



Antigenic Drift Defines a New D4 Subgenotype of Measles Virus

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ABSTRACT The measles virus hemagglutinin (MeV-H) protein is the main target of protective neutralizing antibodies. Using a panel of monoclonal antibodies (MAbs) that recognize known major antigenic sites in MeV-H, we identified a D4 genotype variant that escapes neutralization by MAbs targeting the neutralizing epitope (NE) antigenic site. By site-directed mutagenesis, L249P was identified as the critical mutation disrupting the NE in this genotype D4 variant. Forty-two available D4 genotype gene sequences were subsequently analyzed and divided into 2 groups according to the presence or absence of the L249P MeV-H mutation. Further analysis of the MeV-N gene sequences of these 2 groups confirmed that they represent clearly definable, sequence-divergent D4 subgenotypes, which we named subgenotypes D4.1 and D4.2. The subgenotype D4.1 MeVs were isolated predominantly in Kenya and Ethiopia, whereas the MAb-resistant subgenotype D4.2 MeVs were isolated predominantly in France and Great Britain, countries with higher vaccine coverage rates. Interestingly, D4.2 subgenotype viruses showed a trend toward diminished susceptibility to neutralization by human sera pooled from approximately 60 to 80 North American donors. Escape from MAb neutralization may be a powerful epidemiological surveillance tool to monitor the evolution of new MeV subgenotypes.

IMPORTANCE Measles virus is a paradigmatic RNA virus, as the antigenic composition of the vaccination has not needed to be updated since its discovery. The vaccine confers protection by inducing neutralizing antibodies that interfere with the function of the hemagglutinin protein. Viral strains are indistinguishable serologically, although characteristic nucleotide sequences differentiate 24 genotypes. In this work, we describe a distant evolutionary branch within genotype D4. Designated subgenotype D4.2, this virus is distinguishable by neutralization with vaccine-induced monoclonal antibodies that target the neutralizing epitope (NE). The subgenotype D4.2 viruses have a higher predominance in countries with intermediary levels of vaccine coverage. Our studies demonstrate that subgenotype D4.2 lacks epitopes associated with half of the known antigenic sites, which significantly impacts our understanding of measles virus evolution.

KEYWORDS virus evolution, antibody-mediated neutralization, antigenic variation, measles virus hemagglutinin, viral epitopes, measles virus genotypes, immune evasion

Measles ranks as one of the most deadly diseases in the history of humankind (1, 2). The advent of an effective measles virus (MeV) vaccine 46 years ago dramatically reduced the number of measles deaths (3), making the MeV live-attenuated vaccine one of the most successful public health interventions ever undertaken (4, 5). Nonetheless, measles is still a leading cause of death globally for children younger than 5 years of age and was responsible for 134,200 deaths in 2015 (6). All 194 WHO member states have committed to reducing measles deaths, and the Global Measles and Rubella

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Strategic Plan set the goal of measles elimination by 2020 through increased vaccination coverage (7). In this regard, measles cases among vaccinated people have raised concerns about waning immunity in vaccinees and the occurrence of antigenic changes in currently circulating strains, raising concerns as to whether MeV elimination can be achieved (8–12).

MeV belongs to the family *Paramyxoviridae* and carries a nonsegmented negative-strand RNA genome tightly encapsidated by a helically arranged nucleocapsid (MeV-N) protein and packaged in a lipoprotein envelope (13). Two transmembrane glycoproteins are found in the virion, the MeV hemagglutinin (MeV-H) protein and the MeV F (MeV-F) protein. The former is responsible for receptor attachment and has a fusion support function when coexpressed with the latter (14, 15). Neutralizing antibodies to either of these antigens inhibit MeV infection by preventing the interaction of the MeV-H protein with its cellular receptor(s) and by blocking fusion activity (16, 17). Although both cellular and humoral immune responses are important during MeV infection, they have different effects. Antibodies to MeV-H and MeV-F are essential for protection, as recently demonstrated by the absence of protection in macaques with an MeV-specific T-cell response but without neutralizing antibodies (18).

Despite the fact that *ex vivo* studies confirm that the MeV polymerase mutation rate is high, similarly to other RNA viruses, MeV is considered antigenically stable and has only 1 serotype (19–21). Nevertheless, sequence analysis of the two most variable MeV genes, MeV-H and MeV-N, in naturally occurring field isolates has enabled MeV to be classified into 24 genotypes (22). During a recent genotype B3.1 measles outbreak, we observed a gradual nucleotide divergence in the MeV-H gene over a 6-month period and estimated a mutation rate of 2.66×10^{-3} substitutions per site per year (23), similar to the rates of antigenic drift in other RNA viruses (24, 25). In contrast, no changes were observed in the hypervariable carboxy end of the MeV-N gene or the gene encoding the other important surface antigen, MeV-F (23). This observation could open the question of immune-driven evolution into the major surface antigen MeV-H. Certainly, the introduction of the live-attenuated MeV vaccine not only has led to a dramatic decrease in MeV incidence but also has been accompanied by changes in the global distribution of measles virus genotypes (26, 27).

Currently, six MeV genotypes (genotypes B1, C1, D1, E, F, and G1) are considered extinct, and five other genotypes (genotypes D2, D3, D10, G2, and H2) have not been detected since 2006. Furthermore, all MeV isolates detected in the past 5 years belong to only seven genotypes and show some geographic restriction: genotypes B3, D4, D6, D8, D9, G3, and H1 (27–29). Because measles vaccination with a genetically restricted strain (genotype A) has been used throughout the world for 50 years, changes in genotype circulation patterns might reflect the immune selection of “fitter” viruses (30, 31). Antigenic differences of MeVs of various genotypes have been detected by using monoclonal antibodies (MAbs) and polyvalent antisera from vaccinees to identify differences in neutralization titers against certain wild-type viruses (32–36; M. A. Muñoz-Alía, J. Carabaña, A. Serrano-Pardo, R. Porrás-Mansilla, C. Santiago, J. M. Casanovas, M. L. Celma, and R. Fernández-Muñoz, presented at the X Spanish National Conference on Virology, Salamanca, Spain, 21 to 24 June 2009). Nevertheless, despite these subtle differences, all contemporary wild-type strains of MeV are readily neutralized *in vitro* with polyclonal serum from vaccinees (34, 37, 38).

During our studies on antigenic variation across MeV genotypes, we identified a difference in the neutralization sensitivities of two viruses belonging to the D4 genotype. Subsequent genetic and antigenic analyses of a larger number of genotype D4 viruses identified two definable D4 subgenotypes, which we named D4.1 and D4.2. In contrast to subgenotype D4.1 viruses, subgenotype D4.2 viruses are not neutralized by antibodies targeting the neutralizing epitope (NE), indicating that they lack three of the six known antigenic sites. Perhaps more significantly, the two subgenotypes differ in their susceptibilities to neutralization by pooled human sera from 60 to 80 North American donors.

