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Mechanism of Vaccinia Virus Release and Its Specific Inhibition by N_1-Isonicotinoyl-N_2-3-Methyl-4-Chlorobenzoylhydrazine

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The release of vaccinia virus from RK-13 cells and its specific inhibition by N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine (IMCBH) was studied. Intracellular naked vaccinia virus (INV) was wrapped by intracytoplasmic membranes, forming an intracellular double-membraned virion. Wrapped virions migrated to the cell surface, where the outer virion membrane presumably fused with the plasma membrane, releasing virus surrounded by the inner membrane, referred to as extracellular enveloped vaccinia virus (EEV). At no time was there any evidence that vaccinia virus acquired an envelope by budding of naked virus from the cytoplasmic membrane. Naked virus and double-membraned virus each constituted about one-third of intracellular virus at 8 and 12 h postinfection (p.i.). Beginning at 16 h p.i., the proportion of intracellular virus occurring as double-membraned virus steadily decreased to 1% at 24 h while the proportion of naked virus rose to 87%. IMCBH inhibited the formation of the double-membraned virion and the appearance of EEV while not affecting the production of INV. IMCBH had no effect on INV infectivity or polypeptide composition, on vaccinia virus-specified membrane-associated proteins or glycoproteins, or on hemadsorption. The presence of IMCBH until 4 h p.i. did not decrease the amount of EEV at 48 h p.i., whereas less than 10% of the normal 48-h EEV yield was obtained if the drug was present during the first 16 h p.i. Cell cultures infected at very low multiplicities showed a rapid virus dissemination in the absence of the drug, whereas the presence of IMCBH very effectively inhibited this spread. We conclude that vaccinia virus is liberated via a double-membraned intermediate as an enveloped virion and that it is this extracellular enveloped virus that is responsible for dissemination of infection.

Extracellular vaccinia virus has been shown to be surrounded by an envelope not present on intracellular virus (1, 5). Only sporadic attention has been directed toward studying the sequence of events following the appearance of intracellular naked vaccinia virus (INV) that lead to the release of enveloped virus from infected HeLa cells (2, 4). Intracellular virus was observed to associate with intracytoplasmic membranes, resulting in the appearance of the virion surrounded by a double-membraned structure. Thereafter, such particles migrated to the plasma membrane and fused the outermost membrane of the virus particle with the plasma membrane, resulting in the release of a virus particle surrounded by a single membrane or envelope. Electron micrographs of released virus showed disrupted envelopes, which was construed as evidence for the final release of vaccinia virus in the naked form (2, 4). INV was also proposed to be released after cell disruption (4). Such interpretations do not agree with density gradient studies (5, 6) showing that 95% of extracellular virus is in the enveloped state. No quantitative data were provided on the appearance of the intracellular double-membraned vaccinia virion (2, 4). The process of extracellular enveloped vaccinia virus (EEV) release is apparently very inefficient in HeLa cells, since only about 5% of the total virus is released (5, 6). We have recently found that 25 to 35% of vaccinia virions are released from RK-13 cells (5). The RK-13 cell system is, thus, a more easily studied model than HeLa cells for the characterization of EEV release.

It was previously reported that N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine (IMCBH) inhibited the release of infectious vaccinia virus but had no effect on the appearance of intracellular infectious vaccinia virus (3).

There have been no further investigations on the mechanism of this remarkably specific inhibition. We report in this paper the mechanism of vaccinia virus release from RK-13 cells, its significance in virus dissemination, and the stage at which IMCBH inhibits this release.

MATERIALS AND METHODS

Cells and virus. The rabbit kidney established cell line RK-13 was passaged into 25-cm^2 plastic flasks in minimal essential medium-5% fetal calf serum. The cells were confluent on day 3 postpassage and, unless otherwise stated, infected with 0.3 ml of IDH-J vaccinia virus at a multiplicity of infection of 3 PFU per cell. After a 1-h incubation at 37°C, the unattached virus was removed and the cells were washed three times with 3 ml of minimal essential medium-1% fetal calf serum. Each flask was then further incubated at 37°C with 10 ml of minimal essential medium-1% fetal calf serum.

Compound. IMCBH was kindly provided by Hans J. Eggers (Institut für Virologie der Universität zu Köln, Cologne, West Germany). The compound was dissolved at a concentration of 10 mg/ml in dimethyl sulfoxide and stored at −20°C. In experiments with IMCBH, the drug was added to cell cultures at 1 h postinfection (p.i.) at a final concentration of 10 μg/ml. Control flasks were treated with an equal volume of dimethyl sulfoxide lacking IMCBH.

Virus quantitation. Intracellular and extracellular virus were both plaque assayed on RK-13 cells after one cycle of freeze-thawing and subsequent sonication as previously described (5). Four petri dishes were used at each 10-fold dilution step.

