories). To examine the growth of infectious virus, Vero cell monolayers were infected with the MV strains AIK-C and Edmonston as well as the virus strains rescued from the]
infectious clones (approximate m.o.i. of 0·001). Tissue culture fluid was obtained for infectivity assays in B95a cells. Infectivity was expressed as TCID<sub>50</sub>/ml.

**Results**

**Deduced amino acid differences in the F protein and fusion inducibility**

The expression plasmids pAIK-F01 and pAIK-H were constructed from the MV vaccine seed strain AIK-C. pEdm-F and pEdm-H were constructed from the wild-type parental strain Edmonton. We determined the consensus nucleotide sequences of the F and H genes of the AIK-C and Edmonton strains. Direct sequencing revealed that amino acid 278 of the F protein was either Leu or Phe in the AIK-C strain. We constructed four expression plasmids, three with Phe and one with Leu at position 278 of the F protein. Thus, we assumed that the seed strain was a mixed population. The deduced amino acid differences among the expression plasmids are shown in Fig. 2. Six amino acid changes (A237V, L278F, Y362S, L435S, E471V and C494S) were observed between pAIK-F01 and pEdm-F. Five amino acid changes (R302G, T338P, D374N, N484T and E492G) were observed between pAIK-H and pEdm-H.

Expression plasmids were transfected in different combinations and the results of fusion inducibility are shown in Fig. 2. When vTF7.3-infected B95a cells were transfected with F or H expression plasmids separately, and thereafter the cells were mixed, no fusion was observed. When vTF7.3-infected B95a cells were co-transfected with pEdm-F and pEdm-H, extensive fusion was observed, as in the co-transfection experiments with pEdm-F and pAIK-H. Small syncytia involving several cells occurred when pAIK-F01 was used as the expression partner for the F protein. Thus, we speculated that fusion inducibility was dependant on the plasmid expressing the F protein.

**Fusion inducibility of recombinant F protein expression plasmids**

Amino acid differences and a diagram of the methods used for the construction of recombinant F gene DNAs are shown in Fig. 3. Using Sall (position 814 from the ATG codon of the F protein-coding region), Bst1107I (position 935) and PshAI (position 1343), we constructed six recombinant plasmids. Expression of the recombinant F plasmids (B95a cells was ascertained by immunofluorescence (data not shown). These recombinant plasmids were transfected into B95a or Vero cells infected with vTF7.3 or MVAT7pol. The results of fusion inducibility are shown in Fig. 3. Three amino acid changes were retained in pMV-F-SVS and pMV-F-VFS after exchanging the DNA fragments digested with PshAI. pMV-F-VFS induced fusion strongly and so, using Bst1107I, pMV-F362Ser and pMV-F-VF were constructed. We constructed pMV-F278Phe and pMV-F237Val by exchanging DNA fragments between pAIK-F01 and pMV-F-VF after digestion with Sall. After co-transfection with pAIK-H as the expression partner for the H protein in B95 cells, extensive syncytium formation was observed with pEdm-F, pMV-F-VFS, pMV-F-VF and pMV-F278Phe, whereas pAIK-F01, pMV-F-SVS, pMV-F362Ser and pMV-F237Val induced very few syncytia (Fig. 3). The fusion inducibility in B95a, HeLa and Vero cells was the same. Few fused cells were observed after transfection with recombinant plasmids with Leu at position 278 of the F protein, whereas plasmids with Phe at position 278 induced extensive cell fusion.

The recombinant plasmid pEdm-F278Leu was constructed by substituting the DNA fragments of pEdm-F and pAIK-F01 after digestion with Sall/Bst1107I. Site-directed mutations were introduced by PCR. pEdm-F278Tyr and pEdm-F278Arg were constructed using the same procedure. The results of fusion inducibility in Vero cells are shown in Fig. 4. Small syncytia were observed after co-transfection with pAIK-F01 and pEdm-H, but extensive fusion was induced after co-transfection with pEdm-F and pEdm-H. When pEdm-F278Leu was co-transfected with pEdm-H as the expression partner, cell fusion decreased. The recombinant plasmid pEdm-F278Tyr induced extensive cell fusion, as did pEdm-H and pMV-F278Phe, but not pEdm-F278Arg. Phe or Tyr at 278, as an aromatic amino acid, was essential for extensive syncytium formation, whereas Leu at 278 resulted in poor syncytium formation, as seen with the AIK-C strain of MV.