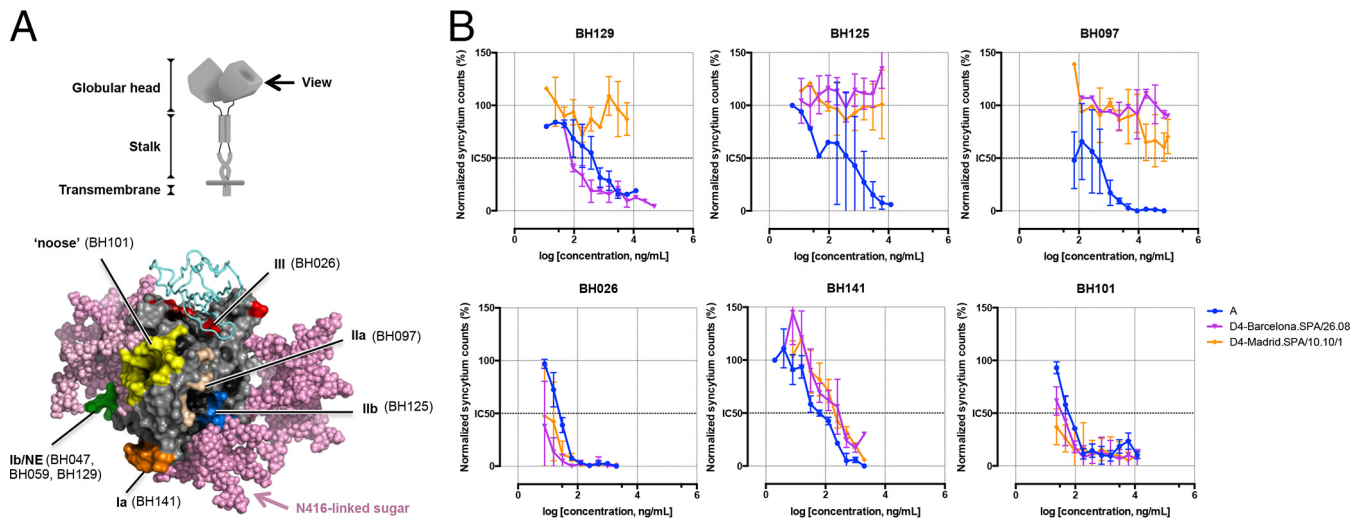


FIG 1 Neutralization activity of a panel of anti-MeV-H MAbs against 2 genotype D4 viruses. (A, top) Diagram of the MeV-H homodimeric structure. (Bottom) Radiographic crystallographic structure of the MeV-H globular head showing the location of the 6 antigenic sites. Antigenic sites are color-coded (orange, Ia; green, Ib or NE; wheat, IIa; blue, IIb; red, III; yellow, noose), indicating a representative antibody target. The signaling lymphocytic activation molecule immunoglobulin V domain and modeled N-linked sugars are shown as cyan ribbons and pink spheres, respectively. A side view is shown, as indicated at the top. The N416-linked sugar present in genotype D4 is highlighted. (B) Virus neutralization assay. Recombinant measles virus expressing 2 different MeV-H genotype D4 sequences or genotype A (vaccine strain) was incubated in the absence or presence of the indicated neutralizing antibody for 1 h at 37°C. Two days later, the numbers of enhanced green fluorescent protein-expressing foci in the presence and absence of MAbs were counted and compared. Neutralization is plotted as a percentage of the control for residual infection (y axis) by MAb concentration (x axis). For a given MAb-virus pair, the data represent the geometric means of results from at least 2 independent experiments performed in quadruplicate. NE, neutralizing epitope.

RESULTS

Discrimination between genotype D4 viruses using antibodies targeting the NE. During our studies on antigenic variation between MeV genotypes, we noted that two MeV strains isolated in Spain and defined as belonging to genotype D4 showed distinct sensitivities to antibody-mediated neutralization. To ascertain a role of substitutions in MeV-H, we generated enhanced green fluorescent protein (EGFP)-expressing recombinant MeV in which the MeV-H gene was replaced with that of two Spanish genotype D4 isolates, Barcelona.SPA/26.08 and Madrid.SPA/10.10/1. We again tested their neutralization sensitivities with a panel of anti-MeV-H antibodies against known major antigenic sites in MeV-H (Fig. 1A). The results presented in Fig. 1B show that the BH026, BH141, and BH101 MAbs completely inhibited infection by both genotype D4 viruses and the vaccine strain (genotype A), used as a control for neutralization. In contrast, discrimination between the MeV vaccine strain and the genotype D4 viruses was observed when the BH125 and BH097 MAbs were tested instead. Surprisingly, the two genotype D4 viruses showed markedly different neutralization profiles when the NE-targeting BH129 MAb was used. Although the BH129 MAb completely blocked infection by the Barcelona.SPA/26.08 virus, it was ineffective against the genotype D4 Madrid.SPA/10.10/1 virus.

Two sequence-divergent D4 subgenotypes defined within genotype D4. Having shown that the two genotype D4 Spanish isolates showed differing neutralization patterns by anti-MeV-H MAbs, we sought to determine the broader significance of this finding for other members of genotype D4. A new MeV genotype is typically defined by 2.5% and 2.0% nucleotide divergences in the MeV-N and MeV-H genes, respectively (85). We therefore retrieved both MeV-N and MeV-H gene sequences available for MeV isolates classified as belonging to genotype D4 from GenBank ($n = 41$) (Table 1). After removing redundant sequences, we reconstructed phylogenetic trees for both genes and rooted them on the reference strain for genotype A (MVi/Maryland.USA/0.54). Phylogenetic analysis showed that the two Spanish genotype D4 viruses were indeed representative of two definable subgenotypes, which were supported by high bootstrap values (Fig. 2). The average number of base substitutions per site for the MeV-N gene between the 2 subgenotypes was 4.0% ($\pm 0.8\%$), ranging from 2.5% to 6.5%. The

