Inefficient Measles Virus Budding in Murine L.CD46 Fibroblasts

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Infection of mouse L.CD46 fibroblasts with measles virus resulted in a poor virus yield, although no defects in the steps of virus binding, entry or fusion, were detected. Two days post-infection, the level of expression of the viral F protein was found to be similar on the surface of infected L.CD46 and HeLa cells using a virus multiplicity enabling an equal number of cells to be infected. After immunofluorescence labelling and confocal microscopy, L.CD46 cells also displayed a significant increase in the co-localisation of the N protein with the cell surface H and F proteins. Immunogold labelling and transmission electron microscopy demonstrated the accumulation of numerous nucleocapsids near the plasma membrane of L.CD46 cells with little virus budding, in contrast to infected HeLa cells which displayed fewer cortical nucleocapsids and more enveloped viral particles. Purified virus particles from infected L.CD46 contained a reduced amount of H, F and M protein. Altogether, these data indicate that, in L.CD46 cells, the late stage of measles virus assembly is defective. This cellular model will be helpful for the identification of cellular factors controlling measles virus maturation.

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INTRODUCTION

Measles virus (MV) belongs to the Mononegavirales order. It encodes six structural proteins in the following order starting from the 3’ end: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin (H) and polymerase (L). The genomic RNA, encapsidated by N, and associated with the two RNA polymerase sub-units L and P, forms the ribonucleoprotein (RNP) core of the virus. The H and F proteins, with M protein underlying the phospholipid bilayer, constitute the virus envelope. The initial step of MV infection is the H-mediated binding onto the cellular receptor CD46 (Devaux et al., 1996) followed by fusion of the virus envelope with the cell membrane, a process mediated by the F protein (Wild and Buckland, 1995). The RNP released in the cytoplasm behaves as a template for the transcription of viral mRNA. As newly synthesised viral proteins accumulate, the same RNP is used as a template for the replication of negative strand genomes via the synthesis of a positive strand antigenome intermediate. A number of studies have highlighted the important role of the M protein in virus assembly due to its ability to interact both with the RNP (Hirano et al., 1993) as well as with the cytoplasmic tail of the F protein (Cathomen et al., 1998; Cathomen, Naim, and Cattaneo, 1998; Spielhofer et al., 1998). After intracellular assembly of these complexes, infectious particles are released by budding out from the cell membrane. In addition to the spread of infection via the formation of extracellular enveloped particles, viral genomes also spread from cell to cell via syncitia formation, a process induced by the interaction of the surface glycoproteins from an infected cell with CD46 on uninfected cells.

Since humans are the only natural hosts for MV, the study of MV pathogenesis has been hampered by the lack of a convenient animal model. The identification of human CD46 as a cellular receptor for MV (Dörg et al., 1993; Naniche et al., 1993) has led to the development of CD46 transgenic animals as a possible animal model. However, CD46 transgenic rodents/mice are not permissive for MV infection after intranasal, intravenous or intraperitoneal injection (Blixenkrone-Moller et al., 1998; Horvat et al., 1996; Niewiesk et al., 1997). In the case of intracerebral inoculation of MV, the expression of CD46 in transgenic mice enhances the severity of the infection (Blixenkrone-Moller et al., 1998; Rall et al., 1997), but this route of infection obviously does not reflect the normal way of measles virus transmission. The ability of cells harvested from different tissues of CD46 transgenic rodents to support MV infection in vitro was variable according to the cell type (Blixenkrone-Moller et al., 1998; Horvat et al., 1996; Niewiesk et al., 1997; Thorley et al., 1997) and the release of infectious virus was never as efficient as from human cell counterparts. MV replication was also found to be variably restricted in murine cell lines that stably expressed CD46 (Korte-Sarfatty et al., 1998; Naniche et al., 1993; Yanagi et al., 1994) as well as in human monocytes (Helin et al., 1999). Thus, MV rep-
lication involves the participation of specific intracellular host factors. Tubulin, actin and specific kinases have been implicated in virus replication (Das et al., 1995; Gombart, Hirano, and Wong, 1995; Moyer and Horikami, 1991). To gain a better understanding of the steps in MV multiplication that require host factors we have further investigated the viral life cycle in the mouse fibroblast cell line L.CD46. Our studies demonstrate a defect in a late step of virus maturation.