Preparation of material for electron microscopy. Cells were fixed in situ with 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2). Fixed cells were washed, postfixed in 1% osmium tetroxide, and dehydrated in alcohol. The cell sheet was cut with a razor blade into squares, which loosened from the plastic surface during propylene oxide treatment. The squares were then embedded in epoxy resin (Epon 812). Thin sections were stained with lead citrate and uranyl acetate. Virus extracted from infected cells was negatively stained with uranyl acetate before electron microscopy.

RESULTS

Electron microscopy of vaccinia virus-infected RK-13 cells. Electron microscopy of vaccinia virus-infected RK-13 cells revealed a number of distinct phases in vaccinia virus morphogenesis (Fig. 1 through 3). The first morphological evidence of vaccinia virus infection was the appearance of virus factories between 4 and 6 h p.i. Late in this period, de novo-synthesized viral membranes became evident in these factories. Viral membrane formation proceeded for several hours, during which immature particles matured to virus particles with the usual dumbbell-shaped nucleoid associated with mature infectious vaccinia virions. Fig. 1 shows all three morphogenetic phases. It is particularly noteworthy that viral factories with their attendant viral membranes were most often topologically separated from the INV. The latter particles were often localized to areas rich in intracellular membranes. Some of these membranes were morphologically identifiable as Golgi membranes. At this point, mature INV became associated to such cell membranes (Fig. 2a). In this way, large numbers of INV virions were wrapped in double-membraned sacks (Fig. 2b). Wrapped virions migrated away from INV particles toward the plasma membrane (Fig. 2c). Only a few INV virions were observed in the vicinity of the plasma membrane, whereas none were found directly associated with the plasma membrane. We observed only a few wrapped virions subadjacent to the plasma membrane (Fig. 2d). These were presumably in the process of fusing the outer virion membrane with the plasma membrane. Some particles were observed at the cell surface partially surrounded by the plasma membrane (Fig. 2e). Two features of such particles are of particular interest. First, there was usually an electron-dense material coating the cytoplasmic side of such emerging virions. Second, the portion of the virion in contact with the extracellular environment was wrapped in a single membrane. This single membrane is the envelope present on large numbers of extracellular vaccinia virions (Fig. 3) that appear between 8 and 16 h p.i. Extracellular naked virus was only rarely observed. By 16 h p.i. large numbers of INV particles were visible, many of which were associated with the periphery of vacuoles or multivesicular bodies (Fig. 3). At no time was there any evidence of INV particles budding into vacuoles or through the plasma membrane.

The presence of double-membraned virions in thin-sectioned preparations prompted a search for such particles in infected-cell extracts. Material extracted by Dounce homogenization from infected cells and negatively stained with 1% uranyl acetate was examined for the presence of virus particles. Figure 4 shows two morphological entities, INV (A) and double-membraned virions (B). The latter particle often showed damage to the outer membrane or both membranes. Efforts to purify these double-membraned virions in cesium chloride were not successful. Virus in the extracellular phase (Fig. 4C) was observed to be EEV.

Kinetics of appearance of INV and double-membraned virions. Previous work demonstrating the existence of double-membraned vaccinia virus particles (2, 4) in infected HeLa cells was not quantitative. Table 1 shows the

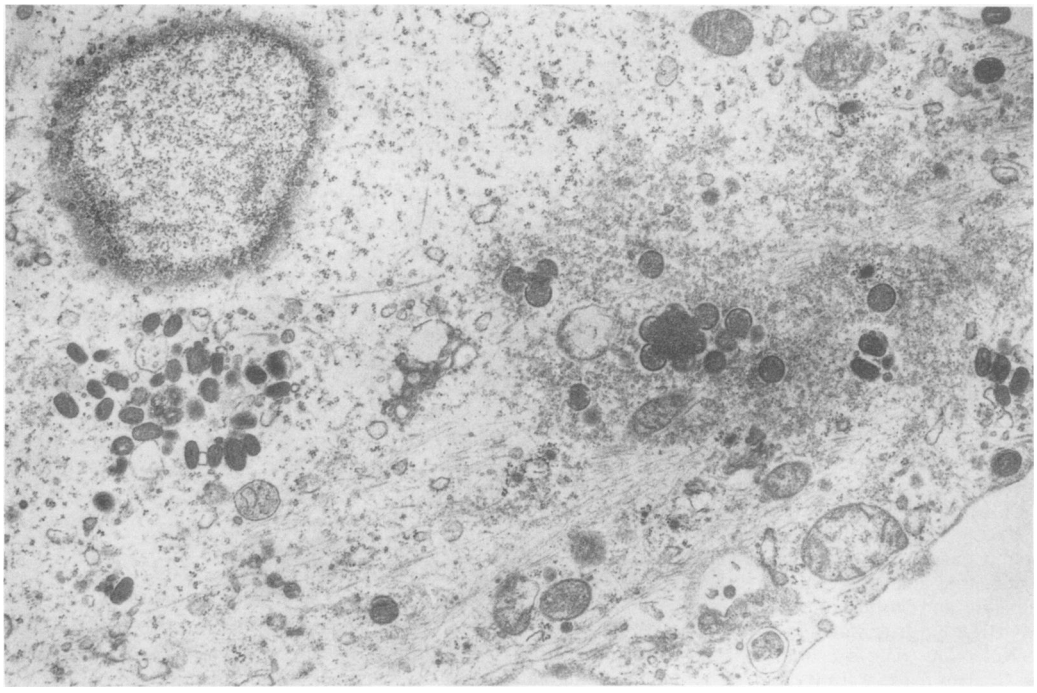

FIG. 1. *Electron micrograph of a vaccinia virus-infected cell, illustrating the morphogenesis of INV. The viral factories and INV occupy separate parts of the cell.*