TABLE 1 List of MeV sequences used in the course of this work

Genotype	WHO strain designation	Material	GenBank accession no.		Reference
			MeV-N	MeV-H	
A	MVi/Maryland.USA/0.54	Cell culture	U01987	U03669	80
D4	MVi/Montreal.CAN/08.89	Cell culture	U01976	AF079554	80
D4	MVi/Montreal.CAN/12.89	Cell culture	AF410990	AF410975	81
D4	MVi/Montreal.CAN/25.89	Cell culture	AF410991	AF410976	81
D4	MVs/Brighton.GBR/49.11	Cell culture	KT732227	KT732227	82
D4	MVs/London.GBR/20.12	Oral fluid	KT732229	KT732229	82
D4	MVs/London.GBR/20.11/2	Oral fluid	KT732226	KT732226	82
D4	MVs/London.GBR/19.11/5	Oral fluid	KT732225	KT732225	82
D4	MVi/Treviso.ITA/03.10/1	Cell culture (urine)	KC164757.1	KC164757.1	E. Franchin et al. ^a
D4	MVi/New York/26.09/3	Not specified	JN635402.1	JN635402.1	E. F. Kirkness et al. ^b
D4	MVi/Florida.USA/19.09	Not specified	JN635403.1	JN635403.1	E. F. Kirkness et al. ^b
D4	MVi/Central.KEN/23.02	Not specified	AY249250	AY249260	46
D4	MVi/Nairobi.KEN/23.02	Not specified	AY249251	AY249261	46
D4	MVs/Coast-Kilifi.KEN/24.02	Not specified	AY249252	AY249262	46
D4	MVs/Central.KEN/24.02	Not specified	AY249253	AY249263	46
D4	MVs/Nyanza.KEN/24.02/1	Not specified	AY249254	AY249264	46
D4	MVs/Nyanza.KEN/24.02/2	Not specified	AY249259	AY249269	46
D4	MVi/Coast-Kwale.KEN/31.02/1	Not specified	AY249255	AY249265	46
D4	MVs/Eastern.KEN/35.02	Not specified	AY249257	AY249267	46
D4	MVi/Coast-Malindi.KEN/26.02	Not specified	AY249258	AY249268	46
D4	MVs/Zagreb.CRO/30.06 (SSPE)	Brain tissue	FJ475060.1	JX126962.1	83
D4	MVs/Bedelle.ETH/5.99	Oral fluid	AF280800	AF280805	84
D4	MVs/AddisAbaba.ETH/2.99	Oral fluid	AF280802	AF280807	84
D4	MVs/Ontario.CAN/3.15/	Urine	KU218405.1	KU218406.1	J. Hiebert
D4	MVs/Saint-Jean-de-Maurienne.FRA/24.09	Oral fluid	KJ183791.1	KJ183852.1	J. Dina
D4	MVs/Suresnes.FRA/20.10	Oral fluid	KJ183846.1	KJ183910.1	J. Dina
D4	MVs/Saint-Etienne.FRA/19.10	Oral fluid	KJ183840.1	KJ183904.1	J. Dina
D4	MVs/Limoges.FRA/18.10	Oral fluid	KJ183838.1	KJ183902.1	J. Dina
D4	MVs/Douai.FRA/18.10	Oral fluid	KJ183837.1	KJ183901.1	J. Dina
D4	MVs/Ajaccio.FRA/18.10	Oral fluid	KJ183836.1	KJ183900.1	J. Dina
D4	MVs/Rabastens.FRA/16.10	Oral fluid	KJ183833.1	KJ183896.1	J. Dina
D4	MVs/Dax.FRA/17.10	Oral fluid	KJ183834.1	KJ183898.1	J. Dina
D4	MVs/Bordeaux.FRA/16.10	Oral fluid	KJ183831.1	KJ183894.1	J. Dina
D4	MVs/Tours.FRA/13.10	Oral fluid	KJ183821.1	KJ183882.1	J. Dina
D4	MVs/Nantes.FRA/25.09	Oral fluid	KJ183795.1	KJ183856.1	J. Dina
D4	MVs/Flers.FRA/31.09/	Oral fluid	KJ183818.1	KJ183879.1	J. Dina
D4	MVs/Vichy.FRA/31.09/	Oral fluid	KJ183819.1	KJ183880.1	J. Dina
D4	MVs/Clermont-Ferrand.FRA/13.10	Oral Fluid	KJ183824.1	KJ183885.1	J. Dina
D4	MVs/Tarbes.FRA/30.09	Oral fluid	KJ183813.1	KJ183874.1	J. Dina
D4	MVs/Vannes.FRA/14.10	Oral fluid	KJ183826.1	KJ183887.1	J. Dina
D4	MVi/Barcelona.SPA/26.08	Cell culture	KY524301	KY524302	This work
D4	MVi/Madrid.SPA/10.10/1	Cell culture (urine)	KY524303	KY524304	This work

^aE. Franchin, F. Dal Bello, M. Pacenti, R. Cusinato, E. Lavezzo, L. Barzon, G. Palu.

^bE. F. Kirkness, R. Halpin, J. Bera, N. Fedorova, L. Overton, T. Stockwell, P. Amedeo, B. Bishop, H. Chen, P. Edworthy, N. Gupta, D. Katzel, K. Li, S. Schobel, S. Shrivastava, V. Thovarai, S. Wang, B. Bankamp, L. Byrd, W. Bellini, P. Rota.

nucleotide sequence divergence for the MeV-H gene was 2.3% ($\pm 0.2\%$) (range, 1.5% to 3.0%). These results show high intragenotypic variation for the sequences assigned to genotype D4 but do not support the designation of 2 different genotypes. Here, we distinguish subgenotype 1 (D4.1) and subgenotype 2 (D4.2).

Evolving residues in the MeV-H protein define a subgenotype classification.

MeV-neutralizing antibodies are directed mainly against the MeV-H protein (16). Because of our identification of two subgenotypes in MeV genotype D4, we further investigated the differences observed in the MeV-H gene at the level of the resulting protein.

The predicted amino acid differences between the MeV-H proteins of the two subgenotypes were 2.0% ($\pm 0.2\%$), ranging from 1.2% to 4.0%. Of note, both subgenotypes were characterized by 2 well-defined consensus sequences with variations at positions 212, 240, 249, 303, 316, 359, 412, and 481 (Fig. 3). These residues, together with residues 305 and 562, were also identified as directionally evolving sites through convergent evolution under a directional evolution of protein sequences test (Bayes factor, $>10^5$) (86). Likewise, 5 pairs of