RESULTS

L.CD46 cells are infected by MV but produce little virus progeny

Murine L.CD46 cells were reported to be non permissive for MV infection (Naniche et al., 1993) despite being able to bind MV and to fuse into syncitia upon expression of the H and F proteins, i.e., to permit the initial step of MV replication. To delineate the step in the virus cycle impaired in these cells, the infection in L.CD46 cells was compared to that occurring in the permissive human HeLa cell line.

HeLa and L.CD46 cells were infected with the Hallé strain of MV at 0.1 and 1 m.o.i. and the kinetics of the cell surface expression of H protein was determined. Over 70% of HeLa cells became infected 1 day p.i. at 1 m.o.i. and 2 days p.i. at 0.1 m.o.i. with all cells infected the next day p.i. (Fig. 1a). The kinetics of MV H protein expression on L.CD46 was slower with around 72% of L.CD46 cells infected 2 days p.i. at 1 m.o.i. and 68% 3 days p.i. at 0.1 m.o.i. Accordingly, HeLa cells produced a higher virus yield than the L.CD46 cells (Fig. 1b-d). For example, 3 days p.i. and at 1 m.o.i., HeLa cells and L.CD46 cells released $3.1 \times 10^4$ and $3.1 \times 10^2$ TCID$_{50}$/ml respectively. Likewise, HeLa and L.CD46 infected at 1 m.o.i. produced $1.2 \times 10^5$ and $6.7 \times 10^3$ infectious cell associated virus 3 days p.i. Moreover, 2 days p.i., whereas a similar % of HeLa and L.CD46 cells were expressing H protein when infected at 0.1 m.o.i. and 1 m.o.i. respectively (see Fig. 1a), the amount of virus released from and cell-associated with L.CD46 was threefold lower (Fig. 1, compare b and d, c and e). Such a low virus progeny in L.CD46 cells was repeatedly observed in 4 different experiments, with a mean fivefold reduction in released virus and a mean 10-fold reduction in cell-associated virus when compared to HeLa cells. Similar results were observed when another MV strain, Edmonston was used. Moreover, the infection of L.CD46 with 1 m.o.i. of recombinant chimeric MV, encoding the foreign envelope G protein of the vesicular stomatitis virus (VSV) instead of the H and F glycoproteins (MGV) or a hybrid G protein with the cytoplasmic tail of the MV F protein (MG/FV) (Spielhofer et al., 1998), resulted in no detectable virus progeny and $1.8 \times 10^2$ TCID$_{50}$/ml, respectively, 6 days p.i. Similarly infected HeLa cells produced $6.7 \times 10^7$ and $3.1 \times 10^5$ TCID$_{50}$/ml, respectively. This deficiency was MV specific since infection of HeLa and L.CD46 cells with 0.1 m.o.i. of another virus belonging to the Mononegavirales order in the Rhabdoviridae family, VSV resulted in similar high virus titers 3 days p.i. ($6.7 \times 10^7$ and $1.8 \times 10^8$ TCID$_{50}$/ml, respectively).

In MV infected L.CD46 cells, the expression of the H glycoprotein in most cells indicated that the first steps of infection, virus binding, fusion and initiation of transcription and translation of at least this viral protein were occurring. Therefore, we searched for a limiting step occurring later during the virus cycle in L.CD46 cells.