**Characteristics of rescued virus strains**

We constructed full-length (Leu at position 278) and recombinant (Phe at position 278) cDNA clones of AIK-C to
confirm the significance of this amino acid in the F protein. B95a cells were transfected with pIC-MVAK-278Leu (original AIK-C) or pIC-MVAK-278Phe (mutant AIK-C), as reported previously (Schneider et al., 1997). B95a cells were cultured at 32.5 °C in 5% CO₂ until syncytia appeared. Tissue culture fluid was obtained and passaged two times in B95a cells. MVAK-278Leu was rescued from pIC-MVAK-278Leu and MVAK-278Phe from pIC-MVAK-278Phe. The results of the plaque assay are shown in Fig. 5. The vaccine seed strain AIK-C produced a mixture of medium- and small-sized plaques at 33 °C but no plaques at 39 °C, whereas the parental Edmonston strain produced large plaques at both 33 and 39 °C. MVAK-278Leu produced small plaques and MVAK-278Phe produced large plaques at 33 °C. Neither strain produced plaques at 39 °C in Vero cells. Virus growth in Vero cells is shown in Fig. 6. Strain AIK-C grew at 33 °C as well as the Edmonston strain, but did not grow at 39 °C. Neither was virus growth observed at 39 °C after culture of the rescued strains of MVAK-278Leu and MVAK-278Phe. Growth of MVAK-278Phe was greater than that of MVAK-278Leu on
Discussion

The results of this study are important to the functional mapping of the F protein, which modifies fusion inducibility, as well as to the identification of the molecular basis for the attenuation of the AIK-C vaccine strain of MV. For virus infection, the enveloped virus must attach to the cellular receptor(s) before the envelope protein can fuse to the cellular membrane lipid bilayers. As for MV, recombinant adenovirus expressing the MV F protein was reported to induce syncytia when highly expressed (Alkhatib et al., 1990). But the expression system of the MV F protein alone in recombinant vaccinia virus did not induce cell fusion, whereas co-expression of F and H proteins did (Taylor et al., 1991; Wild et al., 1991). Binding between the envelope protein and the cell receptor is thought to trigger conformational changes in the F protein and the subsequent protruding of the fusion peptide to initiate the fusion process (Horvath & Lamb, 1992; Sergel et al., 1993; Lamb, 1993).

The F protein is a type I membrane protein that is initially synthesized as the F0 precursor. F0 is transported to the Golgi apparatus where it is cleaved by a host protease into two disulfide-linked subunits, F1 and F2. Several functional domains of the F protein have been clarified. The well-known cleavage motif RHRK follows the N-terminal 20 residues of F1 (fusion peptide), which is a highly conserved hydrophobic region among paramyxoviruses and which intercalate into the target membrane (Alkhatib et al., 1994; Horvath & Lamb, 1992). The processed F protein reaches the cell membrane as an oligomer, possibly a trimer (reviewed by Griffin & Bellini, 1995; Lamb, 1993). Interaction between two heptad repeats was reported for F protein oligomerization of simian virus type 5 (SV-5) (Ghosh et al., 1997, 1998; Dutch et al., 1999; Joshi et al., 1999). In MV, one repeat is located in the amphipathic α-helix region of the C terminus adjacent to the fusion peptide (heptad repeat A) and the other extends from positions 455 to 490 (heptad repeat B). A synthetic peptide corresponding to the heptad leucine repeat B region inhibited MV fusion, whereas the peptide corresponding to positions 148 to 177 (heptad repeat A) did not (Buckland et al., 1992; Wild & Buckland, 1997). In addition to these domains, a limited part of the cysteine-rich region of the MV F protein was required for interaction with the H protein in recombinant F gene construction experiments using MV and canine distemper virus (CDV), which has a similar cysteine-rich region (Wild et al., 1994). The cytoplasmic tail and Cys at position 506 or 519 located in the transmembrane domain were suggested to participate in cell fusion (Caballero et al., 1998). Modifying the interaction of F, H and membrane (M) proteins to promote assembly (Cathomen et al., 1998).