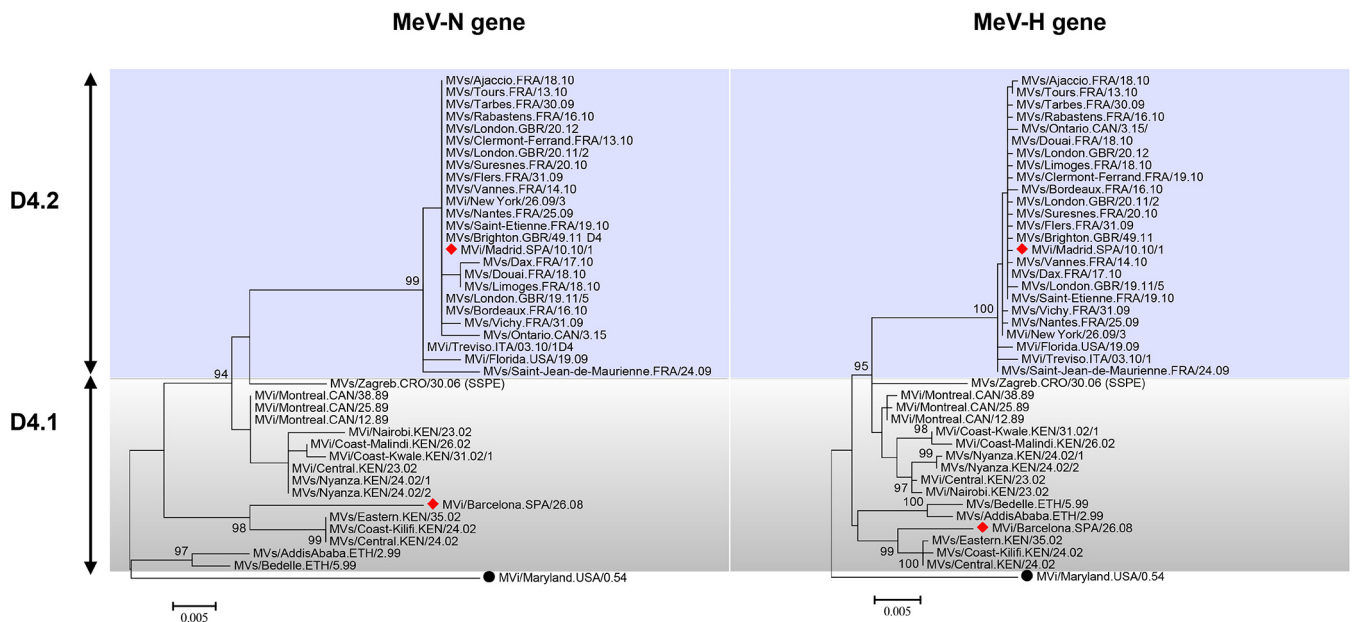


FIG 2 Phylogenetic relationships of the MeV-N gene (left) and MeV-H gene (right) of measles virus genotype D4. The evolutionary history was inferred by using the maximum likelihood method based on the general time-reversible model. Measles virus genotype A (MVi/Maryland.USA/0.54) was used for rooting the tree. Evolutionary analyses were conducted with MEGA 6.06. Statistical support for grouping is shown as bootstrap values (1,000 replicated). Values of >80% are indicated at the deep node. The bar represents the genetic distance. The subgenotype classification is illustrated. Sequences were retrieved from the National Center for Biotechnology Information (NCBI) GenBank database and are named according to World Health Organization guidelines (Table 1).

interactions were detected ($P < 0.05$): N238T \longleftrightarrow P276L, R34K \longleftrightarrow T307A, F476L \longleftrightarrow S590T, M333L \longleftrightarrow I564L, and S318N \longleftrightarrow F571S. All sites except position 571 were exposed on the protein surface (not shown). However, none of them showed close proximity in the two crystallographic forms resolved so far for MeV-H (form I and form II [not shown]) (39).

These results prompted us to analyze selection pressures on the MeV-H gene among genotype D4 viruses. To estimate positively and negatively selected sites, we used the following four methods: single-likelihood ancestor counting (SLAC), fixed-effects likelihood (FEL), internal fixed-effects likelihood (IFEL), and mixed-effect model of evolution (MEME). Four positively selected sites were estimated by using the MEME method (Table 2), which detected residues under both pervasive positive selection and episodic selection (40): E85Q, L172M, V562F/A, and Q574K. In addition, 15 sites were under negative selection, 5 of which were common to all four methods (Table 2): 3P, 113N, 256E, 342D, and 347D. Except for sites at positions 172 and 256, they were located on the protein surface (not shown). We also calculated the combined synonymous and nonsynonymous rate to be 0.22 (95% confidence interval [CI], 0.17 to 0.29) with the SLAC method. Overall, these results establish a basis for the distinction of D4 subgenotypes.

An L249P amino acid mutation in MeV-H is the main driver for subgenotype D4.2 escape from NE-specific antibodies. Apart from the BH129 MAb, the BH047 and BH059 MAbs have also been shown to bind a sequential epitope encompassing amino acids 240 through 250 of MeV-H (41). To confirm that antigenic drift of the NE site defines D4 subgenotypes, we studied the neutralization capacity of the other NE-targeting MAbs. We additionally tested different recombinant MeVs encoding the genotype-specific MeV-H genes of genotypes B3.1, C2, D4, D6, D7, D8, D9, G3, and H1 (Fig. 4A). As expected, none of the NE-targeting MAbs (BH129, BH047, and BH059) were able to inhibit infection by subgenotype D4.2 viruses, whereas all of them inhibited viruses of subgenotype D4.1. Surprisingly, these antibodies did not inhibit infection by recombinant MeV possessing the MeV-H gene of genotype D6, although all other genotypes were neutralized.

To understand in greater detail the neutralization selectivity of these NE-targeting antibodies, we aligned and compared the MeV-H amino acid sequences for all the

