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Adaptation of wild-type measles virus to CD46 receptor usage

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Summary. Vaccine strains of measles virus (MV) use CD46 as receptor and downregulate CD46 from the surface of infected cells. MVs isolated and passaged on B-lymphoid cells (wild-type MVs) seem to use another receptor and do not downregulate CD46. In the present study, we found that isolation of MV on human or marmoset B-lymphoid cells did not alter the MV haemagglutinin (H) protein relative to that in the patient. The wild-type isolates were adapted to the human epithelial HEp-2 cell line or the monkey fibroblast Vero cell line. All HEp-2 cell adapted viruses and 1 out of 4 Vero cell adapted viruses acquired the capacity to use CD46 as receptor, as measured by their ability to infect murine cells expressing human CD46. Adaptation to CD46 receptor usage was coupled to substitution of amino acid 481 of the MV H protein from asparagine to tyrosine but not to CD46 downregulation. The present study demonstrates that CD46 receptor usage can be induced by adaptation of wild-type MV to cells that do not express a wild-type receptor and suggests that a similar mechanism acted on the progenitor viruses of the present MV vaccine strains during their isolation and attenuation.

Introduction

Measles virus (MV) vaccine strains and most MV laboratory strains use CD46 (membrane cofactor protein) as a receptor, and their receptorbinding haemagglutinin (H) protein downregulates the expression of CD46 on the surface of infected cells [9, 15, 22, 25, 30]. MVs isolated and passaged solely on human or marmoset B-lymphoid cells (wild-type MV), appear to use another yet unidentified receptor, and generally their H proteins do not downregulate CD46 [3, 8, 17, 30, 31, 36].

The amino acid residues of the MV H protein that determine its CD46 downregulating properties have previously been investigated [2, 21]. In these studies,

the roles of amino acid differences between the H proteins of CD46 downregulating strains and CD46 non-downregulating wild-types were analysed using site-directed mutagenesis and vaccinia virus expression systems. Amino acid 481 was found to be far the strongest determinant, and this amino acid position alone determined the ability of the H protein to adhere to CD46 in a binding assay [17] and in a cell fusion assay [41]. Epitope mapping and peptide scanning have also suggested that a region of the H protein containing amino acid 481 is involved in receptor binding [18, 26].

In retrospect, the passage history of individual MV strains appears to influence the affinity of their H proteins to CD46. Thus, MVs, which do not downregulate CD46, were most often isolated and passaged solely on B-lymphoid cells and have asparagine at position 481 of the H protein, whereas CD46 downregulating strains have been passaged on non-lymphoid cells and have tyrosine at position 481 of the H protein [2, 8, 21, 30, 31]. These observations have led to the hypothesis that the CD46 receptor usage of MV vaccine strains is a cell culture phenomenon induced by adaptation of MV to cells that do not express a wild-type receptor [8, 21].

In the present study, we have examined whether such a mechanism is possible. Wild-type isolates that do not use CD46 as receptor and do not downregulate CD46 were adapted to HEp-2 or Vero cells and assayed for CD46 receptor usage and CD46 downregulation.

Materials and methods

Cell lines and viruses

The origin and cultivation of JP, B95-8, HEp-2, the murine hCD46-positive and the murine hCD46-negative cell lines were previously described [4, 6]. Vero cells were grown in Medium 199 supplemented with 5% fetal calf serum.

The Edmonston strain of MV was obtained from American Type Culture Collection and passaged in HEp-2 cells. Wild-type MV was isolated in 1996 from a Danish patient on TT cells, a human marmoset EBV-transformed B-lymphoblastoid cell line (B-LCL), or on B95-8 cells, a marmoset B-LCL, as shown in Fig. 1. For adaptation to the HEp-2 (human epithelial) and Vero (monkey fibroblast) cell lines, cultures of these cells were cocultivated for 72 h with JP (a human B-LCL) or B95-8 cells that had been infected with wild-type MV 96 h before the start of cocultivation. The titers of virus stocks were determined by end-point titration on B95-8 cells.