percentages of intracellular virus in RK-13 cells that was INV in a free or vacuole-associated state and virus that was partially or completely wrapped. Intracellular virus observed between 8 and 24 h p.i. did not vary greatly in the percentage that was vacuole associated (10 to 20%) or in the percentage that was partially wrapped (<10%). In contrast, the percentages of free INV and completely wrapped virus varied markedly with time p.i. At both 8 and 12 h p.i., free INV and completely wrapped virus each composed slightly more than one-third of the intracellular virus. By 16 h p.i. the proportion of free INV had increased significantly (to 68%), whereas completely wrapped virus had decreased (to 16%). This reciprocal relationship was even more apparent at 20 h p.i., and by 24 h p.i., 87% of intracellular virus was free INV, whereas only 1% was completely wrapped.

Effect of IMCBH on vaccinia virus-infected RK-13 cells. IMCBH was previously shown to prevent the development of a cytopathic effect in vaccinia virus-infected primary cultures of chicken embryo fibroblasts and monkey kidney cells, but not in continuous mouse L cells (3). The drug was found to inhibit the release of vaccinia virus from chicken embryo fibroblasts, but it did not affect the appearance of intracellular virus. We have recently shown that vaccinia virus-infected RK-13 cells release large amounts of EEV (5). It was therefore of interest to characterize the effect IMCBH had on vaccinia virus-infected RK-13 cells.

Figure 5 delineates the appearances of intracellular and extracellular vaccinia virus in control untreated and IMCBH-treated RK-13 cells. The onset, kinetics, and amounts of intracellular virus production were very similar in control and IMCBH-treated cell cultures. By contrast, the release of infectious virus into the extracellular environment was markedly inhibited in IMCBH-treated cultures. Although the release of virus in control cultures was manifest between 4 and 6 h p.i. and continued until approximately 16 h p.i., in drug-treated cultures no significant increase was detected until 22 h p.i. The proportions of total virus produced at 24 h p.i. and released were 15% for control cultures but only 0.1% for IMCBH-treated cells.

The activity of IMCBH was further characterized by studying the reversibility of the inhibition (Fig. 6). After virus infection cells were IMCBH treated, and at various times p.i. the drug was removed. The cells were then further incubated in drug-free medium until the EEV was harvested, at 48 h p.i. Presence of the drug during only the first 4 h of infection had little effect on the amount of EEV released during the