Expression of virus proteins in L.CD46 cells

The expression of cell surface H and F, or that of the intracellular structural N and M viral proteins, was fol-
ollowed by flow cytometry after immunolabelling. To achieve infection of a similar number of cells and to avoid premature death of HeLa cells infected at 1 m.o.i., HeLa cells were infected with MV Hallé at 0.1 m.o.i. and LCD46 at 1 m.o.i. Two days p.i., only cells scored as positive for virus protein expression were taken into account. As shown in Figure 2, under conditions which led to about a 5–10 fold greater virus titer in HeLa cells, viral expression was as high (F protein) or even higher (N, M and H protein) in infected LCD46 cells. This indicated that both transcription and translation of every structural protein was efficient in LCD46 including the L protein because of its major involvement in virus transcription. Therefore, in LCD46 cells, the MV structural proteins were synthesised as well as or even over-accumulated as compared to HeLa cells.

N, P and RNP accumulate underneath the plasma membrane of LCD46 cells

Following translation, the viral proteins are presumably targeted to cell sites favorable for RNP assembly and virus budding. To ascertain this process, the relative distribution of plasma cell membrane H and F proteins and intracellular N, P and M proteins was determined in HeLa and LCD46 cells 2 days p.i. The extent of co-localisation of a pair of viral proteins was measured after dual immunostaining, quantification of fluorescence by confocal microscopy and calculating the percentage of green spots co-localising with red spots and vice-versa. To simplify the analysis, and since virus budding occurs close to the plasma membrane (Bohn et al., 1987; Dubois-Dalcq and Reese, 1975), only surface staining of the envelope glycoproteins H and F was followed.

To assess the validity of our approach, H protein at the cell surface was double-stained with the two non-overlapping antibodies, I29 and 48C16 (Gerlier, Varior-Krishnan, and Devaux, 1995), and the extent of co-localisation was determined. An 85% but not 100% level of co-localisation was observed between red and green spots. This under-evaluation of the co-localisation level of these two antibodies bound to the same protein was most likely attributable to different physical properties of the two fluorochromes and different affinity of the antibodies. However, despite these limitations, significant statistical differences were observed when the co-localisation of several pairs of viral proteins was studied in numerous individual HeLa cells 2 days p.i.

Six dual staining experiments carried out on infected HeLa cells are illustrated in Figure 3. The F and H proteins strongly co-localised, as shown by the yellow color, after superimposition of the two fluorochromes (Fig. 3a). The H+F/M dual staining (Fig. 3b) showed that M protein localised both under the membrane and in a diffuse manner in the cytoplasm. The H/N (Fig. 3c), F/N (data non illustrated) and H+F/P (Fig. 3d) dual staining showed, that, in contrast to M, the N and P proteins were distributed in aggregates within the cytoplasm. The N/M and N/P dual stainings (Fig. 3e, 3f) confirmed the diffuse intracytoplasmic distribution of M and distribution of N and P proteins in aggregates. Corresponding quantification of the co-localisation is given in Figure 4. More than 80% of F and H proteins co-localised (Fig. 4d, 4e), i.e., close to the expected maximum level of co-localisation. 87% of M co-localised with H and F at the membrane (Fig. 4c). 40 to 50% of F and H at the cell surface co-localised with N and P (Fig. 4d, 4e). Only 20 to 30% of N and P co-localised with cell surface F and H (Fig. 4a, 4b). About 65% of M protein co-localised with N, but only 35% of N co-localised with M (Fig. 4a, 4c). N and P were highly co-localised (>70% co-localisation of the two proteins, Fig. 4a and 4b). Surprisingly, we noticed that the percentage of co-localisation obtained was highly variable from cell to cell and the standard deviation of the mean co-localisation was around 10 to 25%. This heterogeneity of viral protein expression in each cell could explain some apparently contradictory results: for example, M highly co-localised with H+F (87%) and with N (65%) although only 15–20% of N co-localised with H and F.

Visualization of the same dual staining performed on infected LCD46 cells suggests that the labelling and intracellular distribution were comparable to HeLa cells. However, quantitative analysis of the co-localisation of virus protein pairs reveals some variations. In LCD46 cells, 31% and 40% of N co-localised with F and H, respectively (Fig. 4f). This was twofold higher than observed in HeLa cells and the difference was statistically significant. Similarly, although the differences were not statistically different because of the limited number of cells analysed, P was found to co-localise more strongly with cell surface H+F in LCD46 (44%, Fig. 4g) than in HeLa cells (27%, Fig. 4b). The co-localisation of other
pairs of MV protein did not reveal any difference with those observed in HeLa cells (compare Fig. 4 h-j with Fig. 4 c-e). These data suggest that in L.CD46, RNP tends to accumulate underneath the plasma membrane, and this was investigated using ultrastructural methods.