For assay of CD46 receptor usage, adherent murine hCD46-positive or negative cells in 24 well tissue culture plates were incubated with 2500 $TCID_{50}$ MV pr. well for 4 h. The cells were thoroughly washed and incubated in fresh medium for 96 h. The cells were then used for cytospins, which were analysed for MV infection by immunofluorescence microscopy.

Immunofluorescence assays

For immunofluorescence microscopy, cells in acetone-fixed cytospins were assayed for MV infection with human anti-MV serum, essentially as previously described [5].

For flow cytometry (FACS), mock- or virus infected JP or HEp-2 cells were harvested 72 h post infection and assayed with anti-MV H MAb I41 [33] or anti-CD46 MAb 122-2 (Cymbus Biotech) as previously described [6].

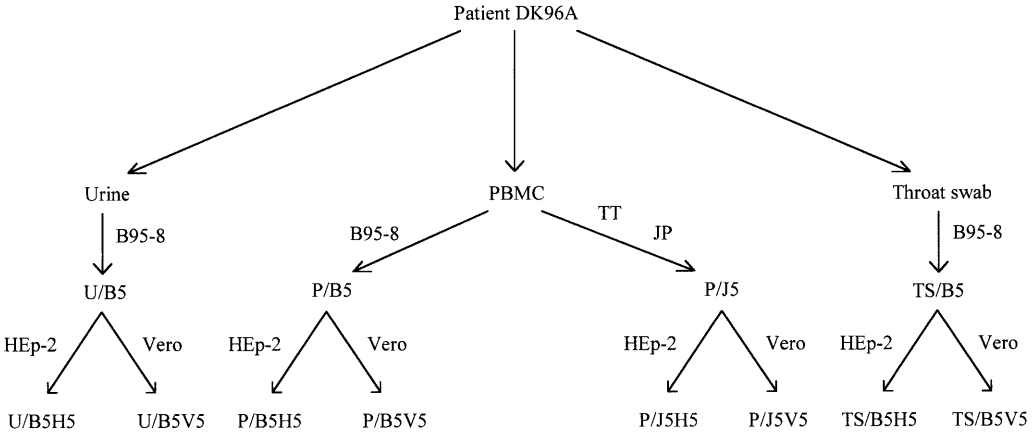

Fig. 1. Flow diagrammatic representation of the isolation and the further adaptation to different cell types of wild-type MV isolated from the PBMC (*P*), urine (*U*), and throat swab (*TS*) of a patient. The cells used were the human B-lymphoblastoid cell lines TT and JP (*J*), the similar marmoset cell line B95-8 (*B*), the human epithelial cell line HEp-2 (*H*), and the monkey fibroblast cell line Vero (*V*). For each passage, the number of passages on the individual cell lines is stated behind the letter of each cell line

RT-PCR and DNA sequencing

Total RNA was extracted from cells infected with the above passages as previously described [6]. Viral RNA was also extracted directly from urine of the patient using the QIAamp Viral RNA kit (Qiagen).

For amplification and sequencing of MV H genes, we used primers of 17 meric oligonucleotides selected from the consensus sequence of Radecke and Billeter [27]. The MV genomic segment between nucleotide (nt.) 2301 of the MV F gene and nt. 117 of the L gene was amplified from the RNA preparations using the One-Step RT-PCR system (Life Technologies). The RT-PCR reactions were incubated at 50 °C for 30 min and then 94 °C for 2 min to inactivate reverse transcriptase. Amplification was carried out by 40 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min followed by a final extension step at 72 °C for 8 min. The correct amplification products were purified from agarose gels with the QIAquick Gel Extraction Kit (Qiagen). The purified RT-PCR products were reamplified with 4 primer pairs resulting in fragments corresponding to nt. 2301 of the F gene to nt. 604 of the H gene, nt. 464 to 1086 of the H gene, nt. 881 to 1555 of the H gene, and nt. 1426 of the H gene to nt. 117 of the L gene. Reamplification was done by 34 of the above cycles with the proofreading Vent DNA Polymerase (New England Biolabs). The reamplification products were purified as previously described [16] and sequenced on both strands with the ABI Prism Bigdye terminator cycle sequencing ready reaction kit (Perkin Elmer) and analyzed in a Prism 310 Genetic Analyzer (Applied Biosystems).