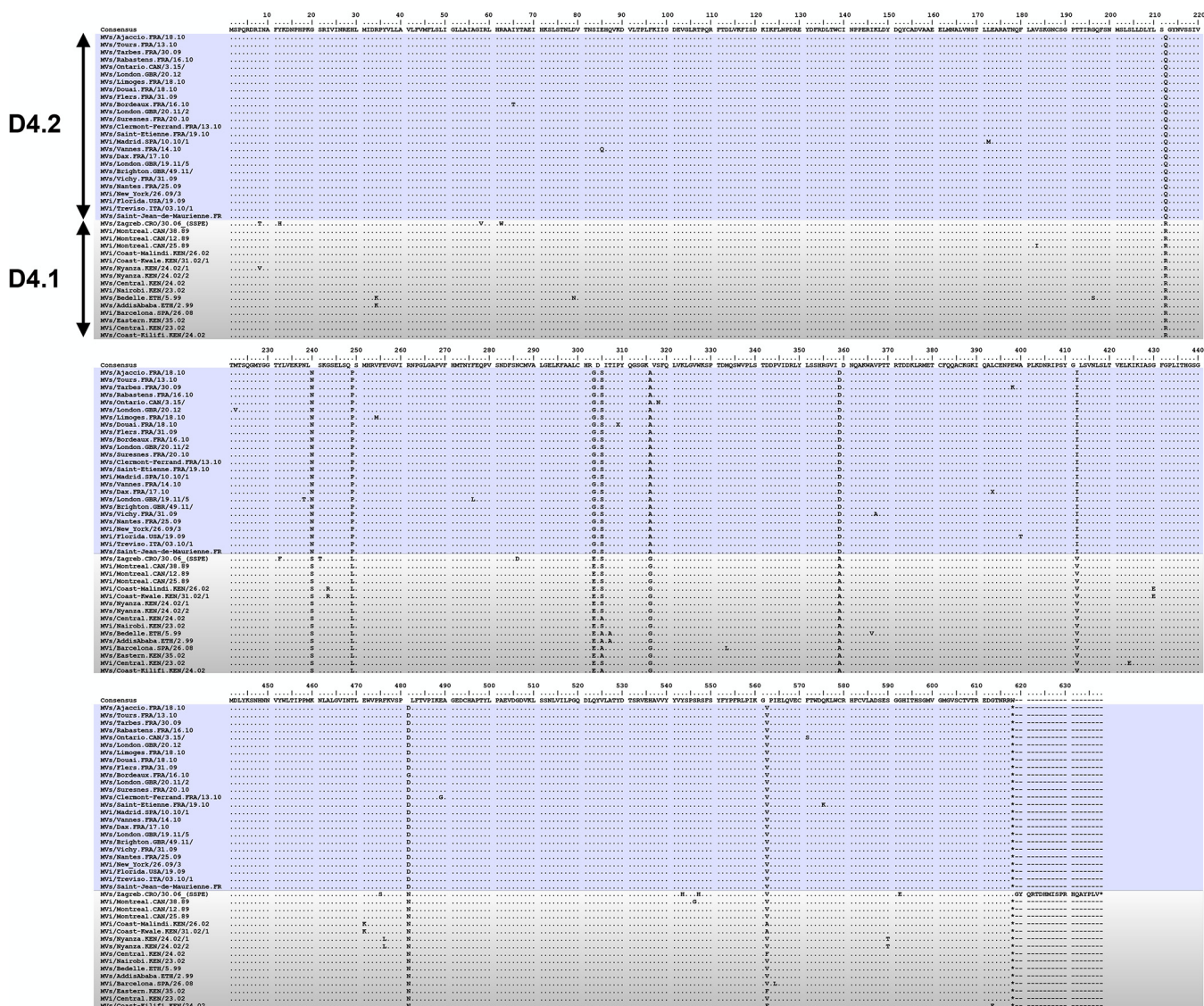


FIG 3 Sequence alignment of the MeV-H genes of genotype D4 viruses. The GenBank accession numbers for the MeV-H gene sequences used in the sequence alignment are shown in Table 1.

viruses that we used (Fig. 4B). Whereas the NE antigenic site is one of the most variable regions of MeV-H, NE-targeting antibodies discriminated only the D4.2 and D6 genotypes. Subgenotype D4.2 viruses showed the single mutation L249P, whereas genotype D6 viruses also showed an S247P mutation. To further elucidate the role of these amino acid changes found within the NE antigenic site, we introduced each of the two single point mutations into MeV-H genotype A and rescued the corresponding viruses. Figure 4C illustrates that an L249P amino acid substitution completely changed the neutralization sensitivity of antibodies targeting the NE, yet the sensitivity of genotype A virus to antibodies remained unchanged with and without the single point mutation S247P. These observations demonstrate that a single point mutation, L249P, but not S247P, allows MeV to avoid neutralizing antibodies targeting the NE.

Viral escape is due to loss of MeV-H binding sites for NE-targeting antibodies.

Virus neutralization requires a certain level of electrostatic and shape complementarity between the viral antigen and neutralizing antibody (42). Since 2 different mutations were found in the NE antigenic site, we further investigated their effect on virus-antibody interactions. To this end, we first produced a soluble form of the MeV-H protein that displays a C-terminal FLAG epitope. This tag enables the control of the

TABLE 2 Selected sites in the MeV-H gene^a

Amino acid	Detection of site by method			
	SLAC	FEL	IFEL	MEME
Positively selected sites				
Glu85Gln ^b				●
Leu172Met ^b				●
Val562Phe/Ala ^b				●
Gln575Lys ^b				●
Negatively selected sites				
3Pro	●	●	●	
113Asn	●	●	●	
173Glu		●		
174Ala		●	●	
256Glu	●	●	●	
342Asp	●	●	●	
347Asp	●	●	●	
393Leu		●		
405Asn		●		
445Lys		●		
497Pro		●	●	
522Leu		●		
572Thr		●	●	
606Cys		●		
610Arg		●	●	

^aAbbreviations: FEL, fixed-effects likelihood; IFEL, internal fixed-effects likelihood; MeV-H, measles virus hemagglutinin; MEME, mixed-effects model of episodic diversifying selection; SLAC, single-likelihood ancestor counting.

^b*P* value of <0.10.

amount of protein coating the surface of the plastic well. As expected, a FLAG antibody equally recognized all FLAG-tagged MeV-H proteins (Fig. 4D). However, NE-targeting antibodies showed defects in binding to those MeV-H proteins whose viruses escaped neutralization. Overall, these results show that an L249P substitution, but not S247P, impairs the binding of neutralizing antibodies that recognize the NE antigenic site, thereby allowing virus neutralization escape.

Subgenotype D4.2 shows diminished sensitivity to neutralization by pooled human sera. The data presented in Fig. 1 show that a naturally occurring MeV strain can escape neutralization by MAbs targeting 3 of 6 antigenic sites of the MeV-H protein. Because 90% of the antibodies of MeV patients are directed against the MeV-H protein (17), we next assessed whether the mutation of the NE antigenic site in the subgenotype D4.2 MeV-H protein translates into a different serum neutralization pattern than those for subgenotype D4.1 and vaccine genotype A viruses.

To obtain a representative sample for population immunity against measles, we used pooled human serum collected from approximately 60 to 80 blood type AB donors in North America. To rule out potential interference by MeV-F-specific antibodies, we first absorbed MeV-F-specific antibodies from pooled human sera using MeV-F of genotype A, the F protein expressed by all of our recombinant MeVs. This depletion was performed by incubation with MeL-JuSo cells expressing the MeV-F protein. Following this depletion procedure, the remaining measles neutralization activity of the depleted serum is mediated exclusively by anti-MeV-H antibodies. Figure 5A shows that incubation with untransfected MeL-JuSo cells results in no depletion of MeV-H/F-specific antibodies, whereas incubation with MeL-JuSo/MeV-F cells results in the elimination of MeV-F-specific antibodies while maintaining the MeV-H-specific antibodies. As an additional control for the presence of residual MeV-F-specific antibodies due to the limit of detection of the fluorescence-activated cell sorter (FACS) analysis, we used an envelope-chimeric MeV/canine distemper virus (CDV) (M. A. Muñoz-Alía and Stephen J. Russell, unpublished data). This envelope-chimeric virus expresses the MeV-F protein in combination with the Onderstepoort strain CDV-H protein possessing a

