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Role of Cell-Associated Enveloped Vaccinia Virus in Cell-to-Cell Spread

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The roles of intracellular naked (INV), cell-associated enveloped (CEV), and extracellular enveloped (EEV) forms of vaccinia virus in cell-to-cell and longer-range spread were investigated by using two closely related strains of vaccinia virus, WR and IHD-J. We confirmed previous results that WR and IHD-J produced similar amounts of INV and formed similar-size primary plaques but that IHD-J produced 10 to 40 times more EEV and spread to distant cells much more efficiently than did WR. Nevertheless, cells infected with WR and IHD-J had similar amounts of CEV, indicating that wrapping and transport of WR virions were unimpaired. A WR mutant with a deletion in VP37, the major outer envelope protein, formed normal amounts of INV; however, the generation of CEV was blocked and plaque formation was inhibited. These results suggested that CEV is the form of virus that mediates cell-to-cell spread. Marker rescue experiments indicated that the differences in EEV production by WR and IHD-J were not due to sequence differences in VP37. The low amount of WR EEV could be attributed to retention of CEV on the cell membrane. In support of this hypothesis, mild treatment with trypsin released as much or more infectious virus from cells infected with WR as it did with cells infected with IHD-J. Most of the virus released by trypsin sedimented with the buoyant density of EEV. Also, addition of trypsin to cells following inoculation with WR led to a comet-shaped distribution of secondary plaques characteristic of IHD-J. These results demonstrated that the release of CEV from the cell surface was limiting for extracellular virus formation and affirmed the role of EEV in long-range spread.

Vaccinia virus, the representative member of the family *Poxviridae*, replicates in the cytoplasm of infected cells, where it orchestrates a complex and still poorly understood series of synthetic and assembly processes leading to the formation of infectious enveloped particles containing a linear double-stranded DNA genome of nearly 200,000 bp (19). Viral RNA and protein synthesis can be detected within minutes after infection, and dense granular regions of the cytoplasm, known as viral factories, become demarcated within a few hours. The first distinctive viral structures, seen by electron microscopy, are spicule-coated membrane crescents which evolve into spherical structures enclosing portions of granular material (8). As internal differentiation proceeds, the particles become dispersed in the cytosol. These infectious particles are referred to as intracellular naked virions (INV), although they are bounded by at least one membrane. Some INV become wrapped by smooth-membraned cisternae and are transported to the cell surface, where fusion of the outer wrapping membrane with the plasma membrane occurs (15, 18, 22). Infectious virions that are released into the extracellular space have an additional membrane, relative to INV, and are called extracellular enveloped virions (EEV). Enveloped virions that remain physically associated with the outside of the cell have been noted (23) and are referred to as cell-associated enveloped virus (CEV) (4). Thick actin-containing microvilli that are induced at late times during the infection have been shown to contain virus particles, possibly providing another mechanism of release from the cell (11, 13, 28).

The roles that INV, CEV, and EEV play in virus dissemination and pathogenesis are poorly understood. There are indications of different roles for INV and EEV. EEV has been shown to be important for long-range virus transmissibility (2, 5, 21). Furthermore, immunization with inactivated INV is not effective for protection in vivo, while the outer proteins of EEV appear to be capable of inducing protective immunity in experimental infections (1, 2, 6, 21, 29). Recent studies further demonstrated that formation of EEV and virus transmission were blocked simultaneously by treatment of infected cells with the drug N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine (10, 22, 26), by repressing the expression of the 14-kDa protein component of INV (25), or by deletion of the gene encoding the outer envelope protein VP37 (4).

There is a remarkable variation in the amount of EEV generated by different vaccinia virus strains. Not surprisingly, the ability of a given vaccinia virus strain to cause long-range spread of infection in vitro (as measured by the comet-shaped distribution of secondary plaques) is directly related to the amount of EEV that it produces (20, 21). However, there is no correlation between the amount of EEV produced and transmissibility to adjoining cells, since vaccinia virus strains which produce widely different amounts of EEV all give primary plaques of approximately the same size. This observation calls into question the role that EEV plays in direct cell-to-cell spread and raises the possibility that other infectious forms (INV or CEV) may be more important in mediating this process. Recent studies, however, suggest that INV are not sufficient to mediate plaque formation (4, 25, 26). This has led to the suggestion that CEV are primarily responsible for cell-to-cell virus spread (4). In this study, we have examined two vaccinia virus strains which differ greatly in their ability to produce EEV in order to clarify the contributions of INV, CEV, and EEV in cell-to-cell and long-range virus spread. The vaccinia virus strains IHD-J and WR were chosen because both were derived by passage of the same New York City Board of Health strain. IHD-J produces large amounts of EEV, while WR produces very little (21). Here, we provide evidence that

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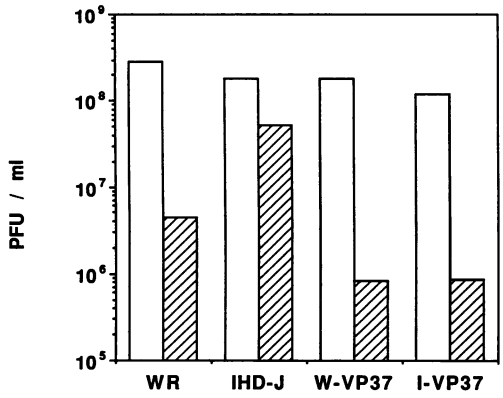

FIG. 1. Extracellular virus production by parental and mutant viruses. RK13 cell monolayers were infected at a multiplicity of 10 and harvested at 48 h after infection. Virus from the cell lysates (open bars) and culture medium (hatched bars) were titrated by plaque assay in BSC-1 cells. Plaques were counted after 2 days for WR and IHD-J and after 7 days for W-VP37 and I-VP37.

plaque formation by WR, like that by IHD-J, is dependent on synthesis of VP37 and the formation of wrapped virions. Moreover, the low production of EEV by WR is not a consequence of diminished wrapping of INV or a decreased fusion with the plasma membrane but rather is due to inefficient release of CEV from the cell surface. These results emphasize the importance of CEV in cell-to-cell transmission and of EEV in longer-range spread.

MATERIALS AND METHODS

Cells and viruses. Vaccinia virus strains WR (ATCC VR-119) and IHD-J (from S. Dales) were routinely propagated, and titers were determined in BSC-1 cells (9). Previously described mutant vRB10 (named here I-VP37) is a vaccinia virus IHD-J mutant in which most of the coding sequence of the VP37 gene was deleted (4). The deletion spans about 93% of the VP37 coding sequence (all except 28 and 35 nucleotides from the 5′ and 3′ ends, respectively, of the coding region of the gene). The same plasmid used to delete the VP37 gene from the IHD-J strain of vaccinia virus was used here to create the same deletion in the WR strain. This mutant virus was called vRB12 or W-VP37.