Radioimmunoprecipitation and glycosidase digestion

MV H protein was immunoprecipitated from lysates of infected HEp-2 cells radiolabeled overnight as previously described [7]. Digestion with 25 mU peptide: N-glycosidase F (PNGase F) (New England Biolabs) was carried out as described for Endo H digestion [7] except that sodium phosphate to a final concentration of 50 mM pH 7.5 was added to the reaction instead of sodium citrate pH 5.5. The reactions were analysed by SDS PAGE and autoradiography.

Results

Isolation of wild-type MV and adaptation to HEp-2 or Vero cells

Wild-type MV was isolated on human or marmoset B-LCLs from peripheral blood mononuclear cells (PBMC), urine, and a throat swab of a Danish patient with acute measles (Fig. 1). We have previously referred to the isolates as DK96A1-A4 [6, 7], but according to the guidelines issued by WHO for naming of MV wild-type isolates [40], the names of the present isolates are MVi/Haderslev/DEN/06.96/1-4[D6]. The isolates were passaged 5 times on the human (JP) or the marmoset (B95-8) B-LCLs to give the wild-type passages P/J5 and P/B5 (isolated from PBMC), U/B5 (isolated from urine), and TS/B5 (isolated from a throat swab) (Fig. 1).

The 4 wild-type passages were adapted to a human epithelial (HEp-2) and a monkey fibroblast (Vero) cell line. Cultures of these cells were cocultivated with JP or B95-8 cells infected with the wild-types P/J5, P/B5, U/B5, or TS/B5. Frozen and thawed HEp-2 or Vero cells deriving from the cocultivations were used for infection of new cultures. This way, the HEp-2 adapted viruses were passaged 5 times each on HEp-2 cells to give the virus passages P/J5H5, P/B5H5, U/B5H5, and TS/B5H5 (Fig. 1). These 4 passages all induced typical MV CPE with extensive syncytium formation comparable to the Edmonston strain in HEp-2 cells (not shown). In a similar manner, the Vero adapted viruses were passaged 5 times on Vero cells to give the virus passages P/J5V5, P/B5V5, U/B5V5, and TS/B5V5 (Fig. 1). These 4 passages produced distinct CPE in Vero cells, but syncytium formation was much less pronounced than with the Edmonston strain (not shown).

CD46 receptor usage of the MV wild-type isolates and their derivates

The wild-type viruses and their HEp-2 or Vero cell adapted derivates were assayed for their ability to use CD46 as a receptor. This was done by examining the capacity of the virus passages to infect murine cell lines. Normal murine cells are generally unsusceptible to MV, but expression of human CD46 can confer susceptibility to MV vaccine strains [9, 25]. For the present study, we used a previously described cell line from the lungs of a transgenic hCD46-positive mouse and a similar hCD46-negative cell line from a normal mouse [4]. The CD46 surface expression of the transgenic murine cell line was similar to those of the human HEp-2 and HeLa cell lines ([4], data not shown). Cytospins of the murine cells were assayed for MV infection by immunofluorescense microscopy.

None of the viruses assayed in the present study infected the hCD46-negative murine cell line. As expected, the Edmonston MV strain infected the CD46-positive murine cells, whereas the 4 wild-type viruses P/J5, P/B5, U/B5, and TS/B5 did not (Fig. 2, Table 1). Interestingly, all 4 HEp-2 adapted virus passages (P/J5H5, P/B5H5, U/B5H5, and TS/B5H5) were also capable of infecting the CD46-positive murine cells (Fig. 2, Table 1). Among the 4 Vero cell adapted virus passages, only P/J5V5 infected the CD46-positive murine cells (Table 1).