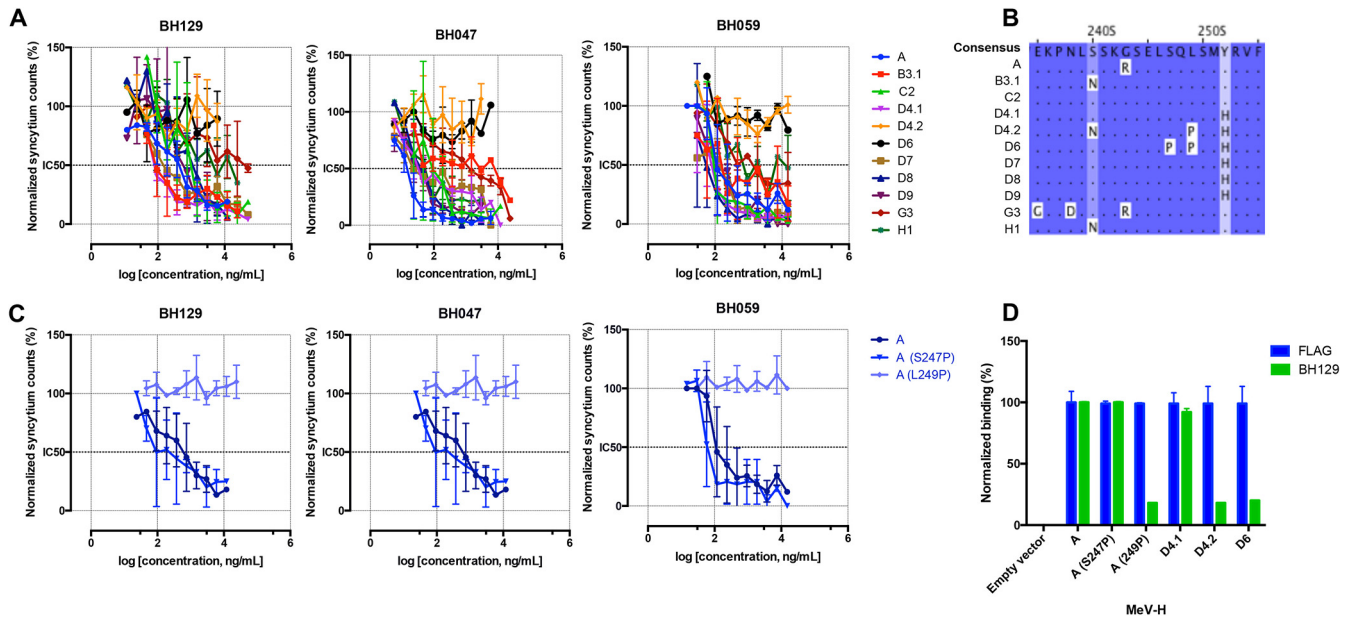


FIG 4 Leu249Pro amino acid change as the driver mutation for escape of MeV subgenotype D4.2. (A) Neutralization activity of the NE-targeting antibodies against MeV genotypes. The neutralization assay was performed and data are represented as indicated in the legend of Fig. 1. Of note, only genotype D6 and the so-called subgenotype D4.2 viruses escape neutralization by all 3 antibodies. (B) Sequence alignment (amino acids 235 to 255) of MeV-H proteins among the MeV genotypes used in this study. (C) Effect of amino acid mutations on NE-targeting antibody neutralization. (D) NE-targeting antibody binding to MeV-H proteins. MAb binding was determined by an enzyme-linked immunosorbent assay after the addition of a nonsaturating concentration of MAbs to microtiter plates coated with the FLAG-tagged MeV-H protein. The amount of anti-FLAG antibody binding was used as a loading control. Binding values were normalized to those obtained with MeV-H genotype A. Means and standard deviations for a representative experiment performed in duplicate are shown.

Y537D point mutation (43). This envelope-chimeric MeV-F/CDV-H showed complete resistance to neutralization by MeV-F-depleted pooled human sera (Fig. 5B). Neutralization assays using recombinant MeVs expressing the two D4 subgenotype-specific MeV-H proteins showed a diminished sensitivity for subgenotype D4.2 (50% inhibitory concentration [IC₅₀] = 33.00 ± 3.50 milli-international units [mIU]/ml) compared to subgenotype D4.1 (IC₅₀ = 30.93 ± 4.31) or the genotype A vaccine strain (IC₅₀ = 30.05 ± 3.35) (*P* = 0.21, according to a paired *t* test). Therefore, the absence of the NE in subgenotype D4.2 MeV-H was associated with a definite reduction (albeit nonsignificant) in neutralization sensitivity compared to those of subgenotype D4.1 and vaccine genotype A viruses.

DISCUSSION

RNA virus populations are well known for their high levels of genetic diversity and high mutation rates, which provide a continuous challenge for investigators who must develop and constantly update vaccines and antiviral drugs (44). However, MeV stands apart from many other RNA viruses because of its limited antigenic diversity. In the present study, we focused on intragenotype variation among genetically and antigenically closely related viruses belonging to genotype D4. Viruses belonging to genotype D4 represent about 20% of the MeV sequences reported in databases (<http://www.who-measles.org/>), reflecting the high current prevalence of this genotype. The abundance of available genotype D4 sequences has allowed us to analyze a considerable number of MeV-N and MeV-H nucleotide sequences to probe their genetic diversity. MeV-H phylogeny analysis was congruent with MeV-N gene phylogeny, in support of the current standard for measles virus genotype identification. It allowed us to distinguish two subgenotypes, D4.1 and D4.2, that were paired with different neutralization sensitivities by anti-MeV-H antibodies targeting the NE antigenic site.

Distinct unrelated subgroups for genotype D4 viruses were previously proposed exclusively on the basis of phylogenetic analysis of the MeV-N gene (45–47). We are aware that the minimum genetic distance observed in the MeV-H genes between the

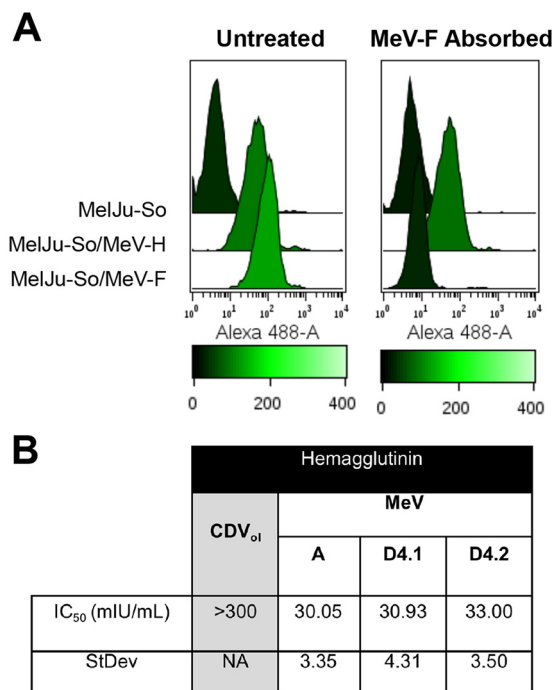


FIG 5 Neutralization of MeV by anti-MeV-H antibodies from pooled human sera. (A) Reactivity of pooled human sera after 0 days (untreated) or 4 days (MeV-F absorbed) of incubation at 37°C with cells expressing MeV-F. Binding reactivity was measured by fluorescence-activated cell sorter analysis using MeL-JuSo cells expressing or not expressing the MeV glycoproteins (MeV-H and MeV-F). Anti-human immunoglobulin G(H+L) Alexa Fluor-488 was used for staining. (B) Neutralization of MeV by anti-H pooled human sera. The IC₅₀s and standard deviations (StDev) were calculated from 3 experiments performed independently in quadruplicate. Values were calculated after nonlinear regression of the corresponding neutralization curves with GraphPad Prism software. CDV, canine distemper virus; NA, not applicable.

proposed D4 subgenotypes (1.5%) does not match WHO criteria for assigning a new genotype (>2%), and the MeV-N minimum genetic distance (2.5%) is at best borderline (>2.5%). Nevertheless, a similar subgenotype classification was proposed for subgenotypes B3.1 and B3.2 without fulfilling the divergence criteria (48, 49). Regardless of the definition for measles virus lineages, the subgenotype B3.1 and B3.2 viruses and the subgenotype D4.1 and D4.2 viruses are antigenically distinguishable within their respective genotypic clades.

