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Characterization of Intracellular and Extracellular Vaccinia Virus Variants: N_1-Isonicotinoyl-N_2-3-Methyl-4-Chlorobenzoylhydrazine Interferes with Cytoplasmic Virus Dissemination and Release

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Infectious vaccinia virus can be purified from whole cells by experimentally induced lysis (intracellular virus) or from supernatant growth medium (extracellular virus). Extracellular virus and intracellular virus differed by buoyant density (1.237 versus 1.272 g/cm^3), phospholipid content and composition, and polypeptide pattern. Differences in structural polypeptides on the virus surface could be detected by lactoperoxidase-catalyzed radioiodination or Brij treatment. Characteristic of extracellular virus was an additional polypeptide, with a molecular weight of 37,000 (37K), which represented 5 to 7% of the total particle protein. Antibodies to the 37K protein detected only some of the cell-associated particles late in normal infection. Upon treatment of infected cultures with N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine, a drug which prevents vaccinia virus release, no particle-associated 37K protein could be detected. In all other properties tested so far, except for a slight difference in phospholipid composition, the virus obtained in the presence of the drug resembled the normal intracellular virus. N_1-Isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine prevented vesicularization of intracellular viral particles. Lack of vesicularization was accompanied by the absence of particle-associated 37K viral protein and seemed to correlate with an inhibition of virus dissemination to the cell periphery.

Several immunological studies (1-3, 24) have pointed to the existence of differences between intracellular and extracellular poxvirus populations. Payne and Norrby (19) have shown that intracellular vaccinia virus and virus released into the growth medium of infected tissue culture cells differ in buoyant density. With the recognition of efficient extracellular virus production in rabbit kidney cells (RK 13) infected with vaccinia virus strain IHD-J (17), experimental conditions have become available to compare intracellular and extracellular vaccinia virions biochemically. Reports on antigenic differences between the two particle populations (2, 3, 24) could be substantiated by differences in virion composition. Working with radioactive precursors, Payne (16) found some additional polypeptides on extracellular particles, whereas some proteins found associated with intracellular virus were missing. In the experiments described herein, we extended the previous characterization of both virus variants by iodination of peripheral proteins and phospholipid analysis. Included in our study was a third particle population, assembled in cells treated with N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine (IMCBH), a drug which prevents virus release from infected tissue cultures (12, 18). The intracellular particle distribution in drug-treated and untreated embryonic chicken cells, as well as the appearance of extracellular virions and their precursor forms, was followed by immunofluorescence microscopy, using antisera against two defined virion proteins. A correlation was found between the vesicularization of progeny virions, the presence of a viral protein with a molecular weight of 37,000 (37K) on intracellular particles, and the final virus dissemination to the cell periphery.

MATERIALS AND METHODS

Cells and viruses. Rabbit kidney cells (RK 13), originally obtained from Flow Laboratories, Inc., Rockville, Md., were grown as monolayer cultures at 37°C in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. After infection with hemagglutinin-positive vaccinia virus strain IHD (American Type Culture Collection, Rockville, Md.) at a multiplicity of approximately 20 elementary bodies per cell, the fetal calf serum concentration was lowered to 3%. Growth medium and cells were har-

vested separately at 36 to 48 h postinfection, when the cells clearly showed cytopathic effects but the majority still remained attached to the substratum. After removal of detached cells and larger debris from the harvested medium by low-speed centrifugation (10 min, 1,000 × *g*), extracellular virus was pelleted (30 min, 35,000 × *g* at 4°C). This crude preparation was further purified either on a continuous potassium tartrate gradient (15 to 35%, wt/wt, 110,000 × *g* for 90 min at 4°C) or on a discontinuous cesium chloride gradient (80,000 × *g* for 90 min at 20°C) formed by prelayering 1.30 (6-ml)-, 1.25 (8-ml)-, and 1.20 (10-ml)-g/ml cesium chloride solutions in a Beckman SW27 rotor tube (see also reference 16). Two consecutive gradient centrifugations were necessary to obtain a homogeneous preparation of extracellular virus. Cell-associated (intracellular) virus was purified from harvested lysed cells as described by Joklik (11). An equilibrium centrifugation in either potassium tartrate or cesium chloride was added as the last step in the purification scheme. Vaccinia virus IHD grown in the presence of IMCBH was obtained from lysed cells by following the scheme for intracellular virus purification. IMCBH was used at 10 μg/ml and added to cultures after the virus inoculum had been adsorbed for 1 h at room temperature.

For morphological studies, chicken embryo fibroblasts prepared by conventional trypsinization techniques were infected with vaccinia virus strain WR as described previously (8, 9).

