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Comparison of virus production in chicken embryo fibroblasts infected with the WR, IHD-J and MVA strains of vaccinia virus: IHD-J is most efficient in *trans*-Golgi network wrapping and extracellular enveloped virus release

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Modified vaccinia virus Ankara (MVA) is an attenuated strain derived from vaccinia virus (VV) Ankara that grows efficiently in primary chicken embryo fibroblasts (CEFs) and baby hamster kidney cells only. MVA produces significantly more of the enveloped forms of VV in infected CEFs compared with VV strain Copenhagen. In the present study, production of the different infectious forms of VV was compared in CEFs infected with MVA or with two well-characterized replication-competent VV strains, WR and IHD-J. In a time-course experiment, the infectivity associated with the extracellular enveloped virus (EEV), the cell-associated enveloped virus (CEV) and intracellular mature and enveloped viruses was determined. Further, the production of the different viral forms was quantified by electron microscopy (EM). The data collectively indicate that IHD-J is most efficient in producing all of the *trans*-Golgi network-wrapped forms and releases the highest titres of EEVs into the extracellular medium, with WR being least efficient. MVA initially replicated with faster kinetics, resulting in more intracellular virus and CEVs between 8 and 24 h post-infection (p.i.). As assessed by EM, the faster growth kinetics of MVA resulted in 3·5-fold more CEVs at the cell surface at 24 h p.i., compared with both WR and IHD-J. Accordingly, we found that despite the presence of two in-frame deletions in the A36R gene of MVA, this virus was able to make actin tails in CEFs.

INTRODUCTION

Modified vaccinia virus Ankara (MVA) is a highly attenuated vaccinia virus (VV) strain that originates from VV Ankara. The latter virus was passaged more than 570 times on chicken embryo fibroblasts (CEFs), which resulted in the loss of part of its genome. Compared with VV Copenhagen, one-third of the genes, in particular host range and immune evasion factors, are deleted or fragmented (Antoine *et al.*, 1998). MVA grows efficiently in CEF and baby hamster kidney (BHK) cells only. The stage at which the morphogenesis of MVA is blocked depends on the cell type (Carroll & Moss, 1997; Drexler *et al.*, 1998). In HeLa cells, for instance, the most commonly used cell line of human origin, viral late proteins are made but virion assembly is blocked and only immature viruses accumulate (Sancho *et al.*, 2002; Sutter & Moss, 1992). Consequently, in human trials in which MVA was tested as a putative vaccine against smallpox, the virus induced an immunological response while having none of the side-effects that can occur with non-attenuated VV strains, even in high-risk groups (Mayr *et al.*, 1978; Stickl *et al.*, 1974). After the eradication of smallpox, MVA emerged as an important candidate for use as a recombinant live vaccine against other pathogens and for cancer therapy (Belyakov *et al.*, 1998; Carroll *et al.*, 1997; Drexler *et al.*, 1999; Gilbert *et al.*, 1999; Hirsch *et al.*, 1996; Schneider *et al.*, 1998; Sutter *et al.*, 1994). Because of the putative application of MVA as a live vaccine and because MVA is an attenuated VV strain, it is important to study MVA host interactions in detail and compare MVA with well-studied replication-competent VV strains. Towards this goal, we have recently compared in detail the different steps of MVA morphogenesis in HeLa cells with the replication-competent VV strain WR (Sancho *et al.*, 2002).

VV assembly is complex and results in the formation of at least two distinct infectious forms, the so-called intracellular mature virus (IMV) and the extracellular enveloped virus (EEV). It is now increasingly accepted that the IMV derives its membranes from the smooth endoplasmic reticulum or the intermediate compartment, and is composed of two tightly apposed cisternal membranes (Risco *et al.*, 2002; reviewed in Sodeik & Krijnse Locker, 2002). A percentage of the IMVs become wrapped in a double membrane derived

from the *trans*-Golgi network (TGN) to form the intracellular enveloped virus (IEV). This form has been shown to move on microtubules towards the plasma membrane, with which it fuses to release the EEV into the extracellular medium (Hollinshead *et al.*, 2001; Rietdorf *et al.*, 2001; Ward & Moss, 2001). After fusion, a variable percentage of the EEVs remains attached to the plasma membrane as cell-associated enveloped virus or CEV. The outer IEV membrane, and its associated membrane proteins, which fuse with the plasma membrane on EEV/CEV release, are able to induce the polymerization of actin tails. This results in the formation of long, plasma membrane-derived filopodia with a CEV attached at the tip (reviewed in Moss & Ward, 2001), a process that is thought to facilitate cell-to-cell spread of the virus. The latter is consistent with the fact that EEV formation has been shown to be required for virus dissemination *in vitro* and *in vivo* (Blasco & Moss, 1991; Payne, 1980). It is thought that the EEV-specific membrane protein A36R is responsible for both microtubule-dependent motility and actin tail formation (Frischknecht *et al.*, 1999; Rietdorf *et al.*, 2001), but a possible role for the F12L gene product has also been proposed (van Eijl *et al.*, 2002).

In a recent study, the production of IEV/CEV and EEV in MVA-infected CEFs was compared with infection with VV Copenhagen (Spehner *et al.*, 2000). The results indicated that MVA was much more efficient at producing all of the TGN-wrapped forms than VV Copenhagen, since MVA infection resulted in 74 % of the total virus being either IEV/CEV or EEV, while this percentage was as little as 22 % following Copenhagen infection. The authors therefore proposed that during the serial passaging of MVA in CEFs the virus had adapted to produce such high amounts of the TGN-wrapped forms. Furthermore, they concluded that the MVA strain may be particularly suitable for the targeting of foreign proteins to the surface of extracellular virions, since MVA yielded relatively high titres of EEV compared with a non-attenuated VV strain (Spehner *et al.*, 2000).