Marker rescue. Monolayers of CV-1 cells were infected with a vaccinia virus deletion mutant (vRB10 or vRB12) at a multiplicity of 1. A 1.9-kb fragment containing the entire F13L gene and flanks of approximately 400 bases was amplified by using the primers 5′-GGACATGCTTATG TACGTAGAAGAA-3′ and 5′-CGTTCTAAAGCTAGTGC TATATCTCCC-3′ and 1 μg of viral DNA template in a polymerase chain reaction (PCR) of 20 cycles (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min). The PCR product was precipitated with calcium phosphate (9) and used to transfect a monolayer of CV-1 cells that had been infected at a multiplicity of 1 with vaccinia virus deletion mutant W-VP37 or I-VP37. At 48 h postinfection, the cells were scraped, harvested, and freeze-thawed three times. The presence of viruses with wild-type phenotype was checked by plaquing in fresh BSC-1 monolayers.

Electron microscopy. For transmission microscopy, rabbit kidney RK13 cell monolayers in T75 flasks were infected at

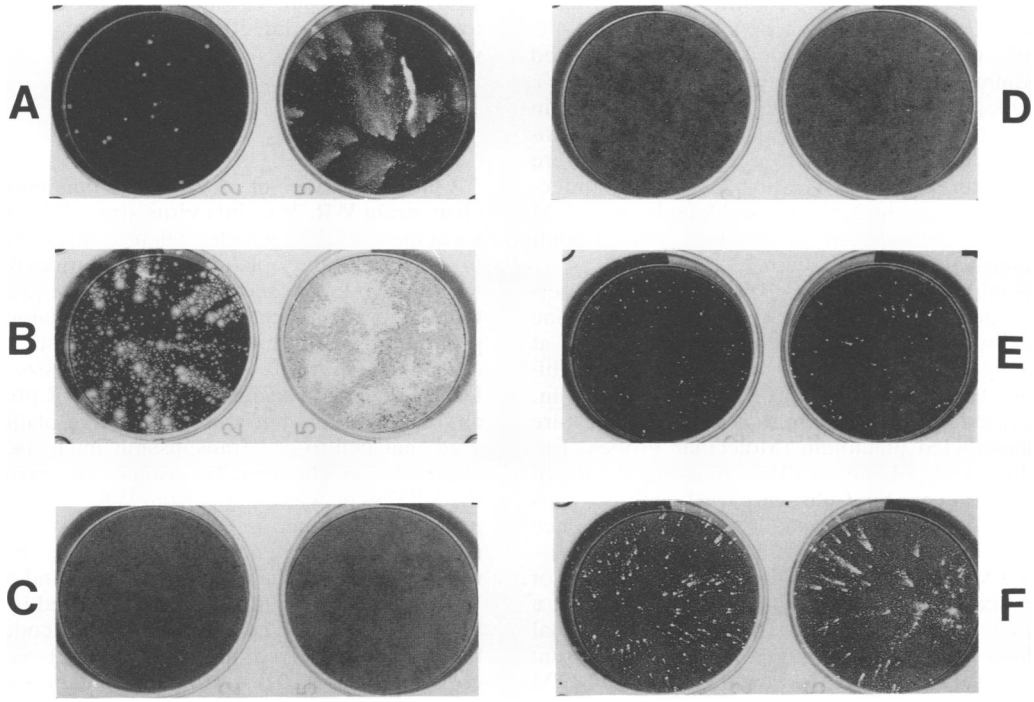

FIG. 2. Visualization of plaques formed by parental and mutant viruses. Monolayers of BSC-1 cells were infected, overlaid with liquid medium, stained with crystal violet at various times, and photographed. The time of staining and virus strains (left and right) are as follows: (A) 2 days, WR and IHD-J; (B) 4 days, WR and IHD-J; (C) 2 days, W-VP37 and I-VP37; (D) 4 days, W-VP37 and I-VP37; (E) 7 days, W-VP37 and I-VP37; (F) 10 days, W-VP37 and I-VP37.

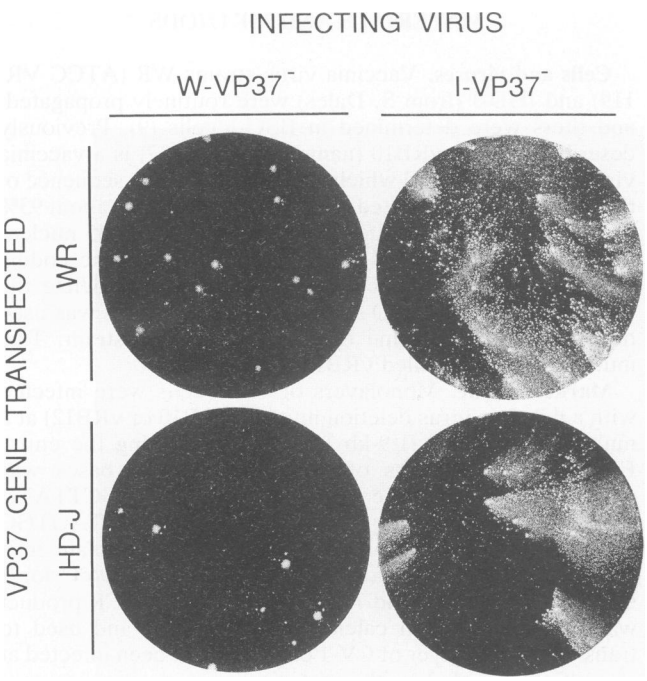

FIG. 3. Marker rescue of VP37 deletion mutants. Cells infected with either W-VP37 or I-VP37 were transfected with a fragment containing the VP37 gene that had been PCR amplified from either WR or IHD-J DNA. Dilutions of the progeny virus were then inoculated onto BSC-1 monolayers, which were stained after 2 days. Photographs are shown.

a multiplicity of 10 with wild-type or mutant viruses. At 16 h after infection, the cells were fixed by the addition of 2.5% glutaraldehyde in 0.13 M sodium phosphate (pH 7.4) at 4°C for 2 h. The cells were scraped, pelleted, and finally washed in 0.13 M sodium phosphate. Cells were postfixed in 1% osmium tetroxide, stained with uranyl acetate, embedded in Spurr's resin, sectioned, and examined in an electron microscope. For scanning electron microscopy, the cells were grown and infected on round coverslips. At 16 h postinfection, the cells were fixed in 2.5% glutaraldehyde in 0.13 M sodium phosphate, dehydrated in ethanol, critical-point dried, and coated with gold palladium.

Fluorescence microscopy. Infected BSC-1 cells, on coverslips, were washed twice with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde for 10 min at 37°C. After being washed with PBS, cells were permeabilized by treatment with 0.1% Triton X-100 in PBS for 5 min. Cells were then incubated for 20 min at room temperature with rhodamine-labeled phalloidin (Molecular Probes, Eugene, Oreg.) dissolved (5 U/ml) in PBS containing 0.1 mg of bovine serum albumin per ml. Cells were washed three times with PBS and dried before examination in a fluorescence microscope.