Iodination. For surface labeling of purified virus variants, suspensions corresponding to 50 μg of total viral protein were incubated with 16 μg of lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.) in 200 μl of phosphate-buffered saline containing 500 μCi of $Na^{125}I$ (15 mCi/μg of I; Amersham Corp., Arlington Heights, Ill.). The reaction was started by the addition of 1.6 μl of 1 mM H_2O_2. After 15 min at room temperature, the reaction mixture was layered on 5 ml of sucrose solution (32%, wt/vol in phosphate-buffered saline) and centrifuged (45 min, 80,000 × *g*, 4°C). The pelleted virus samples showed a specific activity of approximately 200,000 cpm/μg of protein. Aliquots were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and autoradiography.

Lipid extraction and analysis. A sample of purified virus equivalent to 1 mg of protein was suspended in 2 ml of phosphate-buffered saline containing 50 mM EDTA. After addition of 2.4 ml of methanol and vigorous shaking, 1.2 ml of chloroform was added to extract lipids. Chloroform extraction of the methanol-water phase was repeated three times. The pooled chloroform extracts served as starting material for phospholipid separation on thin-layer plates (Silica Gel 60; E. Merck AG, Darmstadt, Federal Republic of Germany). Localization, identification, and quantitation of the different phospholipid classes were performed as described by G. Hiller, H. Eibl, and K. Weber (submitted for publication).

Antibodies. Two vaccinia virus IHD virion proteins (31K and 37K) were isolated, using preparative SDS-polyacrylamide gel electrophoresis as the final purification step. For the preparation of 31K protein, whole, unfractionated virus served as starting material. The 37K protein was enriched in the supernatant obtained after Brij 58 treatment (1% Brij in phosphate-buffered saline, 10 min at room temperature) of extracellular virus and removal of stripped particles by centrifugation (30,000 × *g* for 1 h). Depending on the complexity of the band pattern, between 0.6 and 1.5 mg of total protein was loaded per 2-mm-thick slab gel. After electrophoretic separation, gels were briefly stained with Coomassie brilliant blue and washed for 10 min in water, and the faintly visible bands were excised. The gel pieces were electrophoretically eluted into dialysis tubing, using the standard running buffer system of Laemmli (14). After extensive dialysis against 0.05% SDS, the samples were lyophilized. A portion of each sample was checked for purity and protein concentration, using analytical SDS-polyacrylamide gel electrophoresis. In the case of the 31K protein, a guinea pig was given a total of 100 μg of protein. Antibodies to 37K protein were raised in a rabbit using a total of 130 μg of protein. Proteins were injected both subcutaneously and intraperitonally, with the dosage divided into three or four injections at 3-week intervals. The first injections contained protein and complete Freund adjuvant. Booster injections contained protein and incomplete Freund adjuvant. Appearance and increase of titer were followed by indirect immunofluorescence microscopy, using purified vaccinia virus virions settled on cover slips (8).

Fluorescence and electron microscopy. The indirect immunofluorescence microscopy procedure for cells grown on glass cover slips has been described elsewhere (9, 25). In double-labeling experiments with guinea pig anti-31K-protein serum and rabbit anti-37K-protein serum, we made use of the fact that the two antisera had been raised in different animal species. After fixation, infected cells were incubated with additions made in the following order: guinea pig anti-31K-protein serum, rhodamine-labeled goat anti-guinea pig immunoglobulin G, rabbit anti-37K-protein serum, and fluorescein-labeled goat anti-rabbit immunoglobulin G. After each incubation step the samples were carefully washed with phosphate-buffered saline. Primary antisera were intensively preabsorbed on methanol-fixed uninfected cells and finally used at a 100-fold dilution, whereas fluorescently labeled class G immunoglobulins (Miles-Yeda, Israel) were used at 0.5 to 1 mg/ml. Photographic documentation was as described previously (8).

For thin sectioning, vaccinia virus WR-infected chicken embryo fibroblasts grown on cover slips were fixed in a solution containing 50 mM cacodylate (pH 7.2), 50 mM KCl, 2.5 mM $MgCl_2$, 2.5% glutaraldehyde, and 2% osmium tetroxide for 30 min on ice. After 2 h of postfixation with 2% osmium tetroxide in buffer lacking glutaraldehyde, samples were processed by conventional techniques.

Fixation of cells for examination by scanning electron microscopy was done as described previously (9).