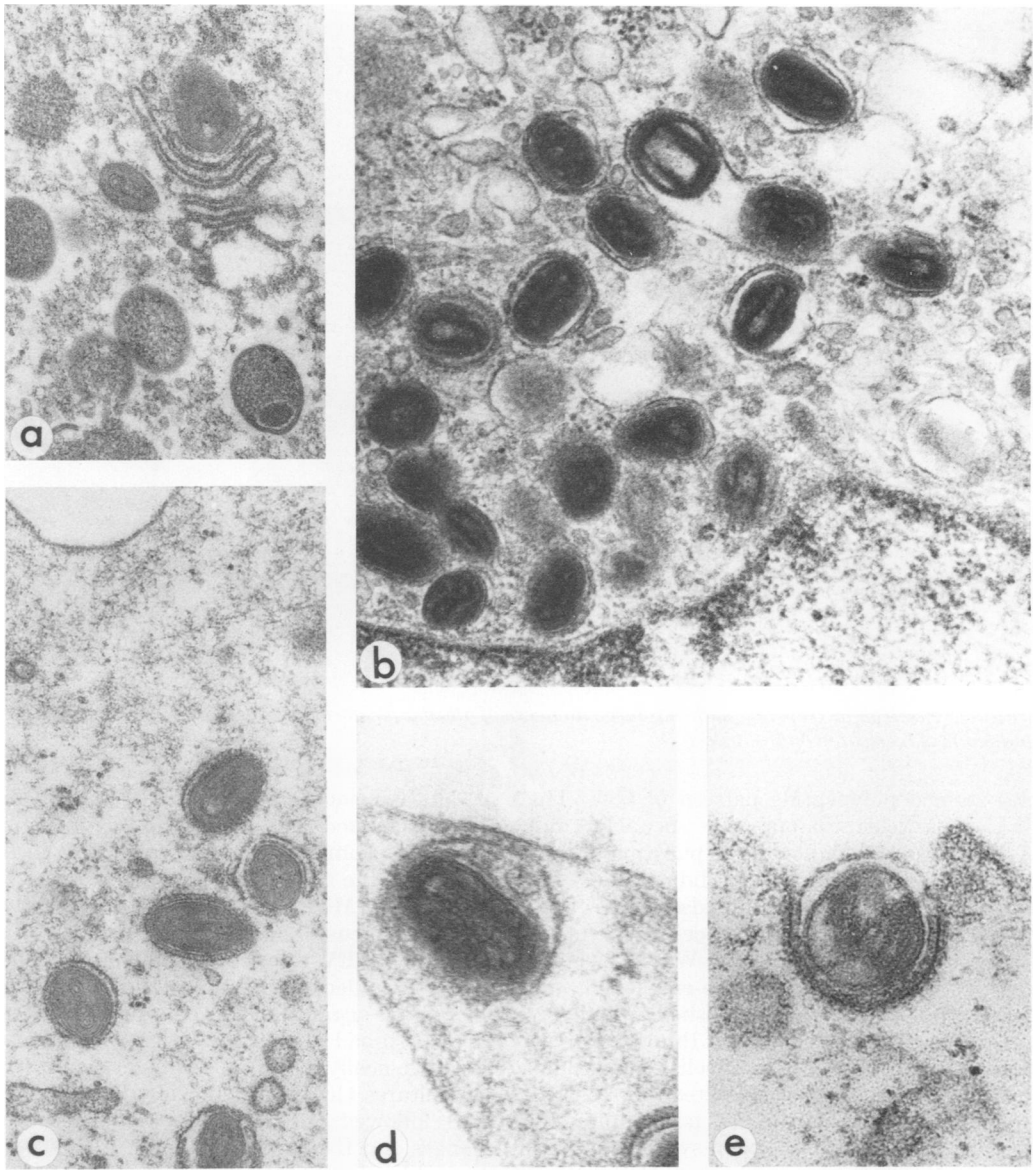

FIG. 2. *Electron micrographs illustrating INV associating with intracellular membranes (a), large numbers of double-membraned virions in the perinuclear area (b), double-membraned virus in the vicinity of the plasma membrane (c), presumed fusion of the outer virus membrane with the plasma membrane (d), and emergence of a single-membraned virion from the cell (e).*

48-h infection. Longer exposures progressively diminished the level of EEV release, until a 16-h drug exposure reduced the quantity of EEV to less than 10% of that obtained from untreated cultures. The release of EEV from untreated cells began at between 4 and 6 h p.i., rose rapidly, and began to plateau at 14 to 16 h p.i.

Vaccinia virus particles and infected cells were examined for IMCBH-induced alterations in their structural-functional properties in an attempt to specify the level at which IMCBH exerted its inhibition (data not shown). Vaccinia virus-specified polypeptides were analyzed by polyacrylamide gel electrophoresis (5). The appearance in cell membranes of [^{35}S]methionine-labeled vaccinia virus proteins and [^{3}H]glucosamine-labeled glycoproteins was not affected. No change was detected in the Coomassie brilliant

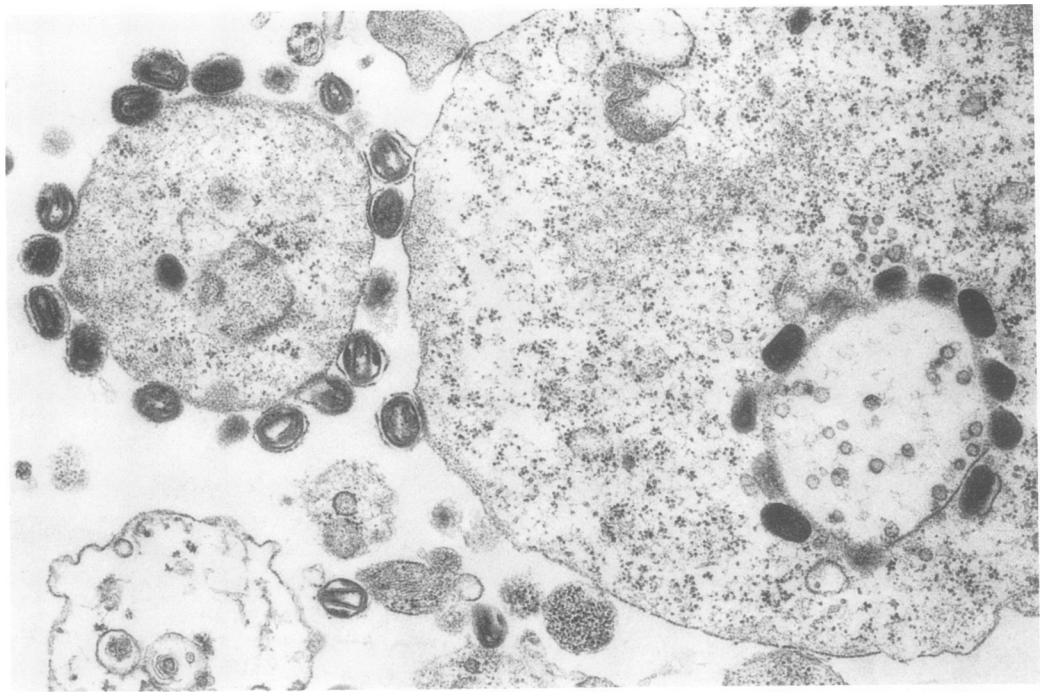

FIG. 3. *Electron micrograph showing large numbers of plasma membrane-associated EEV (left) and INV-studded multivesicular bodies (right).*