A characteristic leucine zipper motif located between two heptad repeats is conserved among paramyxoviruses. This region interacts with two heptad repeat regions to form a stable core (Ghosh et al., 1997, 1998). But positions 255 to 293 of SV-5 lack the classical zipper motif, having two heptad leucines, and the peptide corresponding to this domain did not influence the stable interaction between heptad repeats A and B (Dutch et al., 1999). In comparison with other morbillivirus F proteins, including CDV, peste-des-petits-ruminants virus and phocine distemper virus, the amino acid relevant to position 278 of the F protein was reported to be Phe (Evans et al., 1994). All wild-type MV isolates had Phe at position 278 of the F protein (data not shown). From the results obtained with the T7 polymerase expression system and the recombinant infectious virus cDNA, Leu at position 278 was responsible for the reduced fusion capability and Phe was responsible for extensive cell fusion. Site-directed mutagenesis experiments showed that Tyr at position 278 of the F protein also induced extensive cell fusion, but that Arg did not. Phe and Tyr are aromatic amino acids; therefore, we suppose that this region influences the stable interaction of F protein oligomerization.

Several attenuated live MV vaccines have been used worldwide. However, information on the molecular basis for attenuation is limited. MV isolated in B95a cells was fully pathogenic in cynomolgus monkeys and had lost pathogenicity after adaptation in Vero cells (Takeda et al., 1998). During the adaptation process, several amino acid changes occurred: two amino acid changes in the P and V regions, one in the C protein, three in the H region and two in the L region, but the critical regions were not identified (Takeda et al., 1998).

Fig. 6. Growth of the AIK-C seed, Edmonston and rescued (MVAIK-278Leu and MVAIK-278Phe) virus strains from the full-length and mutated infectious cDNA clones. Virus strains were inoculated into Vero cells at an m.o.i. of 0·001. Tissue culture fluid was harvested on days 1, 3, 5 and 7 of culture and infectivity was assayed in B95a cells. Virus titre at 33 °C and 39 °C (○) is shown.

days 3–5 of culture. The biological characteristics of temperature sensitivity were reproduced by construction of a full-size cDNA clone originating from strain AIK-C. The plaque size of the rescued virus strains reflected the results of the expression experiments with pAIK-F01 and pAIK-F278Phe.
In the present study, the MV vaccine seed strain AIK-C produced a mixture of medium- and small-sized plaques in Vero cells and the consensus sequence of the F gene had either Leu or Phe at position 278. Expression experiments with different recombinant F protein expression plasmids demonstrated that the Leu at position 278 was responsible for reduced fusion capacity and Phe for marked fusion inducibility. We suppose that the AIK-C seed strain has a mixed population of MVAIK-278Leu and MVAIK-278Phe. To assess the contribution of phenotype difference in plaque formation to the attenuation of the AIK-C strain, further in vivo analysis using the recombinant MVAIK-278Leu and MVAIK-278Phe viruses is required. The AIK-C strain has another biological characteristic in that it grows well at 32 °C but not at 40 °C (Sasaki, 1974). The introduction of Phe at position 278 of pIC-MVAIK-F278Leu did not influence the ts marker and we suppose that another mechanism exists for AIK-C. We also examined other regions of the genome to determine the domains of N, P/V/C and L related to genome replication and transcription.

We are very much obliged to Dr B. Moss and Dr G. Sutter for generously supplying the recombinant vaccinia viruses vTF7.3 and MVAT7pol.

References


Received 26 February 2001; Accepted 11 May 2001