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Identification of Two Amino Acids in the Hemagglutinin Glycoprotein of Measles Virus (MV) That Govern Hemadsorption, HeLa Cell Fusion, and CD46 Downregulation: Phenotypic Markers That Differentiate Vaccine and Wild-Type MV Strains

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We have used site-directed mutagenesis of the hemagglutinin (H) glycoprotein of measles virus (MV) to investigate the molecular basis for the phenotypic differences observed between MV vaccine strains and recently isolated wild-type MV strains. The former downregulate CD46, the putative cellular receptor of MV, are positive for hemadsorption, and are fusogenic in HeLa cells, whereas the latter are negative for these phenotypic markers. CD46 downregulation in particular, could have profound consequences for the immunopathology of MV infection, as this molecule protects the cell from complement lysis. Mutagenesis of two amino acids, valine and tyrosine at positions 451 and 481, respectively, in the H protein from the vaccine-like Hallé MV strain to their counterparts, glutamate and asparagine, in the H protein from the wild-type Ma93F MV strain (creating the V451E/Y481N double mutation) abrogated CD46 downregulation, HeLa cell fusion, and hemadsorption. The converse double mutagenesis of the Ma93F H protein (E451V/N481Y) transferred the CD46-downregulating, fusogenic, and hemadsorption functions to this protein. The data provide the first mapping study of the functional domains of MV H. The consequences of these results for MV vaccine design and the role of CD46 in MV infection are discussed.

Forty years ago, Enders and Peebles (3) isolated measles virus (MV) from primary human kidney cells. One of their isolates, the Edmonston strain of MV, was subsequently passaged in monkey kidney cell lines and then adapted to chicken embryo cells for the propagation of a live attenuated vaccine (10), which has since been successfully used worldwide to control MV infection.

Isolation of MV from monkey kidney cell lines such as Vero and CV-1 can take several weeks. However, Kobune et al. (11) recently reported that MV strains could be isolated more rapidly from a marmoset lymphoblastic cell line, B95-8, and its adherent subline, B95a. The adoption of B95a cells and human B-lymphoid cells immortalized by Epstein-Barr virus (this study) for the isolation of MV strains has highlighted phenotypic differences between laboratory-adapted vaccine strains of MV such as Edmonston and the recently isolated wild-type MV strains: the former downregulate CD46, the putative cellular receptor for MV (13, 15), are positive for hemadsorption, and are fusogenic on HeLa cells, whereas the latter lack these functions. It is a matter of conjecture whether these phenotypic differences reflect mutations in the viral proteins as a result of adaptation to nonhuman cells or are simply markers differentiating older strains from their evolved modern counterparts.

As CD46 protects the cell from complement lysis by binding complement proteins C3b and C4b, its downregulation could have important consequences for the immunopathology of the infection: premature lysis of cells infected by vaccine strains could result in insufficient virus being generated to provoke an efficient immune response (25). As wild-type strains do not downregulate CD46 (23, 24), they should, as a consequence, provoke stronger immune responses against MV. If CD46 downregulation could be prevented in vaccine strains, this could be of great importance in terms of MV vaccine design.

To identify the residues on the MV hemagglutinin (H) responsible for these phenotypic differences, we compared the primary sequences of the H proteins from two MV strains: a vaccine-like strain (Hallé) and a wild-type strain freshly isolated in human cells (Ma93F). This comparison revealed that 17 residues, all of which reside in the extracellular domain, differ between the two strains. To investigate which of these 17 amino acids are responsible for the differences in biological activity, we made site-directed mutagenesis in the cDNAs coding for the Hallé and Ma93F H proteins. We show that the substitution of two amino acids in the MV H protein controls the presence or absence of these phenotypic markers.

MATERIALS AND METHODS

Cells. Monolayers of HeLa T4 and rabbit kidney (RK13) cells were grown in RPMI (Gibco) containing 5% fetal calf serum, 0.1 mg of gentamicin per ml, 1 μg of amphotericin B per ml, 2 mM L-glutamine, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer. Vero cells were maintained in Dulbecco's modified Eagle's medium supplemented as for the RPMI medium.

Site-directed mutagenesis. The procedure was carried out with M13 phage as described previously (31).