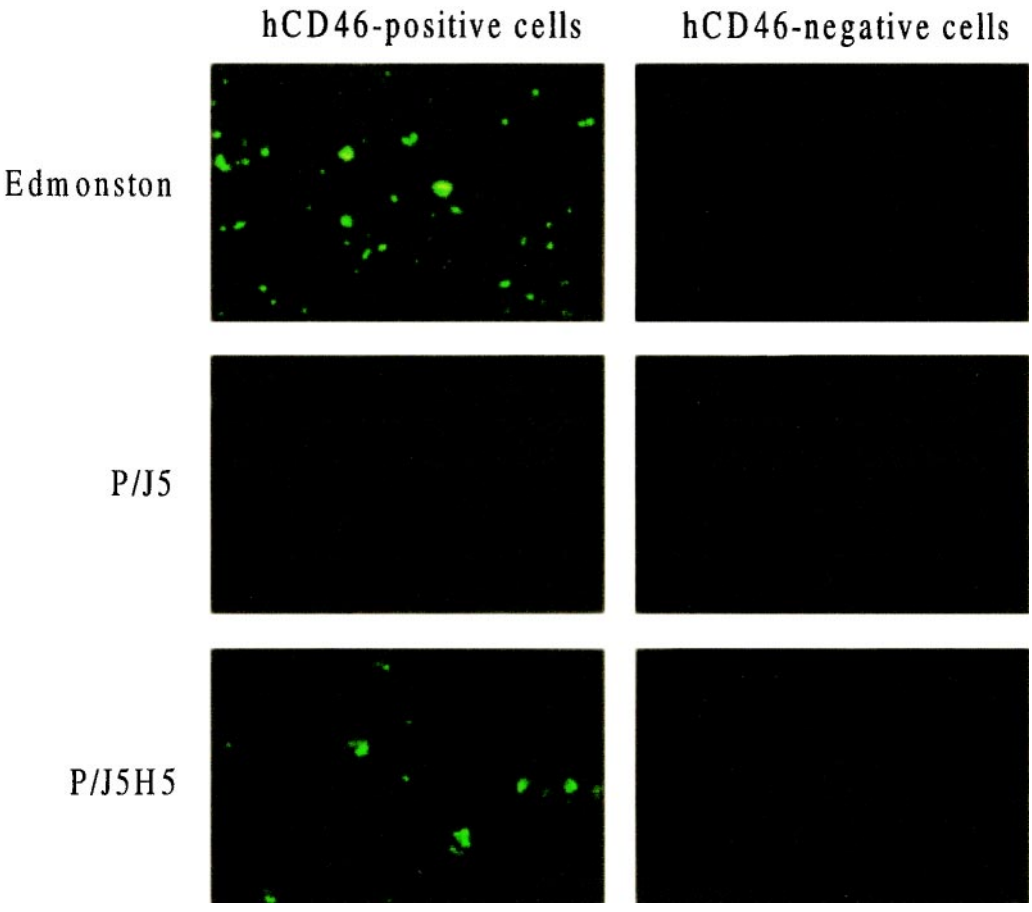

Fig. 2. Immunofluorescence microscopy of cytospins of hCD46-positive or negative murine cell lines 96 h after infection with the Edmonston strain, the wild-type isolate P/J5 or its HEp-2 cell adapted derivate P/J5H5. The cytospins were assayed with anti-MV serum

These data demonstrate that wild-type MV, which do not use CD46 as receptor, can be adapted to CD46 receptor usage upon passage on HEp-2 or Vero cells.

CD46 downregulating properties of the MV wild-type isolates and their derivates

The surface expression of CD46 on JP cells infected with the virus passages were compared with that of mock infected JP cells. JP cells were chosen for this assay because of their susceptibility to infection with both vaccine strain MV and wild-types of MV, possibly because they express both CD46 and one or more yet unidentified wild-type receptor(s). The HEp-2 or Vero cell adapted virus passages were also assayed for CD46 downregulation on HEp-2 cells.

The Edmonston strain induced 75% CD46 downregulation in JP cells (average of 3 experiments), whereas the 4 wild-type passages (P/J5, P/B5, U/B5, and TS/B5) did not downregulate CD46 (Fig. 3, Table 1). Also, none of the 4 HEp-2