**L.CD46 cells are defective for measles virus budding**

The presence of MV proteins at the site of virus assembly was examined by electron microscopy coupled with immunogold labelling. Striking surface labelling of the H protein was observed in L.CD46 cells using 10 nm colloidal gold coupled anti-H antibodies (Fig. 5a and b). However, characteristic MV budding or extracellular viral particles labeled with colloidal gold were rarely detected. Cell blebbing as well as apparent release of heavily immunolabelled blebs were frequently noticed in L.CD46 cells (Fig. 5b). These blebs (400 to 800 nm) were approximately fourfold the size of enveloped MV virions apparent in HeLa cells (Fig. 5d) and they did not contain distinct nucleocapsids. Nevertheless, such blebs may account for the increase in infectivity observed after infection of L.CD46 cells if they enwrap even small amounts of nucleocapsids. Although budding of MV was defective in L.CD46 cells there was extensive accumulation of filamentous nucleocapsids as illustrated within the vicinity of the cell surface in Figure 5c. Immunolabelling with antibodies against N protein (ultrasmall silver enhanced gold particles in Figure 5c) confirmed that these inclusions were actually nucleocapsids. The electron microscopic observations conducted on L.CD46 cells contrasted sharply with the situation in HeLa cells. In the latter case the cells displayed limited cell surface

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**FIG. 3.** Co-localisation of viral proteins in infected HeLa cells after dual fluorescence immunolabelling and confocal microscopy. (a) H and F, (b) M and H+F, (c) H and N, (d) P and H+F, (e) M and N, (f) P and N. Labelling with rhodamine (top), and DTAFT or avidin-FITC (middle) and an overlay of red and green images (bottom) where co-localisation is shown by the yellow colour.
labelling with the anti-H antibodies but large amounts of heavily labeled extracellular viral particles (Fig. 5d). These extracellular particles were 100 to 200 nm in diameter and contained internal filamentous structures corresponding to the viral nucleocapsids (Fig. 5d and inset). These findings suggest that once the viral proteins reached the HeLa cell surface, budding was a rapid event whereas in L.CD46 cells, the glycoproteins accumulated at the cell surface but were not released due to a budding defect.

**FIG. 4.** Level of co-localisation of viral proteins (mean ± standard deviation) after immunolabelling and confocal microscopy. Percentage of co-localisation of N (a, f), P (b, g), M (c, h), F (d, i), H (e, j) with other viral proteins in HeLa cells (a-e) and in L.CD46 cells (f-j). For H and F, only the proteins expressed at the cell surface were detected. (n.d.) Non determined. Statistical differences observed between the two cells are indicated when significant (p values between a and f panels).
Altered protein composition of cell associated MV from L.CD46 cells

Cell associated virus accounts for the majority of MV infectivity in HeLa or LCD46 cells (Fig. 1). To examine its protein composition cell associated virus was purified on sucrose gradients and the relative amount of N, M, F and H proteins was determined using a semi-quantitative dot blot assay. The virus material from LCD46 cells contained a smaller amount of viral proteins compared to that from HeLa cells. The level of viral proteins recovered from LCD46 cells was, in arbitrary units, 3.0 for N, 0.55 for M, 1.9 for F and 1.7 for H, compared with 3.8 N, 1.6 M, 6.0 F and 3.7 H for the virus produced by HeLa cells (Fig. 6a). Since N is associated with the genome probably in a constant proportion, we considered that the amount of N reflected the quantity of ribonucleoprotein particles. Therefore, LCD46 seemed to produce a subnormal amount of ribonucleoprotein particles associated with a more than twofold lower amount of M, F and H proteins when compared to those released from HeLa cells (Fig. 6b). This indicates that in LCD46 cells there is a defect in the wrapping of the nucleocapsid by the virus enve-