In the present study we have reinvestigated the production of the TGN-wrapped forms following MVA infection in CEFs and compared it with the two well-characterized VV strains IHD-J and WR. Our data indicated that IHD-J is best at producing all of the TGN-wrapped forms and releases the highest amounts of EEV into the extracellular medium. WR produced the lowest amounts of the TGN-wrapped forms, while the behaviour of MVA was intermediate.

METHODS

Cells, antibodies and viruses. BHK and BSC-40 cells were grown in DMEM supplemented with 1 % penicillin and streptomycin and 5 % heat-inactivated foetal calf serum. CEFs were prepared from 9–11-day-old chicken embryos and grown in MEM containing 10 % lactalbumin (Invitrogen) and 5 % BMS (Biochrom). Monoclonal antibodies to B5R were a kind gift from Gerhard Hiller (Schmelz *et al.*, 1994). Goat anti-rat–FITC and goat anti-rabbit–HRP were from Jackson Immunochemicals (Dianova). Phalloidin coupled to rhodamine was from Sigma. The polyclonal rabbit anti-vaccinia antibody was from Biogenesis. The following virus strains were used throughout this study; Western reserve (WR); International Health Department-J (IHD-J); and modified VV Ankara (MVA) passage 584, clone F6 (Mayr *et al.*, 1975). For the generation of virus stocks, HeLa cells were infected with WR or IHD-J at a low m.o.i. (0·1), and intracellular virus was isolated and semi-purified at 3 days post-infection (p.i.) as described (Pedersen *et al.*, 2000). MVA stocks were propagated in a similar way on BHK cells. WR and IHD-J were plaque titrated on BSC-40 cells and plaques were visualized at 24 h p.i. using 0·2 % crystal violet, 3 % formaldehyde in PBS. MVA was titrated on CEFs as described (Earl *et al.*, 1998).

Separation of the different viral forms by CsCl gradient centrifugation. Confluent CEFs grown in 7-175 cm^2 flasks were infected at an m.o.i. of 1 and 0·1 for 40 and 50 h, respectively. At the indicated times p.i., EEV, CEV, IEV and IMV were harvested and purified as described (Boulanger *et al.*, 2000).

EM and immunofluorescence. For Epon embedding, infected cells were fixed at the indicated times p.i. by adding an equal volume of 8 % paraformaldehyde and 0·2 % glutaraldehyde in 2× PHEM buffer (120 mM PIPES, 50 mM HEPES/KOH, pH 6·9, 4 mM $MgCl_2$, 20 mM EGTA; van der Meer *et al.*, 1999) to the medium. Cells were fixed for 2 h at room temperature, gently scraped off the dish and collected by pelleting. The fixative was removed and replaced with 8 % paraformaldehyde in 1× PHEM and the samples stored at 4 °C. A piece of the fixed pellet was transferred to another tube and fixed for 1 h at room temperature with 1 % glutaraldehyde. The fixed cells were extensively washed with water before post-fixing with 1 % osmium tetroxide. Epon embedding was then carried out as previously described (Griffiths, 1993). For the quantification, the different viral forms were counted in 50 section profiles of cells that were clearly infected. For immunofluorescence, CEFs grown on coverslips were infected at an m.o.i. of 5 with MVA and fixed at 16 h p.i. The fixed cells were labelled with anti-B5R and goat anti-rat–FITC without prior permeabilization. They were then permeabilized and counter-stained with phalloidin–rhodamine.

RESULTS

The amounts of IEV, CEV and EEV produced in CEFs infected with MVA depend on the conditions of infection

The production of IMV, EEV, IEV and CEV in CEFs infected with MVA using m.o.i.s of 1 and 0·1 at 40 and 50 h p.i., respectively, was first compared. Three different samples were collected: (i) the culture supernatant, which was expected to contain EEV; (ii) CEVs were stripped from cell surface by incubating the cells with trypsin; and (iii) intracellular virus was released by the preparation of lysates of the trypsinized cells. The different virus particles were separated on caesium chloride gradients and the peak fractions determined by A_{280} measurements. Such gradients allow the separation of IMV from all of the TGN-wrapped forms, based on their different sedimentation properties. However, they do not allow the separation of the different TGN-wrapped forms that sediment with the same density.

At 40 h p.i. at an m.o.i. of 1, the majority of virus was found in the intracellular fraction, in two almost equal peaks with densities characteristic of IMV and IEV (Fig. 1C). Negative-staining EM confirmed that the two peaks contained IMV and IEV, respectively (not shown). Substantial amounts of