Table 1. Properties of the present measles viruses

Virus[a]	CD46 downregulation	CD46 receptor usage[b]	H protein amino acid[c]			Genbank acc. no.
			41	481	546	
Edmonston	+	+	V	Y	S	AF172985
Urine[d]			V	N	S	AF172972
P/J5	−	−	V/I	N	S	AF172973
P/J5H5	−	+	I	Y	S	AF172977
P/J5V5	−	+	I	Y	S	AF172981
P/B5	−	−	V	N	S	AF172974
P/B5H5	−	+	V	Y	S	AF172978
P/B5V5	−	−	V	N	S	AF172982
U/B5	−	−	V	N	S	AF172975
U/B5H5	−	+	V	Y	S	AF172979
U/B5V5	−	−	V	N	S	AF172983
TS/B5	−	−	V	N	S	AF172976
TS/B5H5	−	+	V	Y	S	AF172980
TS/B5V5	−	−	V	N	S/G	AF172984

[a]For a description of the various passages, see Fig. 1

[b]Capacity to infect murine CD46-positive cells as shown in Fig. 2

[c]The Edmonston strain and the wild-type passages also differed at amino acid position 187 (N/K), 211 (G/S), 243 (R/G), 247 (S/P), 249 (L/P), 252 (Y/H), 276 (L/F), 284 (L/F), 357 (V/I), 448 (H/Q), 484 (N/T), and 617 (R/G)

[d]Based on viral RNA amplified from urine of the patient

or the 4 Vero adapted virus passages downregulated CD46 in either JP or HEp-2 cells (Fig. 3, Table 1). Based on the frequency of cells that stained positive with anti-MV H monoclonal antibody (MAb) in the FACS assay, 16–88% of the JP cells were infected with the virus passages in these experiments. Previous studies have shown that infection of 1 out of 64 cells is sufficient to induce CD46 downregulation in cell cultures [20, 32]. Thus, the lack of CD46 downregulation by the virus passages of the present study was not caused by inefficient infection of the JP cells. The present demonstration of viruses that use CD46 as a receptor but do not downregulate CD46 add to the growing list of evidence for the uncoupled nature of MV CD46 receptor usage and MV-induced CD46 downregulation [3, 11, 13, 30].

Amino acid substitutions in the MV H proteins before and after adaptation to HEp-2 or Vero cells

The H proteins of the 4 wild-type passages (P/J5, P/B5, U/B5, and TS/B5) were identical to each other and to the H protein encoded by RNA in urine from the patient, except that P/J5 was heterogeneous for amino acid 41 (Table 1). Amino acid 41 belongs to the putative transmembrane region of the H protein [1, 14] and

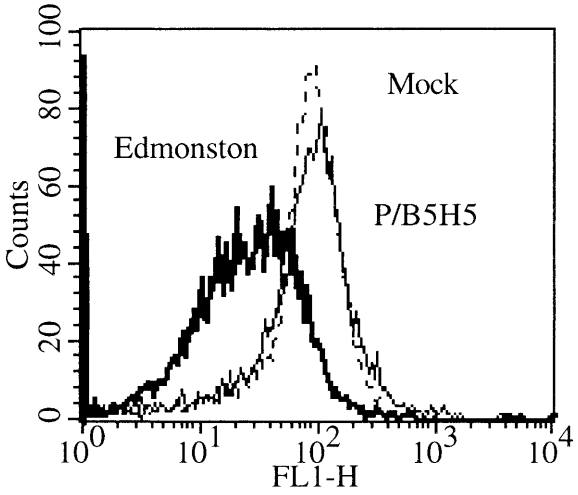

Fig. 3. Surface expression of CD46 measured by flow cytometry (FACS) in mock infected JP cells or JP cells infected with the Edmonston MV strain or the HEp-2 adapted wild-type MV P/B5H5. Similar results were obtained for all virus passages described in this study. In the present experiment, the MV H protein could be detected on the surface of 53% of the Edmonston infected JP cells and 88% of the P/B5H5 infected JP cells

is unlikely to influence the receptorbinding properties of the H protein. Thus, the H protein of the present virulent MV was unaffected by isolation on and passage in both human and marmoset B-lymphoid cells. This is in agreement with the view that B-lymphoid cells are important target cells for MV [24], that these cells express wild-type MV receptors [8], and that B-LCLs are suitable for isolation of wild-type MVs [8, 12, 19, 39].