FIG. 5. Immunolabelling and electron microscopy of MV infected LCD46 or HeLa cells. a and b. Two different regions of infected LCD46 cells showing the cell surface after labelling with 10 nm gold conjugated anti-H antibodies (the arrows in a and the arrowheads in b point toward surface labelling and cell blebs respectively). c. A region of an infected LCD46 cell containing an inclusion of filamentous nucleocapsids (arrows surround the region). The N protein has been immunolabelled with anti-N antibodies which were then detected with anti-mouse antibodies coupled with ultrasmall gold followed by silver enhancement (Nu: nucleus). d. A region of an infected HeLa cell labeled with 10 nm gold conjugated anti-H antibodies (the asterisks are near regions of virus budding displaying underlying nucleocapsid filaments; the arrows point toward a few of the immunolabelled viral particles released from the cell). Note the absence of labelling of cell fragments and of the cell surface. The inset in d shows one immunolabelled measles virus particle produced from HeLa cells and displaying characteristic nucleocapsid filaments. Bar scales are provided for each picture.
lope which may explain both the poor progeny of infectious virus and the budding defect seen under electron microscopy.

DISCUSSION

The data reported show that LCD46 cells are readily infected by MV since all structural proteins are expressed and accumulate but a defect in viral budding results in poor yields. The level of both cell associated and released infectious virus recovered from LCD46 was low as compared to measles virus produced in fully permissive HeLa cells. The cell associated virus from LCD46 cells displayed a reduced level of the MV envelope H, F and M components. Accordingly, MV particles were rarely seen in the vicinity of infected LCD46 cells after electron microscopic examination. In LCD46 cells, nucleocapsids accumulated beneath the plasma membrane as shown by the high level of intracellular N, M and P proteins, the increased co-localisation of these proteins with membrane H and F glycoproteins and the numerous nucleocapsids seen under the electron microscope.

The block in virus budding in LCD46 cells could not be attributed to a defect in one of the key components of the MV envelope such as the lack of M protein expression as previously observed in natural (Billeter et al., 1994) or genetically engineered (Cathomen et al., 1998) M-defective MV virus. Moreover, no obvious post-translational modification of virus proteins was detected, since the MV protein pattern upon western blot analysis was similar in LCD46 and HeLa cells (data not shown). The phosphorylation of N protein has been shown to be required for its assembly into nucleocapsids (Gombart, Hirano, and Wong, 1995) and a lack of phosphorylated N in LCD46 would have resulted in decreased N/P co-localisation and smaller amounts of intracellular nucleocapsids. Similarly, it is unlikely that a defect in the MV polymerase activity in LCD46 would contribute significantly to this budding defect. The high level of expression of all MV proteins tested, indicates that the overall transcription of the MV genome is functional in the murine cells. Furthermore, the intracellular transcription of a CAT-DI minigenome in the presence of the minimal MV polymerase complex associating N, P and L proteins (Sidhu et al., 1995) in LCD46 and HeLa cells was similar (U. Schneider, C. Buchholz and D. Gerlier, unpublished data). In an RNA dot blot using an N gene derived positive DIG-labeled riboprobe, a similar amount of RNA-genomes was found in the mouse and human cells indicating that RNA replication occurred in LCD46 cells (data not shown). We could also exclude a lower specificity of the encapsidation process in LCD46 cells, with encapsidation of irrelevant RNAs occurring (Spehner, Kirn, and Drillien, 1991) as, in this case, one would also expect a decrease in virus mRNA and protein synthesis, since MV/Paramyxoviridae transcription is tightly dependant upon genome encapsidation (Horikami and Moyer, 1995).