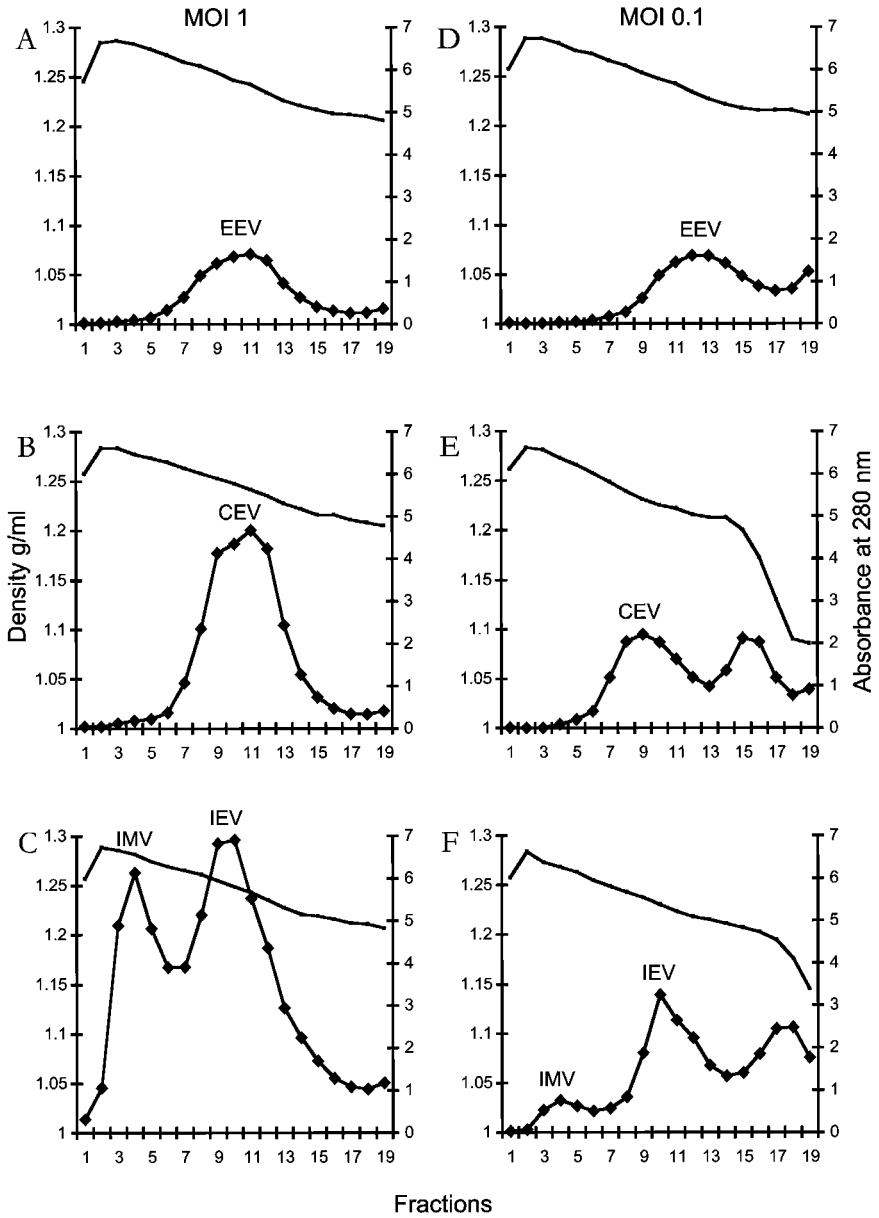

Fig. 1. Separation by CsCl gradients of the different viral forms produced in MVA-infected CEFs. CEFs were infected at an m.o.i. of 1 (A–C) and 0·1 (D–F) and EEV (A, D), CEV (B, E) and intracellular virus (C, F) were harvested at 40 h (A–C) and 50 h (D–F) p.i. The different viral forms contained in all three fractions were separated by caesium chloride gradients in SW40 tubes as described in the Methods. After centrifugation the gradients were fractionated from the bottom to the top of the tube and the fractions containing viral particles determined by A_{280} measurements (indicated on the right of each graph). The density of each fraction was subsequently determined (indicated on the left). Note that in (C) and (F) only half of the total cell lysate was used to avoid overloading of the gradient.

CEV were also detected (Fig. 1B), while the amount of virus that was released into the medium (EEV) was relatively low (Fig. 1A). The infectivity contained in the peak fractions was then determined by plaque assay and calculated as a percentage of the total infectivity measured. The majority of the infectivity was associated with IEV (46 %), followed by IMV (38 %), while only 12 % and 4 % of the total infectivity was associated with CEV and EEV, respectively.

In a similar experiment using an m.o.i. of 0·1 and 50 h p.i., the bulk of the detected virions was also cell-associated. However, in striking contrast to the experiment conducted above, the majority of the intracellular particles now appeared to peak in fractions expected to contain IEV and very few IMVs could be detected (Fig. 1D–F). To ascertain that the small amounts of IMV detected resulted from a high percentage of wrapping under these infection conditions,

the same experiment was carried out in the presence of brefeldin A, a drug known to inhibit TGN wrapping (Ulaeto *et al.*, 1995). Accordingly, we found that under these conditions no EEV or CEV was made and the bulk of the intracellular virus was found at the IMV density (not shown).

These data confirm that MVA infection in CEFs results in the production of substantial amounts of IEV, CEV and to lesser extent EEV. They also show that the amount of IMV detected intracellularly can vary significantly according to the conditions of infection (m.o.i. and the time p.i.; see Discussion).

A time-course experiment of the production of the different infectious viral forms in CEFs infected with MVA, WR and IHD-J

The production of the different viral forms following MVA infection was next compared in a time-course experiment with two other VV strains, WR and IHD-J. Whereas IHD-J infection is known to result in the production of substantial amounts of EEV, WR is thought to be a poor producer of this infectious form (Payne, 1979, 1980).