The H proteins of the 4 wild-type passages had 13 amino acid differences to that of the Edmonston strain MV used in the present study (Table 1). These differences included amino acid 481, which in previous studies were found to be the most important determinant for the ability of the MV H protein to bind and downregulate CD46 [2, 17, 21, 41]. Like most CD46 non-downregulating MVs, amino acid 481 of the present wild-types viruses was asparagine (N), whereas the Edmonston strain had tyrosine (Y) at this position (Table 1), as do many other CD46 downregulating MVs. Among the virus passages adapted to HEp-2 or Vero cells, the viruses capable of infecting CD46-positive murine cells (P/J5H5, P/B5H5, U/B5H5, TS/B5H5, and P/J5V5) all had tyrosine at amino acid 481 of the H protein, whereas the passages that did not infect the CD46-positive murine cells (P/B5V5, U/B5V5, and TS/B5V5) still had asparagine at this position (Table 1). TS/B5V5 was heterogenous at position 546 being either serine (S) or glycine (G), whereas all other virus passages and the Edmonston strain had serine at this position. The HEp-2 or Vero cell adapted virus passages did not exhibit other nucleotide or amino acid differences to their progenitor wild-type passages. Thus, the capacity to use CD46 as receptor was perfectly correlated with tyrosine at position 481.

Amino acid 451 is also believed to influence the CD46 downregulating properties of the MV H protein. Most CD46 non-downregulating MV wild-types have glutamate at this position, whereas most CD46 downregulating MVs have valine [2, 21]. Nevertheless, the present wild-types and their derivates, none of which downregulated CD46, as well as the Edmonston strain, which downregulated CD46, all had valine at position 451.

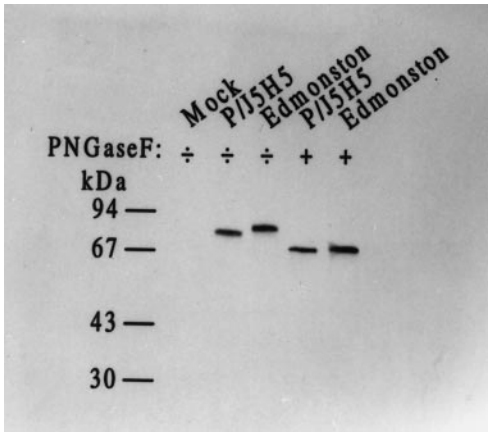

Fig. 4. H protein from the P/J5H5 MV passage compared with that of the Edmonston MV strain. H protein was immunoprecipitated from lysates of radiolabelled HEp-2 cells, incubated with or without PNGase F, and analyzed by reducing SDS-PAGE

The present virus passages all had lysine at amino acid 187, whereas the Edmonston strain and all other previously described MVs have asparagine at this position (Table 1). Therefore, the present viruses lack the asparagine-linked glycosylation site found at position 187 of almost all other MVs. The absence of this glycosylation site due to an amino acid difference at position 189 has previously been described in a Chinese wild-type MV isolate [42]. To examine whether the amino acid difference at position 187 actually influenced the glycosylation, we compared the molecular weight of the H proteins from virus P/J5H5 and the Edmonston strain. The H protein of passage P/J5H5 migrated faster than that of the Edmonston strain (Fig. 4). This difference was mainly caused by the glycosylation of the two H proteins, since they had almost the same molecular weight after incubation with PNGase F, an enzyme which removes all asparagine-linked glycans [37] (Fig. 4).

Discussion

It has been suggested that the use by MV of CD46 as receptor is a cell culture phenomenon induced by adaptation of MV to cells that do not express a wild-type receptor [8, 21]. The present study experimentally confirms that upon passage in certain cells, wild-type MV can acquire the capacity to use CD46 as receptor. In the human epithelial HEp-2 cell line, a strong selective pressure favoring adaptation to CD46 receptor usage appeared to be present. The most likely reason for this pressure is that HEp-2 cells probably do not express the receptor(s) used by wild-type MV. It is tempting to speculate that a similar mechanism acted on the Edmonston MV, when it was isolated in 1954 [10]. This isolate became the progenitor for the presently used vaccine strains (reviewed in [29]), but it still remains to be determined whether CD46 receptor usage and/or CD46 downregulation are attenuating properties.