The budding process of enveloped viruses is not fully understood and only little knowledge is available in the case of MV. Curiously, the production of infectious virus is considerably less efficient for MV than for other Paramyxoviridae (Udem, 1984). Clearly, the M protein acts as the virus assembly organiser mainly through its interaction with the F glycoprotein cytoplasmic tail and nucleocapsids (Cathomen et al., 1998; Hirano et al., 1993; Udem, 1984).
Spielhofer et al., 1998). However, none of the MV proteins seems to have an intrinsic budding propensity as observed with VSV M and G proteins which could promote exocytosis of vesicles when expressed alone (Justice et al., 1995; Rolls et al., 1994), or as observed with retrovirus nucleocapsids that can bud in the absence of envelope proteins (Sakalian and Hunter, 1998). Interestingly, the use of recombinant chimeric measles virus MGV and MG/FV with weaker assembly/budding properties than their parental virus, resulted in the almost complete lack of cell associated (see results) or released virus progeny (data not shown) from infected murine cells. Multiplication of MGV from which MV-M protein is excluded, was the most severely inhibited by at least five orders of magnitude when compared to the virus progeny in human cells.

Quantitative confocal analyses of MV protein expression revealed a large heterogeneity in the MV protein ratios among infected human and murine cells. This was rather unexpected because of the virus gene transcription gradient (Horikami and Moyer, 1995). This lack of a tightly co-ordinated protein expression level in every infected cell could explain in part the relatively low overall yield of virus progeny observed with this *Morbillivirus* even when the target cell is permissive. The relative abundance of viral protein was clearly altered in L. CD46 and this may participate in the misassembly of virus and poor budding.

If MV has poor intrinsic budding properties, its budding should rely mainly on its ability to divert a cell pulling out mechanism such as the vectorial growth of actin microfilaments as previously proposed (Bohn et al., 1987). With respect to their lipid content and polarised budding properties, viruses can be distinguished between those which are enriched in cholesterol and glycolipids and bud at the apical site, such as influenza virus, and others which are mainly made up of phospholipids and bud at the basolateral site, such as VSV (Schellefele et al., 1999). MV has been reported to bud exclusively from the apical site and therefore would be expected to behave as the former. Furthermore, the association of actin and/or tubulin with MV nucleocapsids (Moyer and Horikami, 1991) suggests that the cytoskeleton could be one of the driving forces of the assembly and budding process of MV. Whether L.CD46 has some defect in any of these processes is currently under investigation.

In conclusion, a defect in MV budding has been found in a murine cell line expressing the MV receptor, CD46. This model will be useful to decipher the underlying mechanisms of the cell control of MV budding. Such cellular control may also operate in human monocytes and macrophages which, in *vitro*, display a post-translational defect in MV replication characterised by high intracellular expression of MV proteins and low infectious virus progeny (Helin et al., 1999).

### MATERIALS AND METHODS

#### Cells

African green monkey kidney (Vero) cells, human epithelial (HeLa) cells and murine L.CD46 (Naniche et al., 1993) fibroblasts were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 6% heat-inactivated foetal calf serum (FCS), 10 mM HEPES, 2 mM glutamine, 5 × 10^{-5} M 2-mercaptoethanol and 10 μg/ml gentamicin, at 37°C in the presence of 7% CO₂.

#### Viruses and infections

The Hallé strain of MV and the recombinant chimeric MGV and MG/FV (Spielhofer et al., 1998) were amplified on Vero cells at 33°C using very low m.o.i. to avoid the formation of defective interfering particles. After one cycle of freeze/thawing (−80°C, 37°C) of infected cells, supernatants were collected and used as virus stocks. HeLa and L.CD46 cells, plated out overnight, were infected at 0.1 or 1 m.o.i. (multiplicity of infection), for 1 h at 33°C, washed and incubated at 33°C for various periods of time.

#### Virus titration

Free virus released in the cell supernatant and/or cell associated virus were titrated. Either the supernatant from infected cells was collected or the infected cells were frozen and thawed once, then centrifuged at 400 g for 5 min to discard cell debris. The supernatants were titrated by the TCID₅₀ method (Hierholzer and Killington, 1996) on a Vero cell monolayer.