CEFs were infected with MVA, WR and IHD-J at an m.o.i. of 10 and the three different fractions described above (IMV/IEV, CEV and EEV) were harvested between 1 and 48 h p.i. All infections were carried out in triplicate and each sample was titrated in duplicate.

The results revealed that IHD-J infection resulted in the highest titres of EEV in the extracellular medium at late times of infection (Fig. 2C). MVA replicated with a faster kinetics than the two other viruses; between 8 and 24 h p.i., for instance, the infectivity associated with lysates of MVA-infected CEFs was about 1 log higher than on infection with the two other virus strains (Fig. 2A). This faster growth kinetics of MVA also resulted in 1 and 2 log higher CEV yields at 8 and 16 h. p.i., respectively, compared with WR and IHD-J (Fig. 2B). At later time-points this difference in CEV production was not seen to the same extent and at 48 h p.i. similar amounts of infectious CEV were detected for all three viruses.

Comparison of the relative amounts of infectivity at 24 h p.i. revealed that IHD-J infection resulted in the highest percentage of EEV (13 % of the total infectivity), while this percentage was only 1–2 % in MVA and WR (see Fig. 4A). The relative amount of CEV made in MVA-infected cells was lower than in the two other viruses and instead the bulk of infectivity was associated with intracellular virus (IMV/IEV). Although at first glance these data seemed to contradict the high titres of CEV detected at this time p.i., the relative distribution of the infectivity measured in the different viral forms correlated well with the experiment in Fig. 1(A–C), with 85 % and 84 % of the infectivity being associated with intracellular virus, 15 and 12 % associated with CEV and 2 and 4 % with EEV, respectively.

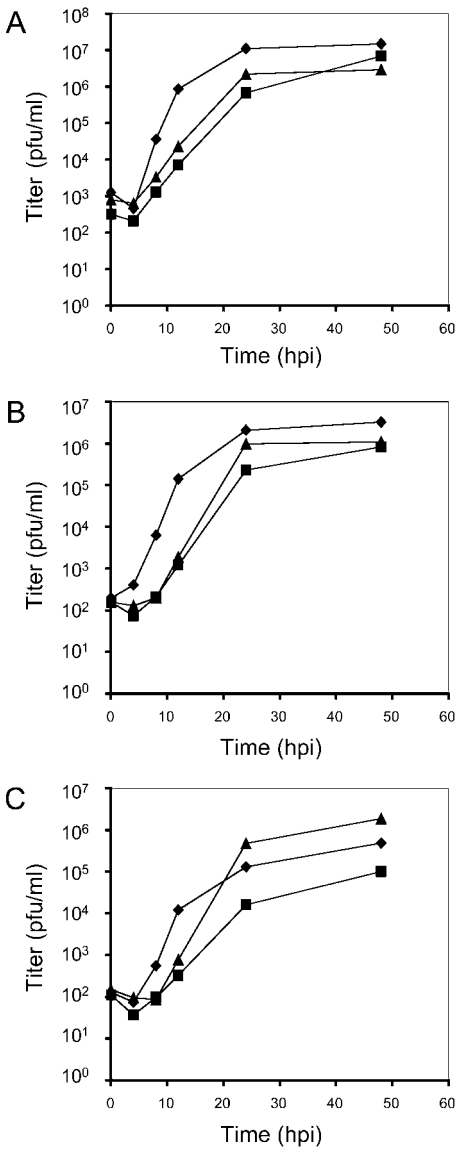

Fig. 2. Time course of the production of infectious particles in CEFs infected with MVA, WR and IHD-J. Monolayers of CEFs were infected for 1 h with MVA (◆), WR (■) and IHD-J (▲) at an m.o.i. of 10. Cells were washed and intracellular virus (A), CEV (B) and EEV (C) were harvested at the indicated times p.i. All fractions were obtained from triplicate samples that were titrated in duplicate and the average infectivity of each time-point was expressed as p.f.u. ml^{-1}.

In conclusion, our data show that in CEFs the IHD-J strain of VV is most efficient in producing EEV. MVA initially tends to produce more intracellular virus and CEV, most likely reflecting the fact that this virus has been adapted to grow efficiently in CEFs.

Electron microscopy quantification of the different viral forms

The infectivity time-course experiment did not allow us to discriminate between the amounts of IMV and IEV produced following infection of CEF cells. Therefore EM was used to distinguish them. CEFs were infected with the three VV strains at an m.o.i. of 10, fixed at 24 h. p.i. and the cells embedded in Epon. In such sections IMV could be distinguished from IEV because the latter form is surrounded by two additional membranes (compare Fig. 3A and B). CEV could be distinguished in a similar manner from IMV (the latter may remain attached to the plasma membrane after the absorption period), because the extra membrane of the CEV that surrounds the underlying IMV was readily discernable (Fig. 3B, C). Moreover, we frequently observed that CEVs apparently attached to specific sites at the plasma membrane that displayed a different electron density compared with the rest of the cell surface (Fig. 3B, C). We assume that these sites where CEVs are attached represent the outer of the two IEV membranes, which fuses with the plasma membrane on CEV/EEV release. Obviously, this EM assay did not allow us to quantify EEV.