Release of CEV with trypsin. Confluent monolayers of BSC-1 cells in six-well plates were infected with virus at a multiplicity of 10. Virus was adsorbed to the cells in minimal essential medium (MEM) containing 2.5% fetal calf serum for 2 h at 37°C. After removal of the inoculum, 2 ml of MEM (no serum) was added, and the incubation continued. After 24 h, the culture medium was removed and clarified by low-speed centrifugation for subsequent titration of extracellular virus. The monolayers were washed with 2 ml of trypsin and incubated at 37°C for 1 h with MEM containing indicated concentrations of trypsin (Worthington Biochemical Corp.). After the incubation period, the medium was harvested, clarified by low-speed centrifugation, and treated with trypsin (125 μg/ml) at 37°C for 30 min, and the titer was determined.

For density analysis of virus, confluent monolayers of RK13 cells in six-well plates were infected with virus at a multiplicity of 10. After the adsorption period, the virus inoculum was removed, and 2 ml of MEM (no serum) was added. At 8 h postinfection, the medium was replaced by complete MEM containing 25 μCi/ml of [^{35}S]methionine, and the incubation continued until 24 h postinfection. Extracellular virus was then recovered from the medium after clarification by low-speed centrifugation. The cells in the monolayer were treated for 1 h with 1 μg of trypsin per ml in MEM. At the end of the trypsin treatment, the medium was removed and clarified. The cells in the monolayer were suspended in MEM, centrifuged, resuspended in 7 ml of 10 mM Tris-HCl (pH 9), Dounce homogenized, and clarified. The viruses recovered (extracellular virus, virus liberated by trypsin, and cell-associated virus) were pelleted through a cushion of 4 ml of 36% sucrose and then loaded on a gradient made by overlaying 2.5, 3.75, and 5.0 ml of CsCl solutions with densities of 1.30, 1.25, and 1.20 g/ml, respectively. Centrifugation was carried out in an SW41 rotor at 32,000 rpm for 60 min at 15°C. Fractions (15 drops) were collected from the bottom of the tube, and aliquots of each fractions were assayed for radioactivity.

Plaque assay in the presence of trypsin. Monolayers of BSC-1 cells in six-well plates were infected with dilutions of virus in 1 ml of MEM. After 1 h of adsorption at 37°C, the virus inoculum was removed, fresh MEM was added, and the incubation continued at 37°C. At 5 h postinfection, the medium was replaced with MEM containing indicated amounts of trypsin. The incubation proceeded at 37°C until a total of 48 h postinfection, when monolayers were stained with crystal violet.

RESULTS

Characterization of a VP37 deletion mutant of vaccinia virus strain WR. Vaccinia virus strain IHD-J produces large amounts of EEV which, when a liquid overlay is used, produce secondary plaques in a cometlike distribution from the primary focus (see Fig. 2 and 3). We previously showed that deletion of the gene encoding the major outer envelope protein (VP37) from IHD-J led to a drastic reduction in the amount of EEV produced and in the size of plaques (4). However, since vaccinia virus strain WR produces very low amounts of EEV but still makes large plaques, we considered that cell-to-cell transmission might occur through an alternative mechanism. To evaluate this possibility, most of the VP37 gene was deleted from WR by using a *gpt* selection protocol, described in detail for construction of the IHD-J mutant, that does not require plaque formation by the mutant virus (4). The genome structure and purity of the WR VP37 deletion mutant were demonstrated by Southern blotting (not shown). The WR mutant was code named vRB12 but will be referred to in this report as W-VP37. The corresponding IHD-J strain VP37 mutant has the code name vRB10 and will be called I-VP37.

The amounts of INV and EEV produced by W-VP37 and I-VP37 and their parent strains were compared. All four viruses produced similar amounts of INV (Fig. 1). As