Viruses. Recombinant vaccinia viruses (VV) expressing parental or mutant MV H were constructed by using the host range selection system described by Perkus et al. (17), with some modifications. Briefly, 10^6 Vero cells were infected with the VV attenuated strain NYVAC (K1L$^-$) (28) at 1 PFU per cell. At 3 h postinfection, cells were transfected with plasmid pCOPAK, containing the MV H gene and the K1L host range gene (6). Transfections were performed by using Lipofectamine (Gibco BRL); 2 μg of plasmid DNA was mixed with 10 μl of

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A

amino acid #	Hallé	Ma93F
211	G	S
233	P	L
243	R	G
246	L	S
258	S	G
303	E	G
332	G	D
339	L	F
367	V	I
399	C	W
400	V	A
405	N	H
451	V	E
481	Y	N
546	G	S
562	V	I
616	R	S

B

FIG. 1. (A) Amino acid differences between the H proteins of the Hallé strain and of the recent MV isolate Ma93F. (B) Schematic representation of MV H protein showing positions of the transmembrane region (hatched), the five potential N-glycosylation sites (Y), and the 17 substitutions (|).

Lipofectamine as instructed by the manufacturer. Two days after transfection, progeny was harvested and plated onto monolayers of RK13 cells. After three plaque selections, stocks were grown on RK13 cells and harvested.

MV Ma93F was isolated in 1993 in Madrid from a patient with acute measles. The virus was isolated in a human B-lymphoid cell line immortalized by Epstein-Barr virus and has been grown exclusively in human B-lymphoid cells since its isolation. Ma93F belongs to the C2 genotype of MV (19).

Viral RNA preparation and sequencing. RNA was extracted from infected cells by the guanidinium isothiocyanate technique. The RNA was then reverse transcribed by using mouse Moloney virus reverse transcriptase (Gibco BRL) and amplified by PCR. PCR products were sequenced directly by the method of Sanger et al. (22).

HeLa cell fusion assay. HeLa T4 cells were infected at 1 PFU per cell with recombinant VV-MVF, expressing the MV Hallé strain fusion (F) protein (30). Three hours postinfection, 5×10^5 cells were transfected with 1 μg of plasmid pgptΔ6 containing a parental or mutant MV H gene. Transfections were carried out by using Lipofectamine (Gibco BRL). Twenty hours after transfection, cells were observed under an inverted light microscope for the presence of syncytia. Cells were then harvested, fixed with acetone, and tested for the expression of mutant proteins by immunofluorescence staining with the anti-MV H monoclonal antibody (MAb) 55 (5).

Hemadsorption assays. The assays were performed on HeLa T4 cells transiently transfected with parental or mutant MV H cDNA. Twenty hours posttransfection, cells were washed twice with phosphate-buffered saline (PBS) and then incubated for 1 h with a 0.2% suspension of vervet erythrocytes (RBC). After three washes with PBS, cells were observed under an inverted light microscope.

Flow cytometry. Forty hours after infection with recombinant VV, HeLa T4 cells were incubated for 30 min on ice with anti-CD46 MAb MCI20.6 (13) or anti-MV H MAb 55, washed once with PBS, and then incubated with anti-mouse immunoglobulin G-fluorescein isothiocyanate for 30 min on ice. After two washes with PBS, flow cytometric analyses were performed with a FACScan (Becton Dickinson).

RESULTS

In our previous studies, we established that the presence of MV H, MV F, and CD46 is required for MV fusion (32) and that antibodies inhibiting the interaction of the MV H and CD46 also block fusion (14). We have used these observations as the basis for analyzing the interaction of mutated H proteins with CD46.

The primary sequence of the Ma93F H protein differs from that of the Hallé H protein by 17 residues. To aid in the identification of the residues in the MV H responsible for the phenotypic differences between vaccine MV strains and recently isolated wild-type MV strains, we sequenced the H gene