The three different viral forms (IMV/IEV/CEV) were counted in 50 randomly chosen sections and the absolute and relative amounts were calculated. Counts of the total amounts of the different viral forms produced at 24 h p.i. revealed that MVA resulted in the highest number of intracellular viruses. In 50 cell profiles, a total of 553 IMVs and IEVs were counted in MVA-infected cells, while this was only 309 and 383 for IHD-J and WR, respectively (Table 1). The most striking difference was observed in the amounts of CEV produced. MVA infection appeared to result in about 3·5-fold more of these viruses at the plasma membrane compared with IHD-J and WR, both of which made similar amounts of CEVs (Table 1). Finally, on IHD-J infection approximately 1·5- and twofold more IEVs could be counted compared with MVA and WR infections, respectively (Table 1).

The relative distribution of the different viral forms was calculated and compared with the relative amounts of infectivity measured at 24 h p.i. These correlated well for WR and IHD-J, but to a lesser extent for MVA (Fig. 4A, B). For instance, on IHD-J infection 60 % of the infectivity was cell-associated at this time of infection, consistent with 66 % of the total particles counted by EM being IMV and IEV, while these numbers were 73 % and 70 %, respectively, for WR. Furthermore, similar percentages of CEV were measured when comparing the infectivities to the EM results (27 % and 34 % for IHD-J and 25 % and 30 % for WR; Fig. 4A, B). For MVA, the EM and plaque assay data correlated less well, since the bulk of the particles seen by EM appeared to be CEV (50 %), while the highest percentage of infectivity was found to be associated with intracellular virus (82 % IMV and IEV). The reason for this discrepancy is not clear at present. A possible explanation is that the trypsin treatment was unable to remove quantitatively all CEV from the plasma membrane. Since MVA infection results in many CEVs at the plasma membrane as assessed by EM, failure to remove all of these viruses from the cell surface perhaps resulted in relatively higher titres associated with the intracellular virus fraction.

By comparing the EM data to the infectivities measured above, a general pattern became discernable (Fig. 4A, B). IHD-J was most efficient in the production of all of the TGN-wrapped forms. At 24 h p.i., 75 % of the virus was either IEV or CEV as assessed by EM (compared with only 62 % and 36 % in MVA and WR, respectively). Moreover, this virus strain resulted in the highest amount of EEV release, as measured by plaque assays. WR appeared to be least efficient in producing the TGN-wrapped forms. This was not only demonstrated by the relatively lower titres for CEV/EEV, but also by EM showing that at 24 h p.i. more than 50 % of all particles were IMV, while this was 38 % and 25 % for MVA and IHD-J, respectively.

MVA is able to make actin tails in infected CEFs

The high amounts of CEV detected by EM at the plasma membrane of MVA-infected CEFs prompted us to ask whether these viruses were able to make actin tails as shown extensively for WR. The IEV-specific gene A36R, encoding the protein thought to be essential for both kinesin- and actin-driven motility of VV (Frischknecht *et al.*, 1999; Rietdorf *et al.*, 2001), contains two in-frame deletions of nine and four amino acids in the MVA gene compared with WR and IHD-J (Antoine *et al.*, 1998). However, these deletions do not cover the regions that have been shown to be essential for the interaction of the protein with conventional kinesin or for actin tail formation.

To detect CEVs at the plasma membrane only, cells were infected overnight, fixed and labelled without permeabilization with antibodies to B5R, an EEV-specific membrane protein. The cells were then permeabilized and labelled with rhodamine–phalloidin to visualize actin. By immunofluorescence, the entire cell surface appeared to be covered with B5R-positive CEVs (Fig. 5A), in agreement with our EM observations (see Fig. 3D). Some of these viruses were clearly present on the tip of an actin tail (Fig. 5B). Attempts to quantify how many of the CEVs were attached to an actin tail by immunofluorescence and to compare this number with WR infection were unsuccessful. We found that the MVA infection resulted in so many CEVs at the plasma membrane that the actin tails were no longer visible and could not be counted accurately.

DISCUSSION

In the present study we have reinvestigated the production of the different infectious forms of VV in CEFs by comparing MVA with two well-characterized replication-competent VV strains, WR and IHD-J. Our data showed,