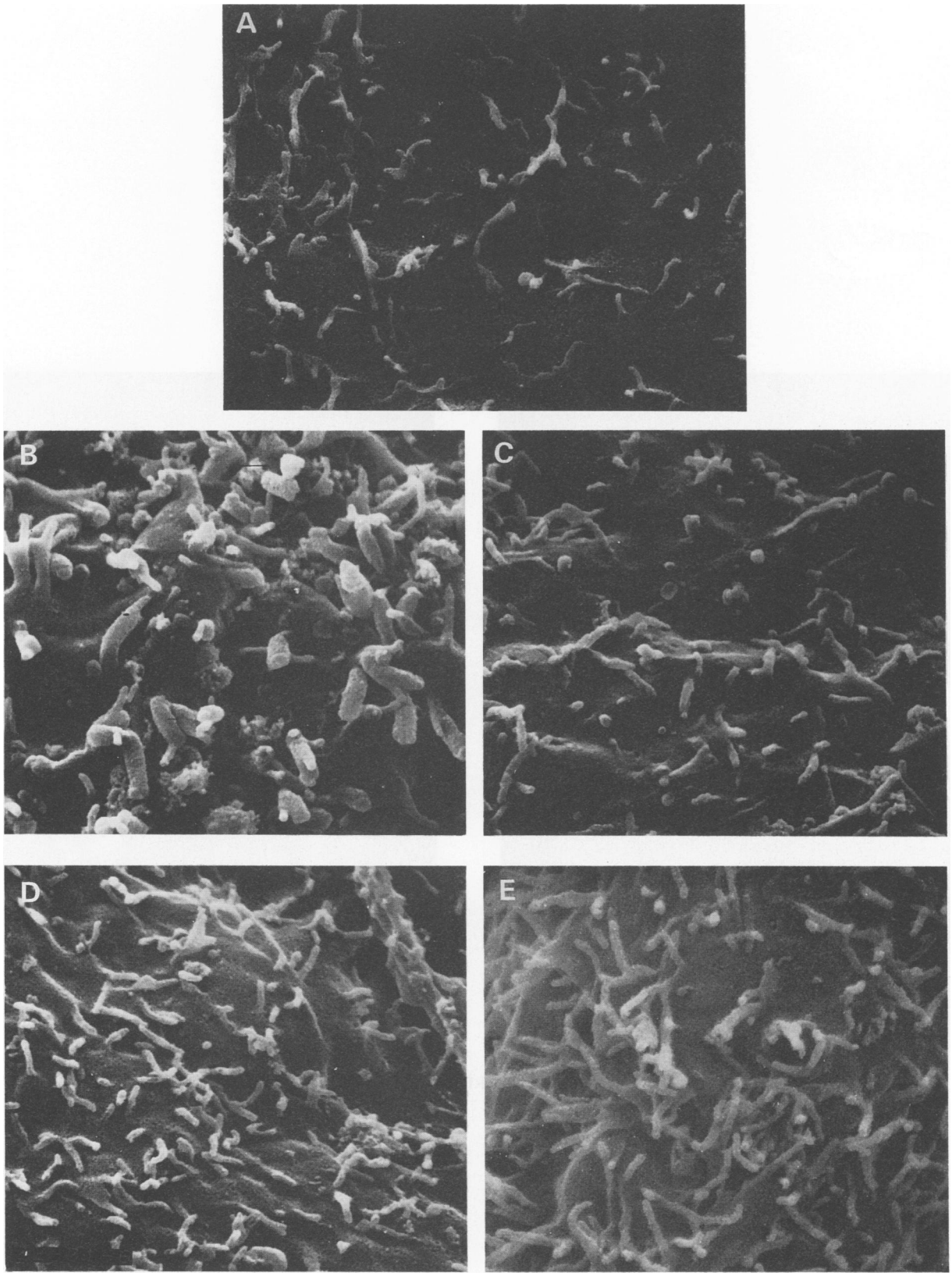

FIG. 4. Scanning electron microscopy of RK13 cells infected with no virus (A), WR (B), IHD-J (C), W-VP37 (D), or I-VP37 (E). Cells were fixed at 16 h postinfection. Note the presence of infection-induced microvilli in WR-infected cells. Magnification, ×9,500.

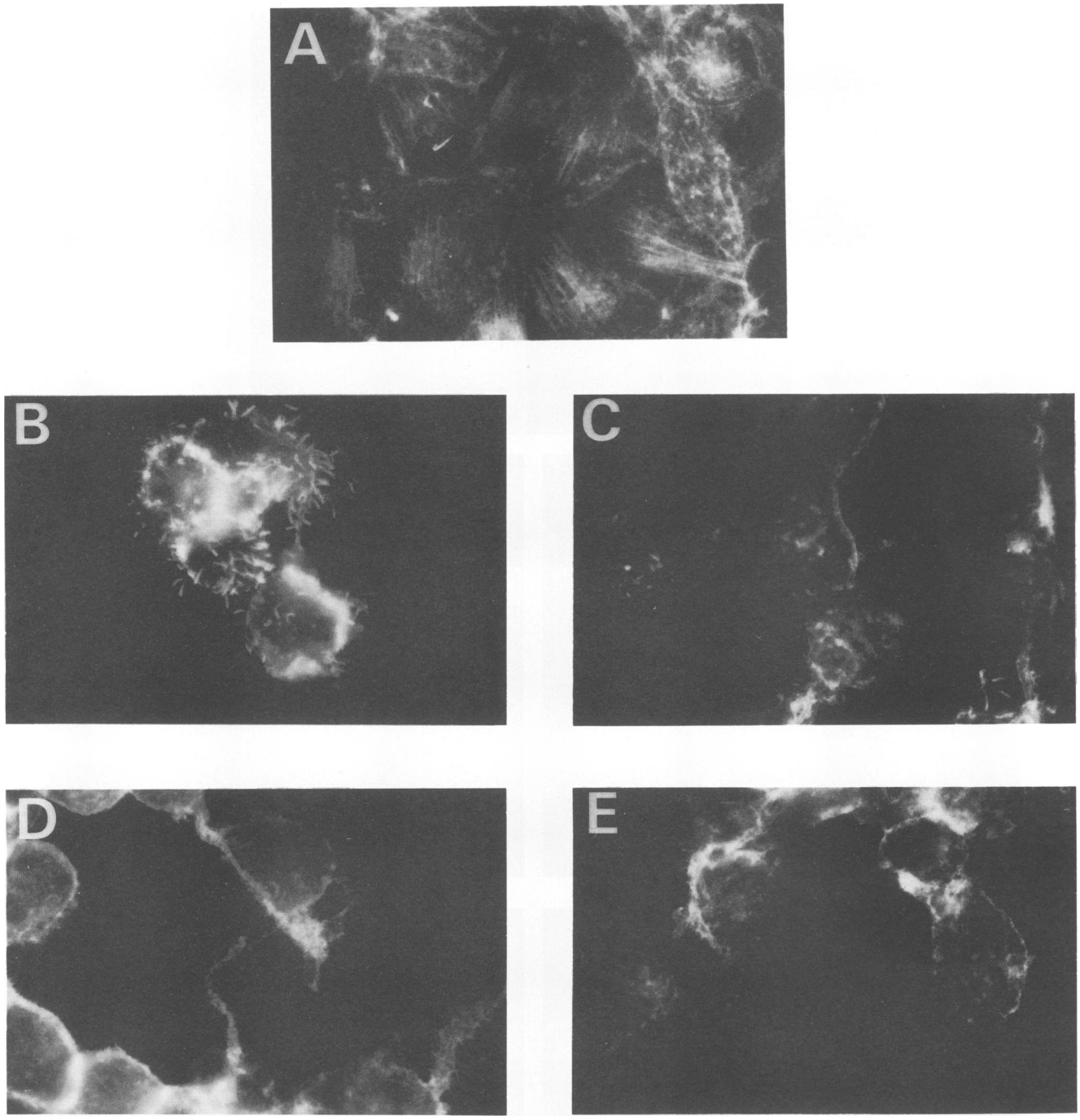

FIG. 5. Staining of filamentous actin with rhodamine-phalloidin. RK13 cells were infected with no virus (A), WR (B), IHD-J (C), W-VP37 (D), or I-VP37 (E).

expected, IHD-J produced 20- to 40-fold more EEV than did WR. However, W-VP37 produced even lower amounts of EEV than did its parent, indicating that expression of VP37 is still an important factor in the formation of EEV by WR. The amounts of EEV produced by W-VP37 and I-VP37 were similar.

We confirmed the difference in the appearance of plaques formed under a liquid overlay by the WR and IHD-J strains of vaccinia virus. After 2 days, WR plaques appeared uniformly round (Fig. 2A, left). At this time, the primary IHD-J plaques were similar in size but had associated numerous secondary plaques caused by the release of EEV that gave the plaques a cometlike shape (Fig. 2A, right). After 4 days, satellite plaques were detected with WR (Fig. 2B, left), but by this time the spread of IHD-J had destroyed the entire monolayer (Fig. 2B, right).