In the present study, adaptation to CD46 receptor usage was linked to a substitution of amino acid 481 in the H protein from asparagine to tyrosine. The pivotal role of this amino acid position for binding of the H protein to CD46 has

also been demonstrated in a recombinant systems [17, 41]. In a previous study, the infectivity of a wild-type MV isolate acquired sensitivity to preincubation of target cells with anti-CD46 antibodies after adaptation to Vero cells [3]. This adaptation was connected to amino acid substitutions at position 192 and 546 of the H protein [28]. In the present study, the H proteins of 2 out of 4 of the Vero cell adapted viruses (P/B5V5 and U/B5V5) did not change relative to the wild-type progenitors, and 3 out of 4 of the Vero cell adapted viruses (P/B5V5, U/B5V5, and TS/B5V5) did not acquire the capacity to use CD46 as receptor. Nevertheless, these viruses grew to the same titers in Vero cells as the P/J5V5 passage, which did obtain the ability to use CD46 as receptor, suggesting that neither changes in the H protein or CD46 receptor usage are required for adaptation of wild-type MV to Vero cells. This raises the question which receptor(s) the present Vero cell adapted wild-type MVs use for entry into Vero cells, as Vero cells do not appear to express the wild-type MV receptors found on B-lymphoid cells [8, 17, 38]. One possibility is that certain wild-type MVs can use the monkey CD46 of Vero cells but not the human CD46 as receptor. Also, as suggested by Takeda et al. [35], changes in the H protein may not necessarily be important for adaptation of wild-type MV to Vero cells. However, even if changes in the H proteins of wild-type MVs are not required for multiplication in Vero cells, they may still give an evolutionary advantage. Thus, further passages of the present Vero cell adapted wild-type MVs on Vero cells may introduce changes in the H proteins of all the Vero cell adapted viruses. The heterogeneity of the present Vero cell adapted TS/B5V5 virus on position 546 may represent the beginning of such a change. In previous reports on adaptation of wild-type MV to Vero cells, the amino acid substitution N481Y or S546G was always observed [8, 17, 28, 34, 35].

Interestingly, the virus passages of the present study that used CD46 as receptor did not downregulate CD46, although their H proteins had valine and tyrosine at position 451 and 481, respectively. In studies employing site-directed mutagenesis and recombinant vaccinia viruses expressing MV H proteins, substitution of amino acid 451 and 481 of wild-type MV H protein to the above combination lead to CD46 downregulation [2, 21]. The reason for this apparant discrepancy is unknown, but several explanations can be suggested. The expression of the H protein alone may not always be sufficient to simulate all factors influencing MV-induced CD46 downregulation. Schneider-Schaulies et al. [30] found that the CD46 downregulating properties of MV H proteins expressed by recombinant vaccinia viruses were identical to those of the entire MVs, thus arguing against a role of other factors than the H protein. Alternatively, the difference can be related to the cell types used, as the cited vaccinia virus studies were carried out in HeLa cells. Since our HEp-2 and Vero cell adapted passages also failed to downregulate CD46 in HEp-2 cells, which like HeLa cells are adherent human epithelial cells, we do not find the different cells to be a likely explanation. Schneider-Schaulies et al. [30] also found that the cell type did not influence the CD46 downregulating properties of different MV strains. A third possibility is differences between the wild-type H proteins of the present and those of the cited studies [2, 21] at other

determinants than amino acid 451 and 481. The absence of glycosylation at amino acid 187 of the present H proteins may be such a determinant.

An alternative to the concept that wild-type MV isolates do not use CD46 as receptor was recently presented by Manchester et al. [23], who demonstrated infection with wild-type MVs in splenocytes from CD46-positive transgenic mice but not in CD46 transfected chinese hamster ovary cells. This suggested that wild-type MVs may use CD46 as receptor in certain cells. Manchester et al. [23] also proposed that isolation on B95-8 cells selects MVs that can use another receptor than CD46. The present study, however, argue against this latter suggestion, since the H genes from the viruses isolated on B95-8 cells were identical to the H gene of viral RNA in the patients urine. Thus, in the present study, isolation on B95-8 cells did not introduce changes in the receptorbinding H protein.

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