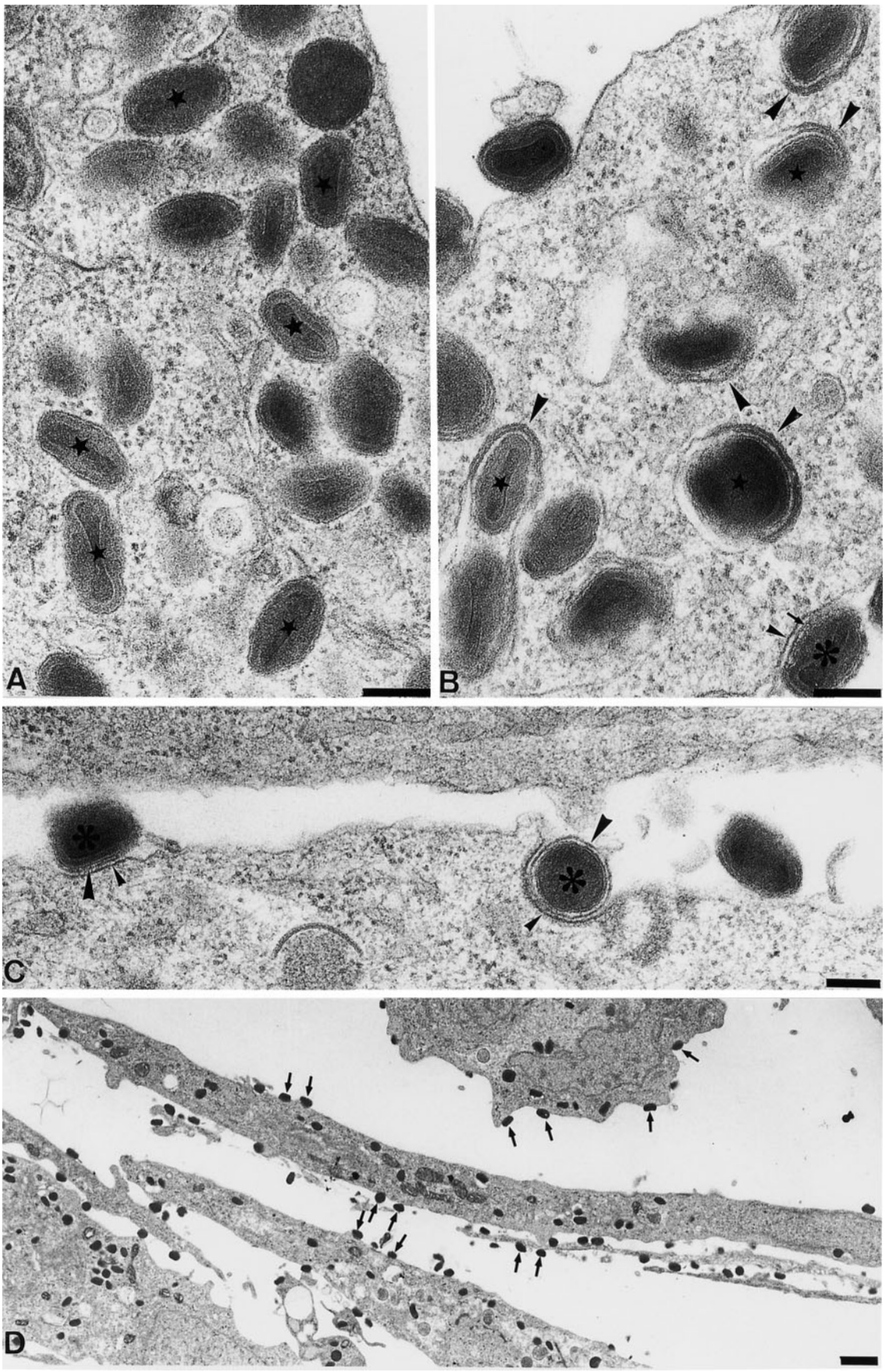
A
B
C
D

Table 1. Amounts of IMV, IEV and CEV (assessed by EM) made at 24 h p.i. in CEFs infected with WR, IHD-J and MVA

	IMV		IEV		CEV	
Strain	Total*	%†	Total	%	Total	%
WR	280	51	103	19	163	30
IHD-J	118	25	191	41	157	34
MVA	416	38	137	12	555	50

*Total amount of IMVs, IEVs and CEVs counted in 50 sections of infected, Epon-embedded CEFs infected at an m.o.i. of 10 and fixed at 24 h p.i.

†The percentage of the indicated viral form made at 24 h p.i. was calculated by dividing the number of these viral particles in 50 sections of infected CEFs by the total of all viral forms counted.

using two different assays, that IHD-J was most efficient at producing all of the TGN-wrapped forms and released the highest amount of EEVs into the extracellular medium. WR was least effective at producing all of these forms, while MVA was intermediate between WR and IHD-J. Another striking observation was that MVA infection resulted in high amounts of CEV at the plasma membrane.

IHD-J is most efficient in the TGN-wrapping process and produces the highest amounts of EEV

Studies by Payne (1979, 1980) revealed that the amount of EEV produced is cell-type and virus-strain specific. RK-13 cells, for instance, produce more EEV than HeLa cells, while the IHD-J strain of VV is significantly more efficient in EEV production than WR. Subsequent studies showed that the amount of EEV released into the extracellular medium is partially determined by whether EEV efficiently detaches from the plasma membrane after fusion of the IEV (see below; Blasco & Moss, 1992). In all of these studies typically the amount of infectivity associated with infected cells, which includes IMV, IEV and CEV, was compared with infectivity released from the cell. No distinction was made between the amounts of IMV, IEV and CEV. In the present study, a detailed comparison of the amounts of all viral forms produced in infected CEFs has been carried out for the first time, comparing three different virus strains. EM analysis suggested that these virus strains may differ not only in the amount of EEV released from the cell, but also in their efficiency of TGN wrapping. IHD-J, a strain known to release EEV efficiently, also released the highest titres of EEV from infected CEFs compared with both WR and MVA. Additionally IHD-J appeared to be most efficient in the TGN-wrapping process as assessed by EM. The reasons for these differences are not clear at present. Efficient EEV release is most likely the result of a combination of an efficient TGN-wrapping process, an efficient microtubule-dependent transport of IEVs and subsequent efficient detachment from the plasma membrane after fusion and release into the surrounding medium (see below). A surprising observation was that MVA seemed to behave in an intermediate way, since it appeared to be more efficient in IEV and CEV formation than WR, but less so than IHD-J. The reasons for this behaviour of MVA are unclear at present. We did not test how VV strain Ankara, the ancestor of MVA, behaved with respect to TGN wrapping and EEV release. A possibility is that VV Ankara behaviour is more closely related to WR but that during the course of repeated passaging and adaptation to CEFs, MVA has evolved to undergo more wrapping and CEV formation than the parental strain. That MVA has indeed adapted to CEFs was demonstrated by its initial faster growth kinetics. Furthermore, we found that MVA produced the highest titres of intracellular and extracellular virus in CEFs compared with BHK and RK-13 cells, using a recombinant MVA virus with an intact K1L gene (Staib *et al.*, 2000; data not shown).