Since the low amount of WR EEV is reflected by the absence of comets rather than the size of primary plaques,

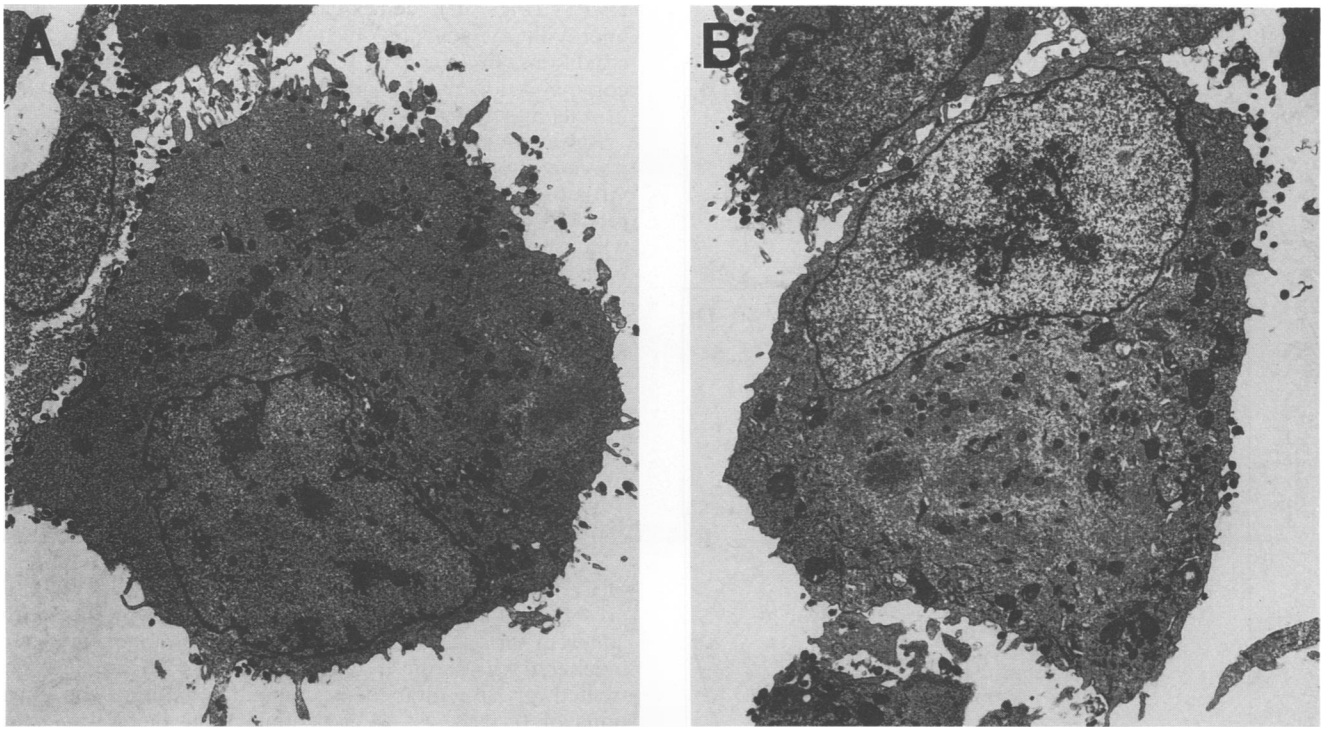

FIG. 6. Transmission electron microscopy. RK13 cells were fixed at 16 h after infection with WR (A), or IHD-J (B). Note the virus particles attached to the plasma membrane in both panels. Magnification, ×4,740.

one might have thought that W-VP37 would still form primary plaques despite the further reduction of EEV. This was not the case, however. After 2 days, neither W-VP37 (Fig. 2C, left) nor I-VP37 (Fig. 2C, right) formed macroscopic plaques. Thus, deletion of VP37 interfered with plaque formation by WR as it did for IHD-J. With longer times, small comets were seen with both W-VP37 and I-VP37 (Fig. 2D to F), evidently because the mutant viruses still produced very low amounts of extracellular virus (Fig. 1).

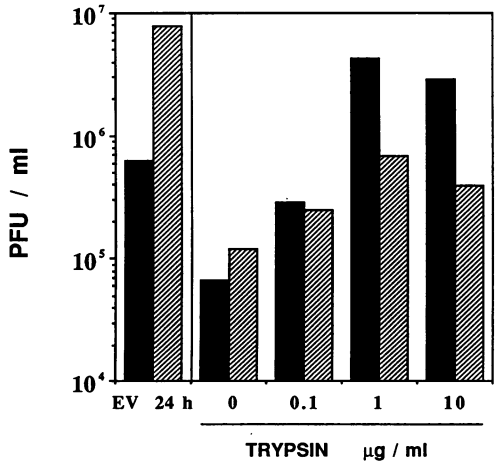

FIG. 7. Release of CEV with trypsin. BSC-1 cells were infected with vaccinia virus WR (black bars) or IHD-J (hatched bars) and incubated for 24 h. The virus present in the medium (EV 24 h) and the virus released to the medium by subsequent 1-h incubation with different concentrations of trypsin are shown.

Marker rescue of the plaque-forming phenotypes of WR and IHD-J. Since VP37 plays a critical role in the production of EEV and the formation of plaques, differences in the amount or sequence of the protein could be responsible for WR and IHD-J strain differences. Previous studies, however, have demonstrated that large amounts of VP37 are made by WR (12, 14). Furthermore, the WR and IHD-J VP37 genes differ by seven nucleotide substitutions, only one of which would result in an amino acid change (26). Nevertheless, to test whether the difference in VP37 could account for the characteristic phenotypes of the virus strains, we carried out marker rescue experiments. The two mutants W-VP37 and I-VP37 contained only 28 and 35 residual nucleotides of the VP37 gene flanking the inserted *gpt* gene. Cells that had been infected with either W-VP37 or I-VP37 were transfected with DNA segments containing the entire VP37 coding region and about 400-nucleotide flanks that had been PCR amplified from WR or IHD-J DNA. In the vast majority of cases, therefore, recombination is likely to occur outside the VP37 gene or its promoter sequences. The progeny virus were then plated on BSC-1 cells, and the plaque morphologies were determined. If a difference determining the comet formation lies within the transfected fragment, we should be able to transfer that characteristic to at least some of the progeny viruses. As expected, both versions of the gene were able to restore the plaque-forming phenotype to the mutant viruses. Incorporation of the IHD-J VP37 gene into the W-VP37 genome resulted in round plaque-forming recombinant viruses; conversely, incorporation of the WR VP37 gene into I-VP37 gave comet-forming recombinant