blue-stained polypeptide pattern of INV. The PFU-to-particle ratio (optical density at 260 nm) of INV was unaffected by replication in the presence of IMCBH. IMCBH did not affect the kinetics of appearance of hemadsorption.

Electron microscopy of vaccinia virus-infected IMCBH-treated cells. We have shown that INV, intracellular double-enveloped, and EEV virions are easily distinguished morphologically. We therefore examined thin sections of vaccinia virus-infected RK-13 cells to determine the morphological stage affected by IMCBH treatment. Cells fixed at 8 h p.i. contained a normal complement of de novo-synthesized vaccinia virus membranes and mature INV virions. A clear difference in drug-treated cells, however, was the virtual absence of INV virions associated with cytoplasmic membranes. It was also very evident in IMCBH-treated cells examined at 8, 12, 16, 20, and 24 h p.i. that there was a complete absence of both double-membraned intracellular virions and plasma membrane-attached EEV. At late times p.i. the intracellular distribution of INV in IMCBH-treated cells was quite different than that observed in control cells. Whereas INV at 24 h p.i. in untreated cells was most often either dispersed in the cell or associated with large vacuoles, the INV in drug-treated cells occurred in large clusters (Fig. 7) and was not at all associated with vacuoles. Uninfected IMCBH-treated cells showed no morphological alterations compared to uninfected cells in the absence of the drug.

Effect of IMCBH on the in vitro spread of vaccinia virus. The very marked IMCBH inhibition of EEV release with unchanged levels of INV provided a novel opportunity to examine the relative significance of INV and EEV for the in vitro spread of vaccinia virus. We examined this biologically important problem by infecting cell cultures (10^7 cells) with only 300 PFU and then followed the appearance of cytopathic changes and INV and EEV. Drug-free cultures showed localized comet-like areas of necrosis at 48 h p.i. (Fig. 8A). By 120 h p.i., there were no cells in the culture not having rounded morphology (data not shown). In contrast, drug-treated cultures (Fig. 8B) showed no difference in cell morphology compared to uninfected cultures (Fig. 8C). There were no differences even at 120 h p.i. (data not shown). The quantity of INV and EEV at 6 h p.i. (Fig. 9) was very nearly the same for IMCBH-treated and control cells. At this time only primary infected cells were synthesizing virus. All primary infected cells in drug-treated and control cultures can be expected to produce equal quantities of INV (see Fig. 5). Nevertheless, at 24 h p.i. vaccinia virus-

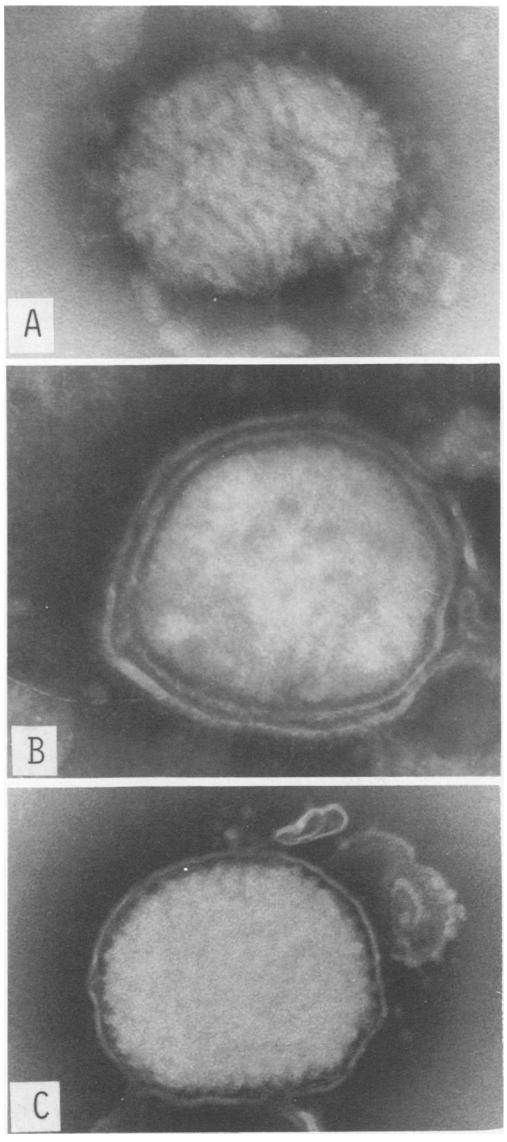

FIG. 4. *Electron micrographs of negatively stained INV (A) and double-membraned virions (B), obtained by examining infected-cell extracts from 8 to 12 h p.i. EEV (C) was found in the extracellular medium.*

infected, drug-free cultures (Fig. 9) contained 40 times as much INV as did similarly infected drug-treated cells. By 48 h p.i. control cultures contained 275 times as much INV as did drug-treated cells. Both control and drug-treated cultures showed only four- to fivefold increases in INV between 48 and 120 h p.i. EEV from control and drug-treated cells showed even a greater difference. Final EEV titers in control flasks were 1,500 times greater than those in IMCBH-treated flasks.