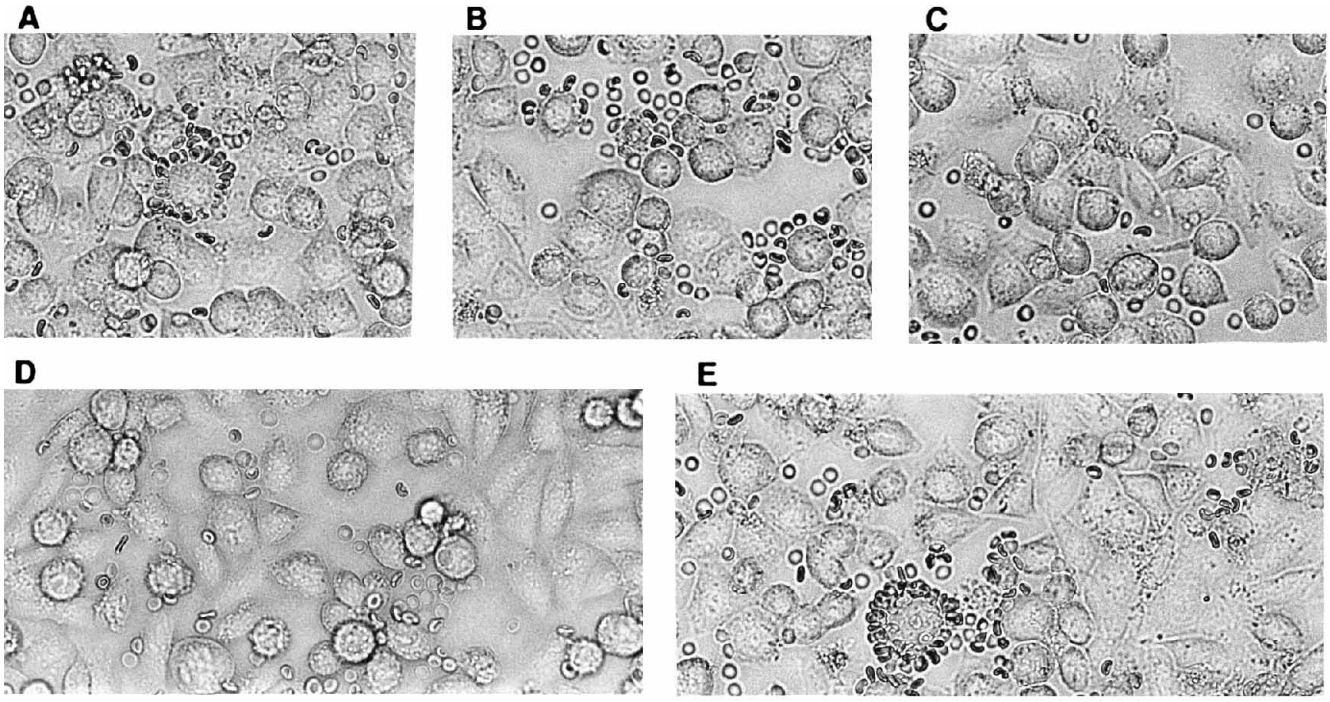

FIG. 2. Hemadsorption on HeLa T4 cells infected with wild-type VV and then transfected with plasmid pgptΔ6 containing the Hallé H gene (A), the mutant Hallé H gene Y481N (B) or V451E/Y481N (C), the Ma93F H gene (D), or the mutant Ma93F H gene E451V/N481Y (E). Twenty hours posttransfection, cells were incubated for 1 h with a 0.2% suspension of vervet RBC. After three washes with PBS, cells were observed under an inverted light microscope. Similar rings of bound vervet RBC are seen on cells expressing Hallé H (A) and mutant E451V/N481Y Ma93F H (F), whereas no bound RBC can be seen on cells transfected with a plasmid containing the Ma93F H gene (D) or the mutant V451V/Y481N Hallé H gene (C). Cells expressing the mutant Y481N Hallé H have a few RBC bound to their surface (B). Note that with transient transfections, not all cells express.