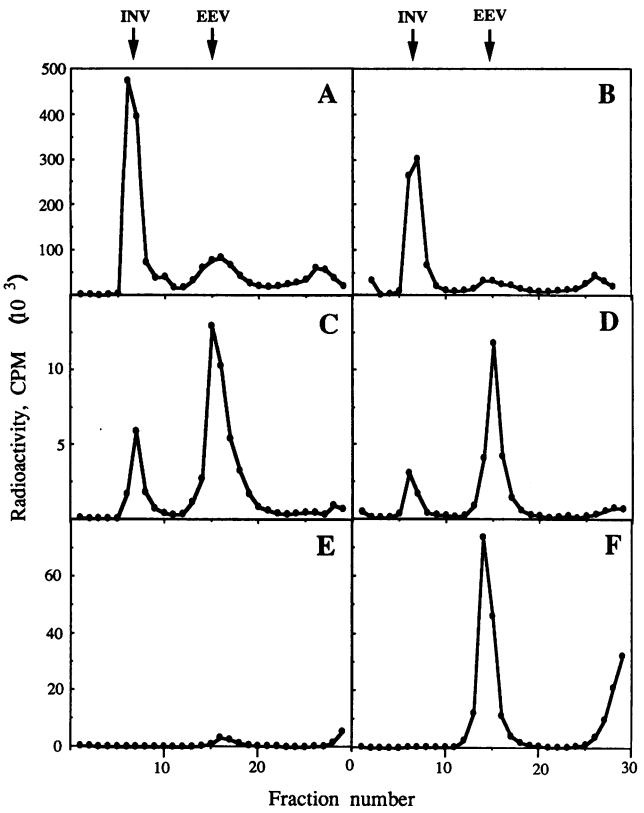

FIG. 8. Buoyant density centrifugation. Cell-associated virus (A and B), virus released to the medium by trypsin (C and D), and extracellular virus (E and F) were isolated from WR-infected cells (A, C, and E) or IHD-J infected cells (B, D, and F) and centrifuged in CsCl gradients as described in Materials and Methods. The positions corresponding to the density of INV and EEV are indicated.

viruses (Fig. 3). Thus, the strain variation must be due to differences in one or more other genes.

Virus-induced microvilli. Two mechanisms have been proposed for the exit of vaccinia virus virions from infected cells. One involves the fusion of double-wrapped virions with the plasma membrane, and the other involves release of virions from the tips of enlarged microvilli. The latter was suggested by high-voltage electron microscopic examinations of cells infected with vaccinia virus strain WR (28). The effect of the deletion of the VP37 gene on the formation of virus-induced microvilli was determined by scanning electron microscopy. Cells infected with WR showed numerous thick microvilli (Fig. 4B) that were absent from uninfected cells (Fig. 4A). The enlarged microvilli were not discerned in cells infected with W-VP37 (Fig. 4D) or I-VP37 (Fig. 4E), indicating that the presence of the protein is required for induction of the microvilli. Unexpectedly, very large microvilli were rare in RK13 cells infected with IHD-J (Fig. 4C).

Virus-induced microvilli have also been visualized by fluorescence microscopy using antibody to actin (13) or rhodamine-labeled phalloidin (3), which binds specifically to actin filaments. In uninfected cells, phalloidin labels stress fibers that constitute the actin cytoskeleton (Fig. 5A). In contrast, actin bundles projecting out of the cell were seen in cells infected with WR (Fig. 5B). However, these bundles were not seen in cells infected with W-VP37 (Fig. 5D) or I-VP37 (Fig. 5E), consistent with our inability to detect microvilli by scanning electron microscopy under these conditions. In agreement with the scanning electron microscopy, very low numbers of large actin bundles were present in cells infected with IHD-J (Fig. 5C).

Release of CEV by trypsin treatment of infected cells. In a previous study (4), we pointed out the presence of wrapped virus on the outer surface of cells infected with IHD-J and referred to them as CEV to distinguish them from EEV, which by definition are free in the medium. Production of both CEV and EEV was blocked in cells infected with I-VP37 (4). Despite the large difference in EEV production by IHD-J and WR, transmission electron micrographs did not suggest a similar difference in CEV (Fig. 6). Indeed, although there was some variability in individual sections, there were typically more CEV in cells infected with WR than in cells infected with IHD-J. As expected, however, CEV production was blocked in cells infected with W-VP37 (data not shown), as previously reported for I-VP37.

The presence of large numbers of CEV on the surfaces of cells infected with WR led us to consider that differences in EEV production might be related to the efficiency of release of CEV into the medium. To quantitate the amount of CEV present at late times in infected cells, we tried to liberate the adherent virions by proteolysis. The cell monolayers were incubated with serum-free medium and at 24 h after infection treated for 1 h with medium containing different concentrations of trypsin. At 24 h postinfection, there was about 12-fold more IHD-J than WR virus in the culture medium (Fig. 7, EV 24 h). Large amounts of virus were released into the medium with trypsin both from IHD-J- and from WR-infected cells (Fig. 7) but not from I-VP37- or W-VP37-infected cells (data not shown). Interestingly, trypsin released more virus from WR-infected cells than from IHD-J-infected cells, thus reversing the normal situation. Actually, more WR virus was released during the 1-h incubation period with 1 μg of trypsin per ml than had been liberated as EEV during the previous 24 h without trypsin. The accessibility of the virus to trypsin also suggested that the CEV were outside the cell membrane. This result suggests that the two virus strains produce similar amounts of CEV and that the difference in EEV reflects the efficiency with which CEV is liberated to the medium.

Characterization of CEV by buoyant density centrifugation. Previous studies have shown that INV has a higher buoyant density than does EEV (20, 24). We characterized the virus from cells that had been incubated in the presence of [^{35}S]methionine during the infection period and treated after 24 h with 1 μg of trypsin per ml for 1 h. The labeled virus in the medium, the virus liberated by trypsin, and the virus remaining cell associated after trypsin treatment were analyzed by centrifugation in cesium chloride gradients. Virtually all of the virus present in the medium of cells infected with IHD-J sedimented as a single peak with a buoyant density of about 1.24 g/ml, which is characteristic of EEV (Fig. 8F). Despite the paucity of virus found in the medium of cells infected with WR, its buoyant density was also that of EEV (Fig. 8E). The majority of the cell-associated virus sedimented with a density of 1.27 to 1.28 g/ml, which corresponds to INV (Fig. 8A and B). In both WR- and IHD-J-infected cells, a fraction of the cell-associated virus (20 to 30%) sedimented at a density of 1.22 to 1.24 g/ml and probably represents intracellular virus wrapped with membranes. Analysis of the virus liberated with trypsin showed that approximately 80% of the virus particles sedimented at the EEV density (Fig. 8C and D).