TABLE 1. *Kinetic relationship of INV and wrapped virus*

Time (h p.i.)	% of total intracellular virus[a]				Total no. of intracellular virions counted
	INV		Wrapped:		
	Free	Vacuole associated	Partially[b]	Completely	
8	37	21	5	37	98
12	37	18	10	34	415
16	68	12	5	16	582
20	65	20	6	9	516
24	87	10	3	1	1,025

[a] Fifty cell profiles were counted at each time point.
[b] Wrapping membranes covered at least one-half of the virion but did not show closure, as seen for completely wrapped virions.

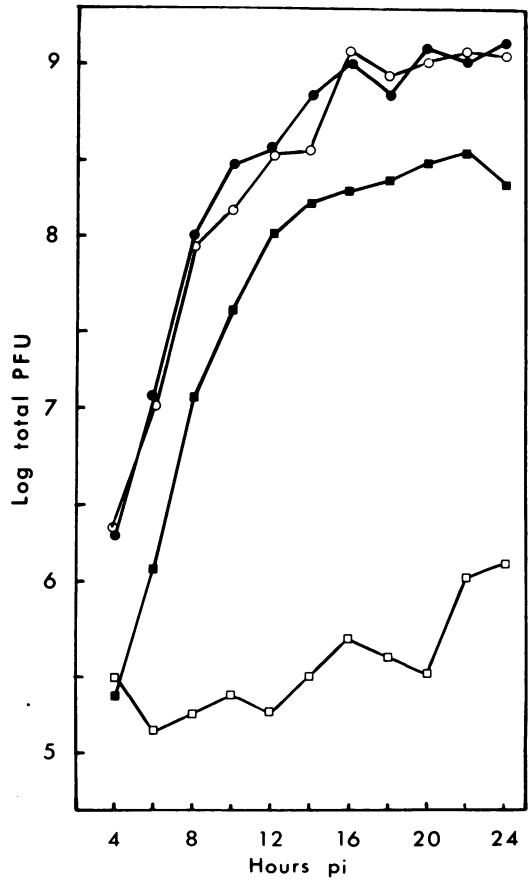

FIG. 5. *Kinetics of INV and EEV appearances in control and IMCBH-treated cell cultures, followed after infection with vaccinia virus at a multiplicity of infection of 3. IMCBH (10 μg/ml) was added to cultures at 1 h p.i. The amounts of infectious virus present as INV (●, control; ○, IMCBH treated) and EEV (■, control; □, IMCBH treated) were measured by plaque assay as described in the text.*

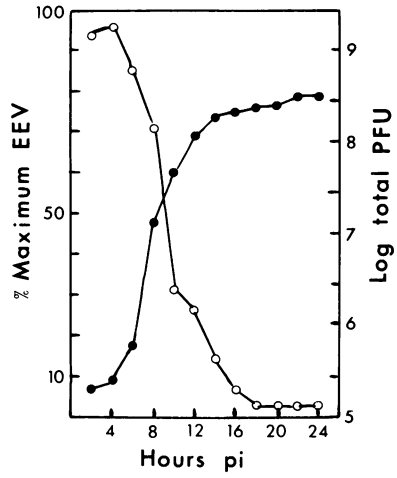

FIG. 6. *Effect of IMCBH reversal on EEV production. Cells were infected with a multiplicity of infection of 3 and treated with 10 μg of IMCBH per ml* (○). *The IMCBH inhibition of EEV release was reversed by removal of the drug at the specified times (2 to 24 h p.i.), and the cells were further incubated with drug-free medium. At 48 h p.i. the released EEV was harvested from all of the reversed flasks. Vaccinia virus-infected but untreated RK-13 cells were monitored for EEV release* (●).

DISCUSSION

We have presented here data on the release of vaccinia virus. The following is a reconstruction of this process. After immature virus particles form in the viral factories, they undergo differentiation to mature virions and migrate away from the factories. INV virions then associate with intracytoplasmic membranes, resulting in particles wrapped in a double-membraned sack. Wrapped virions thereafter migrate to the cell surface, where the outer membrane presumably fuses with the plasma membrane, releasing a virion surrounded by a single envelope. The fact that we did not observe fusion is probably related to the great rapidity of this process. It is particularly noteworthy that we observed no INV virions associated with the plasma membrane and absolutely no evidence of a budding process.