Rapid genetic evolution drives the emergence of viral variants escaping immune surveillance. Mutations of the influenza virus hemagglutinin (HA) protein, the respiratory syncytial virus F protein, and the E2 protein of hepatitis C virus are known to underlie their escape from neutralization by polyclonal antisera (50–53). The MeV-H protein is the primary target of protective anti-measles virus antibodies (16, 17). We observed that subgenotype D4.2 viruses are resistant to monoclonal antibodies targeting three of the six known MeV-H protein antigenic sites (BH129, BH047, BH059, BH125, and BH097). These viruses were nevertheless efficiently neutralized by pooled human sera. The presence of other antigenic sites might mask such an effect.

The NE antigenic site is thought to encompass a linear epitope consisting of the region spanning amino acids 244 to 250, together with the region spanning amino acids 233 to 240, recognized by MAb BH1 (41). Although the region spanning amino acids 240 to 245 has not been visualized in the MeV-H crystal structures because of low electron density (39, 54, 55), it is predicted to display an α-helix where E₂₄₅L-QL₂₄₉ would be located on the protein surface (56). As a consequence, S247 would not be accessible, explaining the lack of an effect on both virus neutralization and antibody binding for the MeV-H S247P mutant. Conversely, a mimotope containing the S247P mutation associates with the NE-targeting BH129 MAb up to 135 times more efficiently

(56). Discrepancies between antipeptide serum binding to the virus and virus neutralization activity have been reported for mimotopes (57, 58), which emphasizes the utility of testing linear epitope-based vaccines in the context of the mutant viruses. The NE antigenic site is immunogenic because MAb BH047 inhibits the binding of 20% of human serum antibodies in measles patients (59). In this regard, the MeV genotype C2 mutant variants S246L and S247L present in 2 of 5 measles isolates sequenced during a Madrid, Spain, outbreak may reflect immune selection (60).

To date, 24 MeV genotypes are recognized with preferential geographic circulation. A Bayesian skyline plot analysis suggested that prevalent genotypes such as genotype D4 are more adaptive to humans (61). The fact that most of the subgenotype D4.1 sequences that we retrieved from GenBank were derived from Kenya and Ethiopia, whereas the MAb-resistant subgenotype D4.2 MeV sequences were derived predominantly from France and Great Britain, might suggest that an intermediary level of vaccine coverage provides an environment more prone to adaptive mutations. Our inability to discern antigenic differences between the 2 subgenotypes by human serum neutralization could be intrinsic to the heterogeneous composition of the human sera used. Their high neutralization potency is indicative of natural MeV infection; therefore, some of the human patients whose sera were included in the pool could have even been infected with a genotype D4 virus strain, potentially masking antigenic differences. The use of vaccine samples with lower neutralization titers could potentially help in discerning this problem.

Nevertheless, the 2 D4 subgenotypes circulated in Spain at the same point, even in the same geographic area. Thus, although our subgenotype D4.1 virus was isolated in the Barcelona region of Catalonia (northeastern Spain) (MVi/Barcelona.SPA/26.08) and our subgenotype D4.2 virus was isolated in the autonomous region of Madrid (central Spain) (MVi/Madrid.SPA/10.10/1), a sporadic case of a subgenotype D4.1 virus circulating in Madrid could be identified (MVs/Madrid.SPA/50.05; GenBank accession no. [EU085473](#)). Interestingly, the sequence is similar to the sequence circulating in the United Kingdom at that time, which was epidemiologically linked to Somalia (45). However, MeV endemicity was established 2 years later in the United Kingdom with a subgenotype D4.2 virus (MVs/Enfield.UNK/14.07), and subgenotype D4.2 viruses have become predominant in Europe since then (47, 62). In 2011, these subgenotype D4.2 viruses were imported from France to the United States in 2011, causing the highest number of measles cases since it was declared eliminated (63).

Finally, we studied the adaptive significance of amino acid substitutions in MeV-H by different methods. We detected 5 pairs of amino acidic interactions. Whether they have functional significance warrants reverse-genetics systems and virus fitness experiments. Of the estimated positively and negatively selected sites in the gene, 5 negatively selected sites were detected by FEL, IFEL, and SLAC methods (3P, 113N, 256E, 342D, and 347D). Of these sites, only 3P has been detected previously by analyzing genotypes D3, D5, D9, and H1 (64).

In summary, we genetically identified 2 different lineages, or subgenotypes, in MeV genotype D4 strains that are antigenically distinguishable by virtue of their neutralization by antibodies targeting the NE antigenic site. This close genetic and antigenic relatedness might indicate selective pressure. Although subtle differences in human sera of MeV patients were detected, the absence of half of the known antigenic sites present in MeV-H warrants close antigenic monitoring in virological surveillance for measles.

MATERIALS AND METHODS

Cells and media. B95a cells (an adherent marmoset B-cell line) were maintained in Roswell Park Memorial Institute medium (RPMI 1640; Corning) supplemented with 10% (vol/vol) heat-inactivated (56°C for 30 min) fetal bovine serum (FBS) (Gibco). African green monkey kidney (Vero) cells stably expressing human SLAM (65) were maintained in Dulbecco's modified minimal essential medium (DMEM) (HyClone; GE Healthcare Life Science) containing 5% FBS and 0.5 mg of Geneticin ml⁻¹ (G418; Corning). The human melanoma cell line Mel-JuSo/wt, transfected or not with the Edmonston MeV-H (Mel-JuSo/MeV-H) or MeV-F (Mel-JuSo/MeV-F) protein, was described previously (16, 66) and cultured in RPMI 1640. In antibody depletion assays, the medium was replaced with human serum diluted 1:10 in

RPMI medium, and cells were cultured for 4 days at 37°C in a humidified atmosphere of 5% carbon dioxide (17). All cell culture media were additionally supplemented with 100 IU penicillin ml⁻¹, 100 µg streptomycin ml⁻¹, and 10 mM HEPES (Gibco). All cells routinely tested negative for mycoplasma contamination.

Viruses. Growth of the Edmonston strain (67) and the MeV primary isolates was performed as described previously (60). The wild-type MeVs used in this work were MVi/Madrid.SPA/15.06/3 (subgenotype B3.1) (23), FV (genotype C2) (60), MVi/Barcelona.SPA/26.08 (genotype D4), MVi/Madrid.SPA.10.10/1 (genotype D4), BCL (genotype D6) (60), MVi/Madrid.SPA/25.03 (genotype D7), MVi/Alicante.SPA/22/03 (genotype D8), MVi/Granada.SPA/32.08 (genotype D9), 65045397 (genotype G3) (68), and MVi/Madrid.SPA/50.10 (genotype H1). Viral stocks were prepared by repeated cycles of freezing and thawing and were frozen in aliquots at -80°C. The MeV titer was determined as PFU by using Vero/hSLAM cells.

Antibodies and virus neutralization assay. Monoclonal antibodies and human sera were heat inactivated and serially diluted in Opti-MEM (Thermo Fisher Scientific). Equal volumes of the respective virus at 30 PFU per well were mixed with the respective antibodies at various 2-fold serial dilutions in 96-well plates (Costar Corp.), incubated at 37°C for 1 h, and inoculated onto 80% to 90% confluent Vero/hSLAM cells. The number of EGFP-expressing syncytia per well was counted under a fluorescence microscope after 2 days of culture and converted to a percentage in relation to infection in the absence of antibodies (100%). Each sample was run in 4 wells, and each experiment was repeated at least twice on different days.