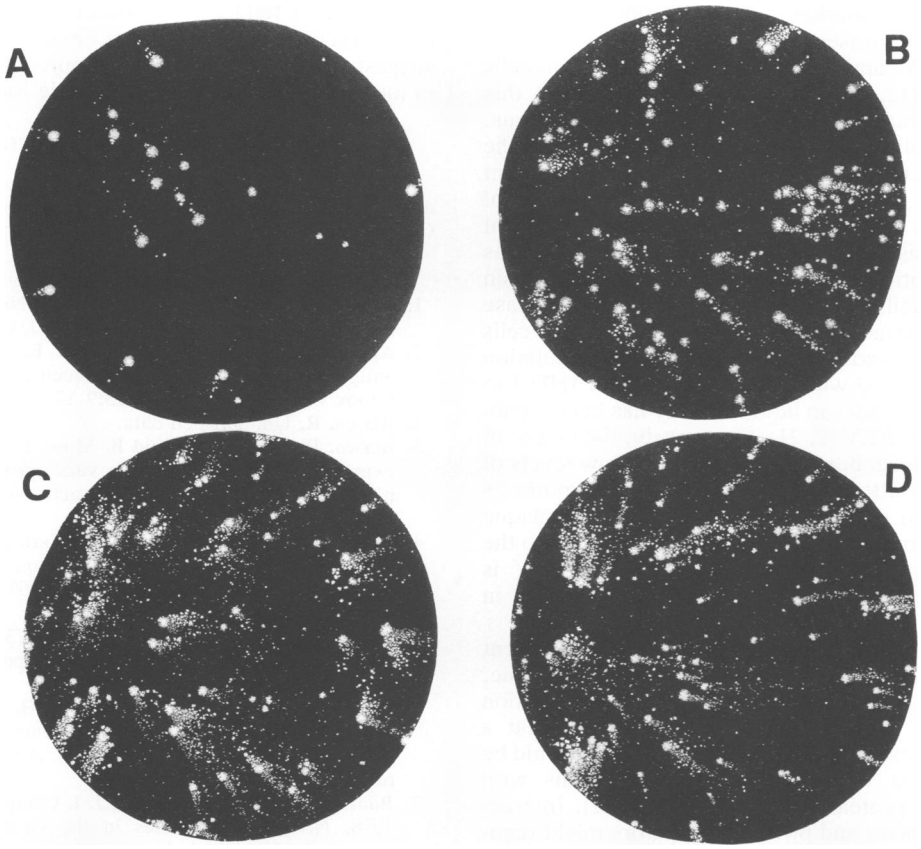

FIG. 9. Comet formation in the presence of trypsin. Monolayers of BSC-1 cells were infected with WR virus and incubated in MEM containing no serum. At 5 h after adsorption, medium was replaced with medium containing no trypsin (A) or trypsin at a concentration of 0.1 μg/ml (B), 0.5 μg/ml (C), or 1 μg/ml (D). Two days after infection, the monolayers were stained with crystal violet and photographed.

Comet formation in the presence of trypsin. The ability of IHD-J to form a comet-shaped distribution of secondary plaques has been correlated with the large quantity of EEV produced. Similarly, the inability of WR to form comets has been attributed to their low levels of EEV. After finding that trypsin could release significant amounts of CEV from cells infected with WR, we thought that WR might be able to form comets if trypsin was included in the liquid overlay. To test this possibility, BSC-1 cells were inoculated with WR and the monolayers were overlaid with serum-free medium. At 5 h after infection, the medium was replaced with fresh medium containing various concentrations of trypsin. The presence of trypsin at late times clearly induced the formation of cometlike plaques by WR virus (Fig. 9). This observation correlated well with the increased virus released by trypsin (Fig. 7 and 8) and reinforced the idea that the phenotypic differences between WR and IHD-J occur at the level of CEV release.

DISCUSSION

The mechanism of vaccinia virus transmission between cells is not well understood. To learn more about this process, we compared two closely related virus strains, IHD-J and WR, with respect to the formation of enveloped virions. First, we confirmed a previous observation (21) that although IHD-J and WR produced similar amounts of INV and formed similar-size primary plaques, IHD-J made 10 to 40 times more EEV than did WR and exhibited much more efficient long-range virus transmission. The possibility that INV is sufficient for plaque formation, however, was not consistent with a number of observations (4, 25, 26). Deletion of the gene coding for VP37, the major protein in the outer envelope of EEV, had no effect on INV production but reduced the already low level of EEV formed by WR and inhibited plaque formation. One explanation for this result is that the low levels of EEV produced by the parental WR virus were just sufficient for efficient plaque formation but that a further reduction was not. We prefer, however, an alternative explanation. Electron microscopic images revealed that there were as many or more CEV on the outer surface of cells infected with WR as on cells infected with IHD-J. As we have previously noted (4), CEV seem to be appropriately located for spread to neighboring cells. Moreover, deletion of the VP37 gene blocked both CEV and EEV formation. Thus, the similarity in primary plaque size of WR and IHD-J is better explained by the similar amounts of CEV than by the disparate amounts of EEV.

The question remained, however, as to why IHD-J produces more EEV than does WR. One possibility was that the difference is related to the amount or structure of VP37 made by the two virus strains. Previous studies, however, demonstrated that large amounts of VP37 are produced in cells infected with WR (12), and sequence data predicted that the VP37 proteins of WR and IHD-J differ by only a single amino acid (26). This substitution is not responsible for functional differences, since we found that exchanges of the WR and

IHD-J VP37 genes by marker rescue did not modify the characteristics of the viruses.

Wrapping of INV appears to occur normally in cells infected with WR (12, 25). We were able to confirm this notion by density gradient analysis of cell-associated virus. Moreover, our finding of large numbers of CEV on the surface of cells infected with WR suggested that the different phenotypes of WR and IHD-J might be a consequence of differences in virus release rather than in earlier steps of virus morphogenesis and transport. This possibility was examined by attempting to artificially create EEV by trypsin treatment of intact cells. Trypsin treatment led to the release of more infectious extracellular virus from WR-infected cells than from IHD-J-infected cells, suggesting an accumulation of CEV on cells infected with WR. The ability of IHD-J to form comet-shaped plaques in liquid overlay has been attributed to the release of EEV (2, 21). Conversely, the failure of WR to form comets has been explained by the low levels of EEV produced. We attempted to test the latter hypothesis by adding trypsin to the liquid overlay during WR plaque formation. Trypsin treatment of WR-infected cells led to the production of comets, reinforcing the idea that EEV is responsible for the long-range spread of vaccinia virus in vitro.

The ability to release CEV by proteolysis is consistent with their adherence to the outside of the plasma membrane. Whether a proteolytic step is naturally involved in formation of EEV, however, is not known. The possibility that a virus-encoded protease is involved in CEV release should be considered. Vaccinia virus encodes three proteins with homology to serine protease inhibitors (7, 16, 27). Interactions between proteases and protease inhibitors might regulate virus release from the cell.

Previous studies demonstrated an association of mature virions with actin-containing microfilaments (13). Late in infection, infected cells display specialized microvilli with a core of microfilaments and a virus particle at their tips (11, 13, 28). Although vaccinia virus encodes a homolog of profilin, this protein plays no apparent role in the formation of the virus-induced microvilli (3). Induction of microvilli is blocked, however, by rifampin, a drug that interrupts an early stage of virus maturation (13). Primarily on this basis, it was suggested that the formation of mature virus particles is required for the induction of microvilli (17). Our present results indicate that mature INV are not sufficient for induction of microvilli and that VP37 is required. It seems likely that induction of the microvilli is dependent on the interaction of INV virions with membranes and that this interaction may be dependent on the presence of VP37. Unexpectedly, we found that the specialized microvilli were much less abundant in RK13 cells infected with IHD-J than in RK13 cells infected with WR, suggesting that their presence is not correlated with formation of EEV. Thus, the induction of enlarged microvilli in WR might be a consequence or a cause of the failure to release CEV. Interestingly, Payne and Kristenson (23) reported that cytochalasin D, a drug known to disrupt cellular microfilaments, prevented the release but not the formation of EEV in IHD-J-infected cells. They proposed that the inhibitory effect of the drug might be mediated by decreased cell motility or membrane oscillations.