Comparison with the results obtained by Spehner *et al.* (2000)

Our data differ significantly from those obtained in the study by Spehner *et al.* (2000) in which it was concluded that MVA produced significant amounts of all of the TGN-wrapped forms (74%), while under the same infection conditions in CEFs the Copenhagen strain of VV produced only 22% of these forms. The major difference between these two studies was the use of WR and IHD-J instead of Copenhagen. The reason for using WR and IHD-J is that these are the most commonly used laboratory strains of VV. Importantly, these viruses have been used extensively to compare the production of EEV/CEV (see for instance Blasco & Moss, 1991, 1992; Blasco *et al.*, 1993; Katz *et al.*, 1997; Payne, 1979, 1980). The data of Spehner *et al.* (2000) suggested that Copenhagen must be a VV strain that is very inefficient in the TGN-wrapping process and consequently the bulk of the virus accumulates as IMV. Another possibility that could account for the differences obtained was that their study used only one m.o.i. and one time p.i. Our study has shown

Fig. 3. Section of Epon-embedded CEFs infected with MVA at 24 h p.i. Monolayers of CEFs were infected at an m.o.i. of 10, fixed at 24 h p.i. and embedded in Epon. In (A) a collection of IMVs are shown, some of which are indicated with stars. In (B) IEVs are shown in which the underlying IMVs (stars) are clearly surrounded by two electron-dense membranes (arrowheads). Note in the bottom right of the image a CEV (small arrow) adjacent to a site at the plasma membrane that is more electron-dense than the rest of the cell surface (small arrowhead). In (C) two CEVs and one IMV are shown. The membrane surrounding the IMV can clearly be seen in the two CEVs (large arrowhead). Note that, as in (B), the plasma membrane adjacent to the two CEVs (small arrowhead) is more electron-dense than the remaining cell surface. In (D) a low magnification view of infected CEFs is given showing many CEVs at the cell surface (small arrows). Bars, 200 nm (A–C); 1 μm (D).

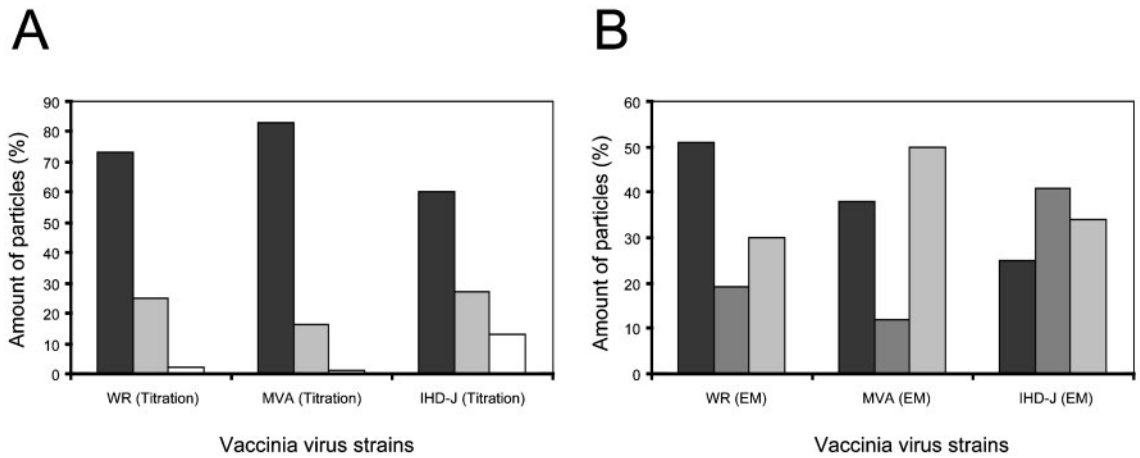

Fig. 4. Comparison of the relative amounts of the different viral forms detected by EM and plaque assay in CEFs infected with WR, IHD-J and MVA at an m.o.i. of 10 at 24 h p.i. In (A) the relative infectivities associated with intracellular virus (black bars), CEV (grey bars) and EEV (white bars) were calculated at 24 h p.i. using the values obtained in Fig. 2. In (B) CEFs were infected with WR, IHD-J and MVA and fixed at 24 h p.i. Fixed cells were embedded in Epon and the different viral forms – IMV (black bars), IEV (dark grey) and CEV (light grey) – were counted in 50 randomly chosen sections of infected cells. The values represent the percentage of the different viral forms relative to the total number of viruses counted.

that the amount of IMV and IEV that could be detected intracellularly varied considerably according to the infection conditions used (Fig. 1). It should be noted that the experiments in Fig. 1 were repeated three times with a similar outcome each time, showing that the results are a reproducible characteristic of MVA infection in CEFs. Apparently, on low m.o.i. infection, TGN wrapping of IMVs is more efficient, resulting in relatively more IEVs at late times p.i. Whatever the reason, we believe that the conclusion that MVA makes substantially more of the TGN-wrapped forms in CEFs compared with replication-competent VV strains, as indirectly implied by the study of Spehner *et al.* (2000), is no longer tenable.