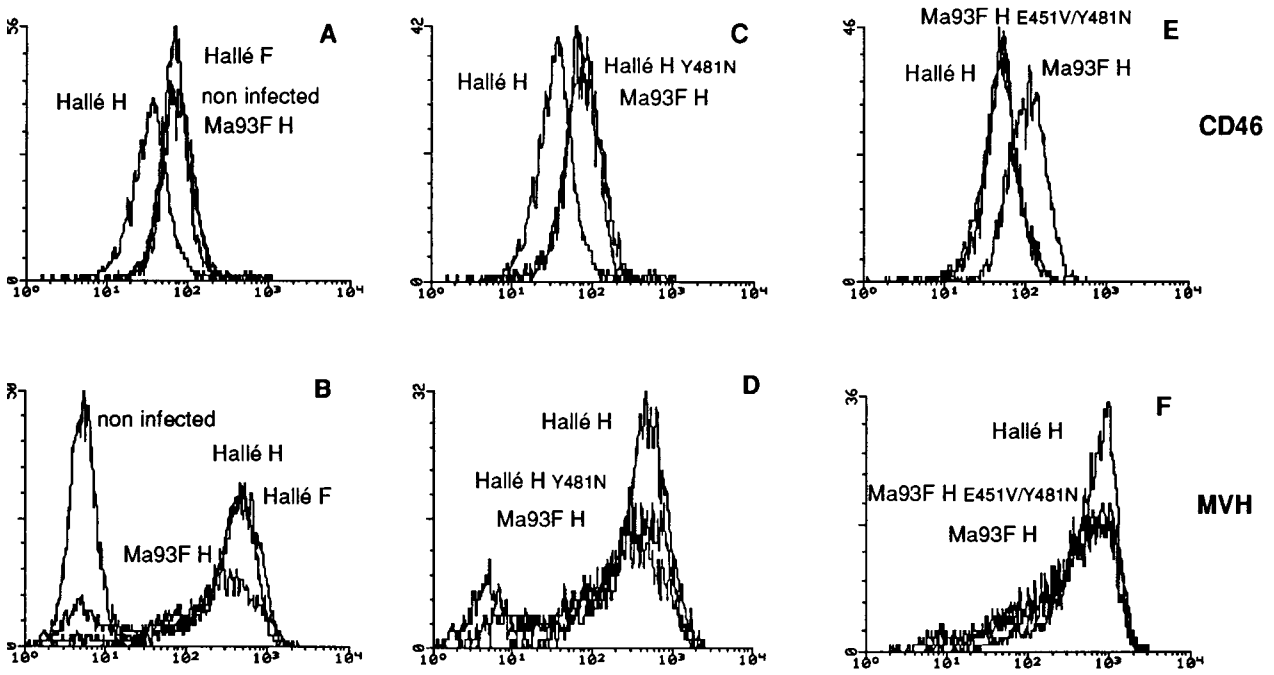

FIG. 3. Differential downregulation of CD46 expression on HeLa T4 cells infected with a recombinant VV expressing parental Hallé H or Ma93F H or Hallé F as control (A and B), the Y481N Hallé H mutant (C and D), or the E451V/N481Y Ma93F H mutant (E and F). Forty hours postinfection HeLa T4 cells were incubated for 1 h on ice with either anti-CD46 MAb MCI20.6 (A, C, and E), anti-MV H MAb 55 (B, D, and F), or anti-MV F MAb 186 (B, cells infected with VV-Hallé F), washed once with PBS, and incubated with anti-mouse immunoglobulin G-fluorescein isothiocyanate for 30 min on ice. After three washes with PBS, flow cytometric analyses were performed on a FACScan (Becton Dickinson).

from Ma93F, a wild-type MV strain that we recently isolated in a human B-lymphoid cell line immortalized by Epstein-Barr virus. Ma93F, which has been grown exclusively in human B-lymphoid cells since its isolation, does not fuse with HeLa T4 cells, is negative for hemadsorption, and does not downregulate CD46 (see below). We compared the Ma93F primary sequence with that from the H protein from the vaccine-like Hallé strain (4). Although the Hallé strain was originally reported (7) to have been isolated from a patient with subacute salerosing panencephalitis, this lytic virus is now suspected to be a laboratory contaminant derived from the Edmonston strain and is classified with the vaccine MV strains (19). The comparison (Fig. 1) shows that 17 amino acids differ between the two H proteins.

To investigate which of the differing amino acids are responsible for these biological functions, we performed site-directed mutagenesis in the cDNAs encoding the H protein from the Hallé and Ma93F strains. Initially, the mutated MV H cDNAs were subcloned into the VV expression plasmid pgptΔ6, which was then transfected into HeLa cells that had already been infected with a VV recombinant expressing the MV F (Hallé strain). When pgptΔ6 expressing the unmutated Hallé H protein is transfected in this system, the result is viral fusion (syncytium formation). Any construction that resulted in the abrogation of fusion was further tested for hemadsorption, and the equivalent VV recombinant was then made for CD46 downregulation studies.