Previous work (2, 4) has also described the release of vaccinia virus from HeLa cells via the series of events reconstructed above. However, in this cell system very few intracellular wrapped virions were observed, and little virus was released. Perhaps a consequence of this inefficient release was the observed association of very large numbers of INV virions with the plasma

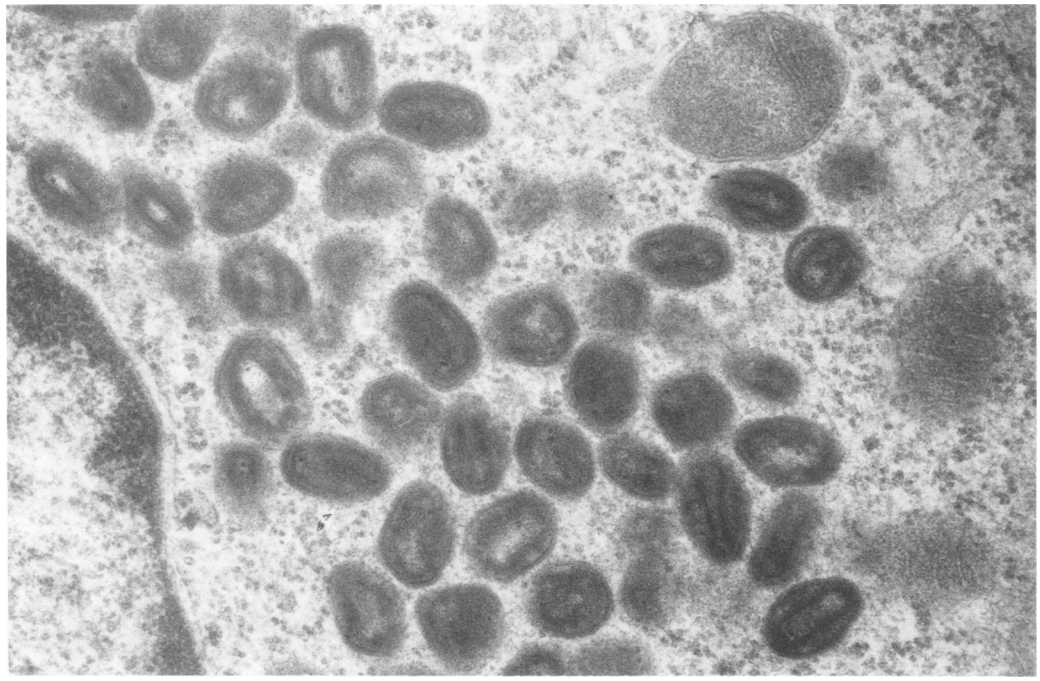

FIG. 7. *Vaccinia virus-infected RK-13 cells treated with IMCBH (10 μg/ml) and fixed at 24 h p.i. for thin-section electron microscopy.*

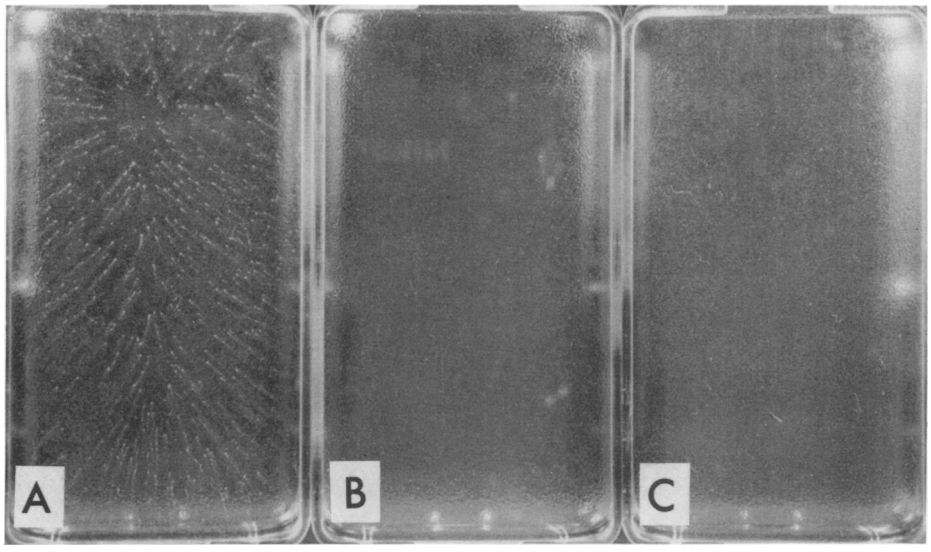

FIG. 8. *Photographs of vaccinia virus-infected, untreated cells (A) and vaccinia-infected, IMCBH (10 μg/ml)-treated cells (B), taken 48 h after infection of each flask with 300 PFU. A control uninfected cell culture (C) is included for comparison.*