The following anti-MeV-H protein MABs used for neutralization were previously described: BH129, BH047, BH059 (69), BH101, BH026 (70), BH141 (71), and BH097 (72). Protein G affinity-purified MABs were used, except for BH141 and BH059. BH141 and BH059 antibody concentrations were determined with a mouse isotype-specific immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) quantitation set (Bethyl Laboratories).

Human serum used in this study was commercially purchased (product no. HP1022, lot no. C80553; Valley Biomedical). This serum represents a pool of 60 to 80 blood type AB healthy donors (communication with the supplier). The antibody concentration that causes a 50% reduction in plaque numbers (IC₅₀) was determined by nonlinear regression using software (Prism version 7.00 for Mac; GraphPad Software). The titers were transformed to international units per milliliter by using National Institute for Biological Standards and Control (NIBSC) reference serum (WHO/BS/06.2031), according to procedures described previously (73).

Viral RNA isolation and generation of recombinant viruses. RNA was extracted from infected B95a cells by using an RNeasy minikit (Qiagen) according to the manufacturer's instructions. A hyper-variable region of the MeV-N gene and the entire region of the MeV-H gene were reverse transcribed by using 200 U of reverse transcriptase (SuperScript II; Invitrogen) and primers (Integrated DNA Technologies) MVN(+)₉₅₈ (TTGGACTGCATGAATTTGCTGG) and V181 (TTAATTAAAACCTTAGGGTCAAGATCATCGA TAATG), respectively. The resulting cDNA was amplified by PCR (HotStarTaq DNA polymerase; Qiagen) with primer pairs V181/H1931 (CACTAGTGGGTATGCCTGATGT) and MVN(+)₉₅₈/MVP(-)₃₃₅₅ (GGTTGGC AGGTAAGTTGAGCT). Amplified DNA fragments were analyzed by electrophoresis in a 0.7% agarose gel and purified from the gel for sequencing (ABI Prism 3730xl DNA analyzer; Perkin-Elmer Applied Biosystems) by using a gel extraction kit (QIAquick; Qiagen). The entire MeV-H gene flanked with SpeI/PacI restriction sites (underlined in the primer sequences) was cloned into the equally restricted pCG vector (New England BioLabs Inc.) (74). At least 4 clones were sequenced, and the direct sequences of the PCR products were used to define the consensus sequences. Only those clones with the consensus sequence were transferred into an infectious MeV genome expressing EGFP upstream of N [MVvac2(GFP)N], using PacI and SpeI restriction sites. The MVvac2(GFP)N coding capacity is identical to those of the Moraten/Schwartz vaccine strains (75). Recombinant viruses were rescued by using standard protocols with modifications (76, 77). The integrity of the insert was verified by sequencing, and the corresponding virus was renamed according to the genotype of the MeV-H gene. The sequence of the MeV-N gene was determined directly from the PCR products.

Cloning and expression of the MeV-H protein. The soluble MeV-H protein was produced by amplification of the MeV-H ectodomain (residues 61 to 617) preceded by the murine IgG κ-chain leader and a Strep-tag II epitope. This construct was cloned in frame with the C-terminal FLAG tag present into the MluI- and EcoRV-restricted pCMV6-AC-IRES-GFP vector by using an InFusion HD kit (Clontech Laboratories Inc.). Expi293F cells (Gibco) were transiently transfected and grown in suspension for 6 days. The culture supernatant was harvested every 3 days, filtered through a 0.45-µm-pore-size filter, concentrated by using a centrifugal filter device (Amicon Ultra 50K; EMD Millipore Corp.), and buffer exchanged with HEPES buffer (10 mM HEPES, 150 mM sodium chloride [pH 7.5]).

FACS analysis. MeV-H- and MeV-F-specific IgG antibody levels were determined as described previously (16, 17, 66), with the following modifications: RPMI 1600 medium supplemented with 2% FBS and 2-mM EDTA (catalogue no. 155575; Thermo Fisher Scientific) was used as a FACS buffer, and cells were stained with human IgG(H+L) Alexa Fluor-488.

Enzyme-linked immunosorbent assay. Coated microplates (Strep-Tactin XT; IBA GmbH) were incubated at 37°C for 1 h with 0.1 ml of soluble MeV-H protein, previously diluted to give a final optical density of 1 under the conditions described above. The plates with the MeV-H protein absorbed through its amino-terminal Strep-tag II sequence were washed 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween), and consequently, 0.1 ml of antibody in PBS (5 µg/ml) was added. In parallel, mouse anti-FLAG M2 antibody (Sigma-Aldrich) was included as a control for the absorbed protein. The plates were incubated for 1 h at 37°C and washed with PBS-Tween as described above. The mixtures were then allowed to react with 0.1 ml of goat anti-mouse IgG(H+L)-horseradish

peroxidase (HRP) (catalogue no. 62-6520; Thermo Fisher Scientific) in PBS-Tween at a dilution of 1:2,000 to 1:4,000 for 1 h at room temperature. The peroxidase bound to the wells was detected by incubation with 1-Step Ultra TMB (3,3',5,5'-tetramethylbenzidine; Thermo Fisher Scientific) according to the manufacturer's protocol. The Epstein-Barr virus VCA IgG titer was assessed by using a commercial ELISA as recommended by the manufacturer (catalogue no. 57351; IBL International GmbH).

Sequence analysis. Sequence data available for MeV-N and MeV-H of genotype D4 MeV strains were retrieved from GenBank and analyzed by using BioEdit version 7.0.9.0. Multisequence alignments were performed with ClustalX (<http://www.genome.jp/tools/clustalw/>), and identical sequences were removed from the data set. Phylogenetic reconstructions were inferred by the maximum likelihood method on the basis of the general time-reversible model available in MEGA version 6.06 (78). To estimate the robustness of the phylogenetic inference, we performed 1,000 bootstrap analyses. Estimation of the evolutionary divergence among sequences was conducted by using the Kimura 2-parameter model in MEGA version 6. The codon positions included were the first plus second plus third plus noncoding positions.

The selective pressure acting on the MeV-H gene was investigated by using 4 different methods: SLAC, FEL, IFEL, and MEME (all available in the HYPHY package on a Web-based interface [DataMonkey]) (79).

Structural modeling. An MeV-H model was generated from the crystallographic structure of the purified protein alone (PDB accession no. 2ZB5) and in complex with SLAM (PDB accession no. 3ALZ). A complex-type N-glycan model was constructed manually by using a complex penta-antennary glycan whose coordinates were obtained online (see <http://www.glycosciences.de/>). Crystallographic structures were analyzed and modeled by using PyMOL software (<http://pymol.org/>).

Accession number(s). The nucleotide sequences determined in this work were deposited in the GenBank database under accession no. KY524301 to KY524304. A list of nucleotide sequences used in the course of this work can be found in Table 1.

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