The Hallé H-protein mutation Y481N severely reduces hemadsorption and abrogates both fusion and CD46 downregulation. In our initial series of single substitutions in the Hallé H protein, only one was found to have an effect on the fusogenicity: the substitution of tyrosine 481 in the Hallé H protein for asparagine (Y481N) abrogated fusion. Furthermore, expression of the pgptΔ6 Hallé H Y481N construction greatly reduced hemadsorption with vervet RBC (Fig. 2B) compared with unmutated Hallé H (Fig. 2A). To determine whether the mutation also affected CD46 downregulation, HeLa cells were infected with VV recombinants expressing either the mutant or parent Hallé H protein and examined by FACScan analysis. The VV recombinant expressing the wild-type Hallé H protein strongly downregulated CD46, whereas the Y481N recombinant was not downregulated (Fig. 3C and D). Thus, a single substitution (Y481N) in the primary sequence of the Hallé H abrogates fusion and CD46 downregulation and severely reduces hemadsorption.

The Ma93F H-protein mutation N481Y results in intermediate CD46 downregulation, but fusion and hemadsorption are still negative. To investigate whether the converse mutation in the Ma93F H protein had an effect on CD46 downregulation, we made a VV recombinant expressing this mutation (N481Y). Although the pgptΔ6 construction expressing the single Ma93F N481Y mutation had been negative in the fusion and hemadsorption assays, the FACScan analysis assay showed that the Ma93F H N481Y VV recombinant downregulated CD46 to a degree intermediate between that induced by the Hallé and Ma93F H proteins (not shown). Thus, residue 481 indeed plays a role in CD46 downregulation, but substituting this single amino acid is insufficient to convert the Ma93F H protein to the downregulating equivalence of the Hallé H; other substitutions must be required. Furthermore, the Ma93F H protein N481Y substitution is insufficient by itself to induce the functions of fusion and hemadsorption.

The Ma93F H double mutation N481Y/E451V induces fusion, hemadsorption, and full CD46 downregulation. To discover which of the other amino acids which differ between the Hallé H and the Ma93F H are required to give Ma93F H the equivalent downregulating phenotype of the Hallé H, we kept the Ma93F N481Y mutation and mutagenized the other het-

TABLE 1. Summary of Hallé H and Ma93F H mutants and their effects on the phenotypic markers HeLa cell fusion, hemadsorption, and CD46 downregulation

Protein	HeLa fusion	CD46 downregulation	Hemadsorption
Hallé			
H	+	+	+
V451E	+	+/−	+
Y481N	−	−	+/−
V451E/Y481N	−	−	−
Ma93F			
H	−	−	−
E451V	−	−	−
N481Y	−	+/−	−
E451V/N481Y	+	+	+

erologous residues one by one. At the same time, single-substitution Ma93F H VV recombinants were analyzed for CD46 downregulation. A VV recombinant expressing the Ma93F H protein with the E451V (Val-451 to Glu) single mutation induced a level of CD46 downregulation similar to that given by the Ma93F H protein (not shown). However, the Ma93F double mutant N481Y/E451V provoked fusion, was positive for hemadsorption, and induced a downregulation of CD46 identical to that of the Hallé H (Fig. 3E and F).

Hemadsorption is completely abrogated in the Hallé H protein double mutant Y481N/V451E. As reported above, mutating a single residue in the Hallé H protein (Y481N) abrogates both fusion and CD46 downregulation, but hemadsorption is still weakly positive (Fig. 2B). To investigate whether substitution of valine 451 in the Hallé H protein to the residue glutamate found in the homologous position in the Ma93F H protein would also affect hemadsorption, we made the double mutant Y481N/V451E. With the Y481N/V451E double mutation, fusion and CD46 downregulation were abrogated as for the Y481N single mutation, but this time the hemadsorption assay was completely negative (Fig. 2C). These results are summarized in Table 1.