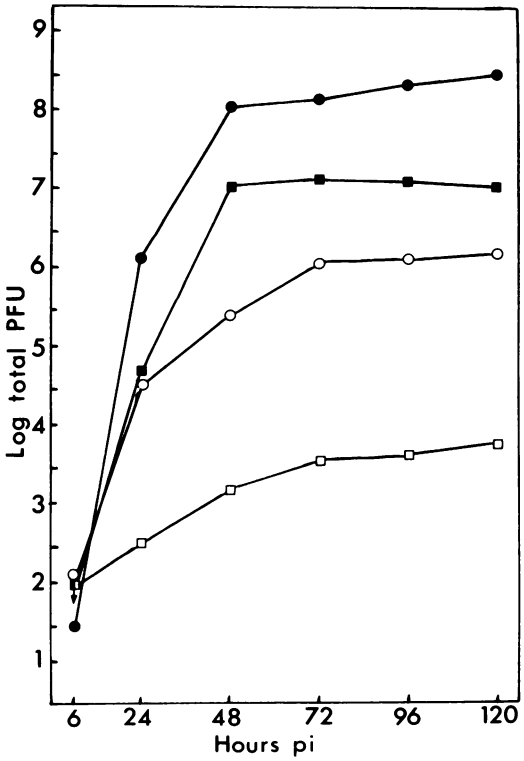

FIG. 9. *Effect of IMCBH (10 μg/ml) on in vitro vaccinia virus dissemination, monitored after infection of RK-13 cells (ca. 10^7 cells per flask) with 300 PFU per flask. INV (●, control; ○, IMCBH treated) and EEV (■, control; □, IMCBH treated) were assayed as described in the text.*

membrane, although no budding was detected (2). The RK-13 cell system described here releases large quantities of EEV (5) via the double-membraned particles and thus made it possible to relate kinetically the presence of double-membraned virions with EEV release. Double-membraned particles were first detected 8 h p.i. and were most prevalent from 8 to 16 h p.i., after which their numbers declined sharply. EEV was first liberated between 4 and 6 h p.i. and reached a plateau at about 16 h p.i. The coincident declines in double-membraned virion formation and EEV release resulted in the accumulation of large numbers of INV virions late in infection. This may be due to a loss of intracytoplasmic membranes to this unique release process, since they presumably are not replaced and therefore their loss would become a limiting factor late in infection.

Vaccinia virus liberation from chicken embryo fibroblasts has been shown to be very effectively inhibited by IMCBH without appreciably affecting the production of intracellular virus (3). We have confirmed and extended this finding in vaccinia virus-infected RK-13 cells. Removal of the drug at 4 h p.i. did not affect EEV yields at 48 h p.i. Increasing exposure time progressively reduced the amount of EEV until finally less than 10% of the normal 48-h EEV yield was obtained by drug exposure during the first 16 h of infection. This period of limited reversibility coincides with the most active time of double-membraned virion production and EEV release. Indeed, electron microscopic examination of

IMCBH-treated vaccinia virus-infected cells showed a complete lack of both double-membraned virions and plasma membrane-associated EEV. It is, thus, quite evident from the IMCBH data that the double-membraned virion is an obligatory intermediate in the conversion of INV to EEV.

We were unable to discern on the molecular level any IMCBH-induced alterations in vaccinia virus-specified membrane-associated proteins or glycoproteins or in the protein composition of INV. Nor were we able to detect any change in the biological functions of hemadsorption or infectivity. IMCBH may, thus, affect vaccinia virus functions in a more subtle way. IMCBH could also simply block a critical intracytoplasmic membrane-related normal cell function necessary for double-membraned virion formation.

It was previously shown that the presence in the growth medium of antibody directed against extracellular virus prevented the spread of an in vitro vaccinia virus infection, whereas antibody against intracellular virus was ineffective (1). This was interpreted as evidence for the importance of extracellular virus for the spread of the infection. The availability of a drug that specifically inhibited EEV release provided us with a means of determining the relative importance of EEV and INV in the in vitro spread of vaccinia virus. Although approximately equal numbers of intracellular virus particles were produced in the infected primary cells during the first 6 h p.i. in control and drug-treated cells, the further evolution of the infection was quite different. Virus quickly spread in control cultures, producing large quantities of both intracellular virus and extracellular virus, whereas in drug-treated cells the infection developed very slowly, with relatively very small amounts of virus being produced. The IMCBH data, thus, agrees with the specific antisera experiments (1) and emphasizes the importance of EEV for the in vitro spread of vaccinia virus. We have previously reported the heavy predominance of EEV in the extracellular environment (5, 6). Rupture of the envelope, resulting in the final release of virus in the naked form (2, 4), and INV release by cell degeneration (4) would therefore seem to be relatively rare events. The present morphological data further emphasize that extracellular virions are almost exclusively in the enveloped state. The weight of the available evidence militates for the conclusion that vaccinia virus dissemination is mediated by EEV released via an intracellular double-membraned virion intermediate.

ACKNOWLEDGMENTS

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