Further comparisons of the WR and IHD-J strains of vaccinia virus may provide additional insights into the mechanisms of poxvirus transmission. We believe that the IHD-J phenotype is dominant, since EEV release occurs in mixed infections with WR (2a). The ability to rescue the phenotype of IHD-J by transfecting total IHD-J DNA into cells infected with W-VP37 and isolating recombinants (2a) suggests that a reverse genetic approach may be successful in identifying the gene(s) responsible for release of EEV.

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REFERENCES

1. **Appleyard, G., and C. Andrews.** 1974. Neutralizing activities of antisera to poxvirus soluble antigens. J. Gen. Virol. **23**:197–200.
2. **Appleyard, G., A. J. Hapel, and E. A. Boulter.** 1971. An antigenic difference between intracellular and extracellular rabbitpox virus. J. Gen. Virol. **13**:9–17.
2a. **Blasco, R.** Unpublished data.
3. **Blasco, R., N. B. Cole, and B. Moss.** 1991. Sequence analysis, expression, and deletion of a vaccinia virus gene encoding a homolog of profilin, a eukaryotic actin-binding protein. J. Virol. **65**:4598–4608.
4. **Blasco, R., and B. Moss.** 1991. Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-dalton outer envelope antigen. J. Virol. **65**:5910–5920.
5. **Boulter, E. A., and G. Appleyard.** 1973. Differences between extracellular and intracellular forms of poxvirus and their implications. Prog. Med. Virol. **16**:86–108.
6. **Boulter, E. A., H. T. Zwartouw, D. H. J. Titmuss, and H. B. Maber.** 1971. The nature of the immune state produced by inactivated vaccinia virus in rabbits. Am. J. Epidemiol. **94**:612–620.
7. **Boursnell, M. E., I. J. Foulds, J. I. Campbell, and M. M. Binns.** 1988. Non-essential genes in the vaccinia virus HindIII K fragment: a gene related to serine protease inhibitors and a gene related to the 37K vaccinia virus major envelope antigen. J. Gen. Virol. **69**:2995–3003.
8. **Dales, S., and B. G. Pogo.** 1981. Biology of poxviruses. Virol. Monogr. **18**:54–60.
9. **Earl, P., and B. Moss.** 1991. Expression of proteins in mammalian cells using vaccinia viral vectors, p. 16.15.1–16.18.10. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Wiley-Interscience, New York.
10. **Hiller, G., H. Eibl, and K. Weber.** 1981. Characterization of intracellular and extracellular vaccinia virus variants: N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine interferes with cytoplasmic virus dissemination and release. J. Virol. **39**:903–913.
11. **Hiller, G., C. Jungwirth, and K. Weber.** 1981. Fluorescence microscopical analysis of the life cycle of vaccinia virus in chick embryo fibroblasts. Virus-cytoskeleton interactions. Exp. Cell Res. **132**:81–87.
12. **Hiller, G., and K. Weber.** 1985. Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. J. Virol. **55**:651–659.
13. **Hiller, G., K. Weber, L. Schneider, C. Parajsz, and C. Jungwirth.** 1979. Interaction of assembled progeny poxviruses with the cellular cytoskeleton. Virology **98**:142–153.
14. **Hirt, P., G. Hiller, and R. Wittek.** 1986. Localization and fine structure of a vaccinia virus gene encoding an envelope antigen. J. Virol. **58**:757–764.
15. **Ichihashi, Y., S. Matsumoto, and S. Dales.** 1971. Biogenesis of poxviruses: role of A-type inclusions and host cell membranes in virus dissemination. Virology **46**:507–532.
16. **Kotwal, G. J., and B. Moss.** 1989. Vaccinia virus encodes two proteins that are structurally related to members of the plasma serine protease inhibitor superfamily. Virol. **63**:600–606. (Erratum, **64**:966, 1990.)
17. **Krempien, U., L. Schneider, G. Hiller, K. Weber, E. Katz, and C. Jungwirth.** 1981. Conditions for pox virus-specific microvilli formation studied during synchronized virus assembly. Virol-

ogy **113**:556–564.
18. **Morgan, C.** 1976. Vaccinia virus reexamined: development and release. Virology **73**:43–58.
19. **Moss, B.** 1990. Poxviridae and their replication, p. 2079–2111. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. Melnick, T. P. Monath, and B. Roizman (ed.), Virology. Raven Press, New York.
20. **Payne, L. G.** 1979. Identification of the vaccinia hemagglutinin polypeptide from a cell system yielding large amounts of extracellular enveloped virus. J. Virol. **31**:147–155.
21. **Payne, L. G.** 1980. Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J. Gen. Virol. **50**:89–100.
22. **Payne, L. G., and K. Kristenson.** 1979. Mechanism of vaccinia virus release and its specific inhibition by N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine. J. Virol. **32**:614–622.
23. **Payne, L. G., and K. Kristenson.** 1982. The effect of cytochalasin D and monensin on enveloped vaccinia virus release. Arch. Virol. **74**:11–20.
24. **Payne, L. G., and E. Norrby.** 1976. Presence of haemagglutinin in the envelope of extracellular vaccinia virus particles. J. Gen. Virol. **32**:63–72.
25. **Rodriguez, J. F., and G. L. Smith.** 1990. IPTG-dependent vaccinia virus: identification of a virus protein enabling virion envelopment by Golgi membrane and egress. Nucleic Acids Res. **18**:5347–5351.
26. **Schmutz, C., L. G. Payne, J. Gubser, and R. Wittek.** 1991. A mutation in the gene encoding the vaccinia virus 37,000-M_r protein confers resistance to an inhibitor of virus envelopment and release. J. Virol. **65**:3435–3442.
27. **Smith, G. L., S. T. Howard, Y. S. Chan.** 1989. Vaccinia virus encodes a family of genes with homology to serine proteinase inhibitors. J. Gen. Virol. **70**:2333–2343.
28. **Stokes, G. V.** 1976. High voltage electron microscope study of the release of vaccinia virus from whole cells. J. Virol. **18**:636–643.
29. **Turner, G. S., and E. J. Squires.** 1971. Inactivated smallpox vaccine: immunogenicity of inactivated intracellular and extracellular vaccinia virus. J. Gen. Virol. **13**:19–25.