DISCUSSION

In this study, we show that the amino acids at 451 and 481 in the primary sequence of the MV H play a crucial role in CD46 downregulation, hemadsorption, and HeLa cell viral fusion. The most probable common denominator for the presence or absence of CD46 downregulation, hemadsorption, and HeLa cell fusion in the phenotype of a given MV strain is the interaction between CD46 and MV H. Although human RBC do not express CD46, the RBC of certain monkey species such as vervets and African green monkeys express the simian homolog: laboratory-adapted MV strains hemadsorb African green monkey RBC but not human RBC. As MV fusion is dependent on the ternary MV F-MV H-CD46 complex (32), fusion is an indicator not only of MV H-MV F interaction but also MV H-CD46 interaction. Fusion induction could therefore be indicative of the latter interaction. The downregulation of CD46 requires only the expression of the MV H to occur, as it can be observed when HeLa cells are infected with a VV recombinant expressing this MV protein (15). Very little is known about the domains on MV H and CD46 responsible for their interaction, but replacing the transmembrane and cytoplasmic regions of CD46 with a glycosylphosphatidylinositol anchor did not perturb downregulation (29), which suggests that interaction is made via the extracellular domains. In support of this view, the MV H binding domain on CD46 has been localized to SCR1 and SCR2 in the extracellular domain (9, 12).

Residues 451 and 481 fall within the region between amino acids 451 and 505 that Hummel and Bellini have suggested forms part of the hemadsorption and hemagglutination domain of the MV H (8). It would appear from our results (Table 1) that residue 481 has the major influence. In support of this view, other non-CD46-downregulating MV strains such as SE (23, 26), JM (20), and AK-1 (21) have asparagine at position 481. Furthermore, the JM strain H protein has glutamate at position 451 (20).

Shibahara et al. (27) reported that three MV strains isolated from B95-8 cells did not bind African green monkey RBC but gained the capacity to do so after 20 passages in Vero cells. Nucleotide sequencing showed that one of the three strains had a single amino acid change compared with the original: at residue 481, asparagine had been substituted for tyrosine. The same substitution was also present in another of the three strains examined. Shibahara et al. invoked the activity of an as yet unidentified influenza virus M2-like MV gene to explain the hemadsorption acquired after passage in Vero cells.

Interpreting our results in the light of those of Shibahara et al. (27), we suggest that during multiple passages in Vero or CV-1 cells, there is a selection for MV isolates with an affinity for CD46. Furthermore, we suggest that this selection occurs via the mutation (primarily) of asparagine 481 to tyrosine in the H protein, which facilitates the interaction with CD46. This interaction then induces syncytium formation, hemadsorption, and CD46 downregulation.

It is evident that this interaction with CD46 must be different from that made during the primary infection by the fresh virus isolate. CD46 was identified as the MV receptor in studies using vaccine strains of the virus (1, 13). Our results show that the MV H protein from freshly isolated wild-type strains which do not grow on certain $CD46^+$ cell lines such as Vero without adaptation do not appear to interact with CD46. This finding suggests that wild-type MV isolates use a different receptor to initiate infection.

Recently, it has been shown that MV can infect CD46-negative murine cells, although viral fusion is not observed (33). Similarly, Katz et al. successfully adapted the Edmonston strain to chicken embryo cells, which do not possess a homologous CD46 molecule (10). This suggests that MV can use other molecules as its cellular receptor. In this regard, moesin has been suggested as an alternative receptor for MV, as antimoesin antibodies block MV infection (2).

CD4, the human immunodeficiency type 1 (HIV-1) receptor, is downregulated during HIV-1 infection, but this phenomenon is not a requirement for virus entry (16). Indeed, the highest levels of HIV infection are usually associated with very limited CD4 downregulation (16). By the same token, downregulation of CD46 could be an attenuation marker for MV vaccine strains. The initial step in viral entry is attachment to the cellular receptor, and this is probably followed by conformational changes in the hemagglutinin that are then transmitted to the fusion protein, which in turn also undergoes changes in conformation leading to fusion. Ideally, this series of events would benefit from a stable expression of CD46 at the plasma membrane rather than its disappearance.

The results presented in this study could have profound consequences in terms of MV vaccine design. As the production of an infectious clone of MV has been recently published (18), it should be now possible to convert vaccine strains of MV to the nondownregulating phenotype exhibited by wild-type strains. This could be a means of dramatically increasing the

immune response that they induce. On the other hand, if CD46 downregulation is responsible for attenuation, such a modification of the MV vaccine strain is undesirable. In this case, specific mutation of a fresh MV isolate that has never been cultured in nonhuman cell lines would perhaps be the optimal basis for a modern vaccine against this virus.

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