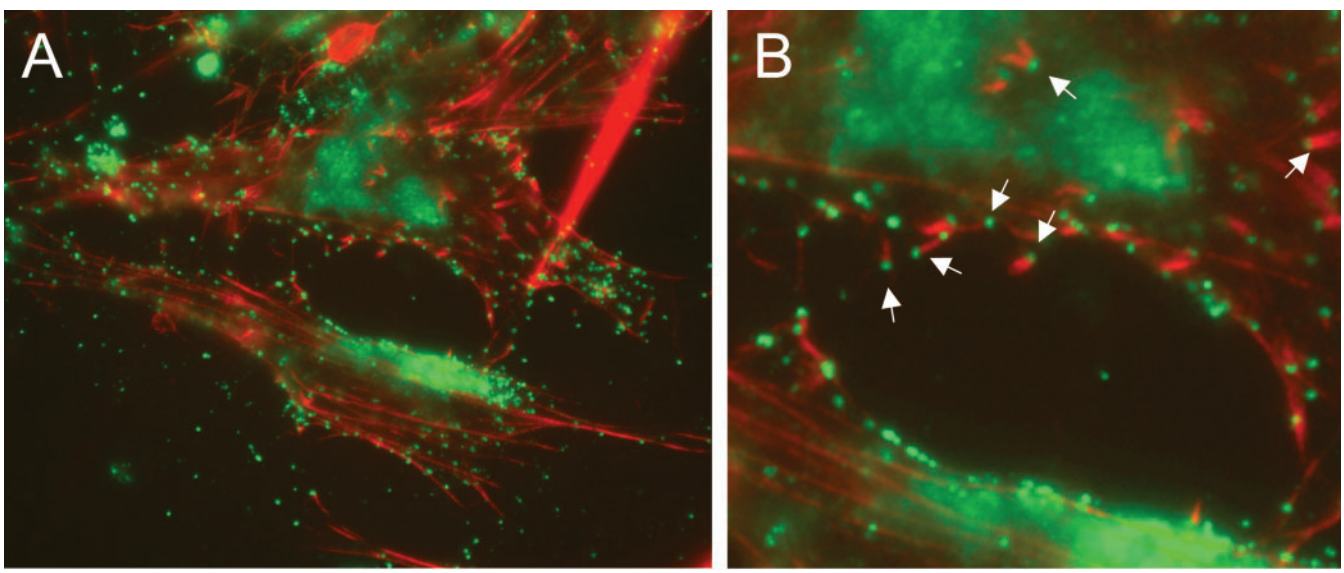

Fig. 5. In infected CEFs, MVA is able to make actin tails. CEFs were infected at an m.o.i. of 10, fixed at 24 h p.i. and labelled with anti-B5R and goat anti-rat coupled to FITC. The cells were then permeabilized and labelled with rhodamine–phalloidin. In (A) an overview is given at low magnification, showing many B5R-positive CEVs (green) on the cell surface of infected CEFs. In (B) a higher magnification of a part of the same image in (A) shows that some of the CEVs are sitting on the tip of an actin tail (arrows).

High amounts of CEVs produced by MVA – a target for foreign proteins?

The most striking observation we made was that MVA resulted in a large number of CEVs at the plasma membrane of infected CEFs. Two studies have shown that CEV adherence or release from the plasma membrane is largely determined by three EEV membrane proteins, A33R, A34R and B5R (Blasco *et al.*, 1993; Katz *et al.*, 2002). A single amino acid in the A34R gene may determine whether CEVs adhere or detach from the cell surface (Blasco *et al.*, 1993). Changing this amino acid at position 151 in the A34R gene of WR to the corresponding residue of the IHD-J gene resulted in the release of substantially more EEVs when compared with wild-type WR infection (Blasco *et al.*, 1993). A recent study by Katz *et al.* (2002) showed that specific amino acids and sequences of the A33R and B5R genes may also determine adherence of CEVs to the plasma membrane. Comparison of the A34R, A33R and B5R genes of MVA with the corresponding genes of WR and IHD-J revealed two main observations. First, the critical residue of the A34R gene that may determine CEV adherence is the same in MVA and WR. These data thus confirm previous studies showing that the amino acid residue 151 of the A34R gene may be an important factor that determines the attachment of CEVs to the cell surface. Secondly, MVA contains several point mutations in the A33R and B5R genes compared with WR and IHD-J, but none of these corresponds to the residues shown by Katz *et al.* (2002) to affect CEV adherence. Therefore, although it cannot be excluded that these point mutations in the MVA EEV proteins are critical for CEV adherence, we speculate that MVA infection results in significantly more CEV because of a combination of initial faster growth kinetics, followed by a relatively efficient TGN-wrapping process and IEV transport to the plasma membrane.

A study by Katz & Moss (1997) suggested that a chimeric human immunodeficiency virus Env protein exposed on either the surface of CEVs or EEVs was equally effective in inducing a humoral immune response. Since the present study shows that MVA infection results in efficient CEV formation, it can thus be expected that chimeric proteins targeted to the TGN-wrapping membranes (and thus to the surface of CEVs) will lead to an efficient humoral response, as proposed by Spehner *et al.* (2000). An important caveat in this reasoning, however, is that MVA assembly may be blocked in most mammalian cells and that the virus therefore fails to form IMV and thus IEV and CEV. Experiments aimed at targeting foreign proteins to the surface MVA CEV therefore require a more thorough investigation, first to determine which cells are the targets of MVA infection for its use as a potential live vaccine, and secondly to study in more detail if, and at what stage, assembly of MVA is blocked in these cells.

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