#### Antibodies

The following antibodies were used: mouse monoclonal antibody (mAb) anti-N, Cl25 and biotinylated-CI25; mouse mAb anti-P, 16AF10; mouse mAb anti-M, an equal mixture of 19CE6, 19DC5, 19EF7, XEF10 anti-M mAb; mouse mAb anti-F, Y503; mouse mAb anti-H, I29, biotinylated-48Cl6 and Cl65; monkey polyclonal serum anti-MV, BMS94. Different secondary antibodies were also used: phycoerythrin or DTAFl or rhodamine-labeled goat anti-mouse IgG (Immunotech); DTAFl-labeled goat anti-human IgG (Immunotech).

#### Flow cytometry analysis

Non confluent monolayer cells were harvested after a short trypsin treatment and washed once in DMEM-6% FCS.

For H or F cell surface detection, 2 × 10⁵ cells were incubated for 30 min at 4°C in a final volume of 60 μl of DMEM-6% FCS-0.05% NaN₃ containing an appropriate dilution of the antibody, in round bottom 96-well microplates. Cells were then washed three times by centrifugation at 280 g for 2 min, and incubated for 30 min with
50 µl of phycoerythrin-labeled or DTAF-labeled goat anti-mouse immunoglobulin. After two washes, labeled cells were fixed in 1% paraformaldehyde (PFA) diluted in ISO-TON II (Coulter) buffer. The fluorescence labelling was then measured by flow cytometry.

To detect intracellular N and M proteins, the initial steps were modified as follows. Cells were fixed 15 min with 0.6% PFA diluted in DMEM-0.05% NaN₃, then washed twice and incubated with the corresponding antibodies at room temperature in DMEM-6% FCS-0.05% NaN₃ containing 0.5% Tween 20 which permeabilized the cell membrane.

Confocal microscopy

For the viral protein co-localisation studies, dual immunostaining experiments and quantitative analyses of fluorescence after confocal microscopy were performed. Different stainings were carried out: H/H, H/F, H+/F/M, H/N, F/N, H+/F/P, N/M and N/P. Briefly, HeLa and L.CD46 cells (2 × 10⁴) were seeded on coverslips and infected or not. After 2 days, cells were fixed 15 min with 3% PFA diluted in PBS, then stained for MV antigens using an appropriate combinations of 2 antibodies (BMS94 for H+/F proteins; I29 and biotinylated-48Cl6 for H protein; Y503 for F protein; an anti-M; biotinylated-Cl25 for N and 16AF10 for P protein) and revealed using combinations of anti-mouse, human DTAF or rhodamine conjugate and avidin-FITC labelling. To avoid non-specific binding of anti-mouse, human DTAF or rhodamine conjugate and 16AF10 for P protein; an anti-M; biotinylated-Cl25 for N and H₁ protein stained in red was determined using the formula:

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\text{Protein virus composition}
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Virus particles produced by infected HeLa and L.CD46 cells were purified on a sucrose gradient and the composition of viral proteins was determined after dot blot and quantification by densitometry. Briefly, after one cy-
ple of freeze/thawing of HeLa and L.CD46 infected cells, supernatants were collected, cleared by centrifugation at 400 g for 5 min and purified on a discontinuous sucrose gradient (30–50% sucrose in PBS (w/v)) as previously described (Gerlier, Garnier, and Forquet, 1988). Gradients were centrifuged in a Beckman SW28 rotor at 25000 rpm at 4°C for two hours. The 30–50% sucrose interfaces containing the virus particles were collected and after serial dilution were absorbed onto a nitrocellulose membrane to allow detection of viral proteins N, M, F and H by dot blot. Membranes were blocked 30 min with 5% non-fat dried milk in Tris buffer saline 1% Tween 20 (TBS-T) and incubated for 30 min with appropriate specific antibodies in TBS-T 1% milk. After washes in TBS-T, membranes were incubated with secondary anti-mouse peroxidase-labeled antibody (Promega). The proteins were revealed by chemiluminescence ECL (Pierce) and the quantification was performed by densitometry.

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