RESULTS

Virus preparations and polypeptide patterns. The difference in buoyant density (1.272 versus 1.237 g/cm^3) allowed quantitative separation of intracellular and extracellular virions by density gradient centrifugation in cesium

chloride (Fig. 1) or potassium tartrate (see reference 19). When virus was grown and harvested as described in Materials and Methods, approximately 15 to 20% of the totally assembled particles were released into the medium. Virus prepared by cell lysis from IMCBH-treated cells showed the same density as intracellular virus from untreated cultures (Fig. 1). In agreement with Kato et al. (12) and Payne and Kristenson (18), both intracellular virus forms were equally infectious. Their PFU-to-particle ratio lay in the range of 1:30 to 1:60 when assayed on African green monkey kidney (BSC-1) cells (data not shown). Fig. 2 shows the Coomassie brilliant blue-stained polypeptide patterns of intracellular virus and extracellular virus in SDS-polyacrylamide gels. The extracellular variant showed one major additional protein, 37K, which accounted for approximately 5 to 7% of the total protein. This polypeptide was also visible in the radioactive amino acid labeling studies of Payne (17), although for unknown reasons its abundance in the particle was not readily apparent.

In an attempt to localize the polypeptides typical of extracellular virus, we labeled the various purified virus variants by lactoperoxidase-catalyzed iodination. Analysis on SDS-polyacrylamide gels (Fig. 3) revealed iodination of a 60K band. The mobility of this polypeptide was slightly decreased when extracellular virus was compared with its intracellular counterpart. The most intensively labeled polypeptide (20K, corresponding to band 10a in the numbering system of Sarov and Joklik [20]) of intracellular virus was significantly reduced in extracellular virus. In addition, an apparently 13K polypeptide was almost completely absent in the extracellular form, which, in contrast, showed labeled material migrating close to the dye front (Fig. 3). Some of the changes in peripheral proteins found by iodination could also be seen on stained gels of total proteins (compare Fig. 2 and 3).

The 37K protein, the only major additional polypeptide of extracellular virus, was not iodinated by our procedure. However, we could confirm Payne's report (16) that the 37K protein is easily detached by gentle treatment with Brij 58. This behavior is typical of a peripheral membrane protein. Thus, our failure to obtain iodination was probably due to unavailability of tyrosine residues.

Vaccinia virus particles assembled in RK 13 cells exposed to IMCBH (10 μg/ml) revealed an iodination pattern very similar to that seen for normal intracellular virus (Fig. 3). The virus particles did not contain the 37K protein typical of extracellular virus.

Phospholipid analysis. Different amounts of particle-associated lipids could possibly contribute to the observed differences in buoyant density between intracellular and extracellular virions. Using a very sensitive phosphate assay (7), we were able to quantitate the different viral phospholipids with only 1.9×10^{11} particles for methanol-chloroform extraction (Hiller et al.,

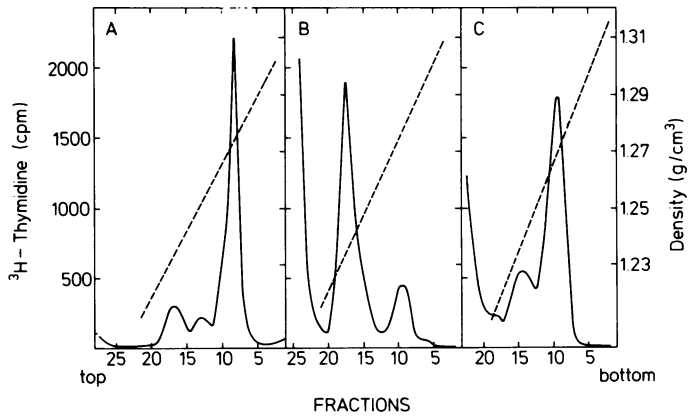

FIG. 1. *Equilibrium density gradient centrifugation of three vaccinia virus variants in cesium chloride. Vaccinia virus IHD-infected RK 13 cells were labeled with [^{3}H]thymidine (20 μCi/ml; specific activity, 5 Ci/mmol) from 2 to 8 h postinfection, either in the presence or in the absence of IMCBH. At 22 h after infection, cells and growth medium were harvested separately (see text). Cell-associated virus was isolated after cell lysis by sucrose gradient centrifugation. Pooled peak fractions of intracellular samples and the crude extracellular vaccinia virus particles were incubated with 50 μg of pancreatic DNase (Sigma) per ml in the presence of 1 mM $MgCl_2$–0.5 mM $CaCl_2$ for 10 min at 37°C. Immediately after incubation, samples were layered on discontinuous cesium chloride gradients and centrifuged. (A) Intracellular virus from untreated cells; (B) extracellular virus from untreated cells; (C) intracellular virus from IMCBH-treated cells.*

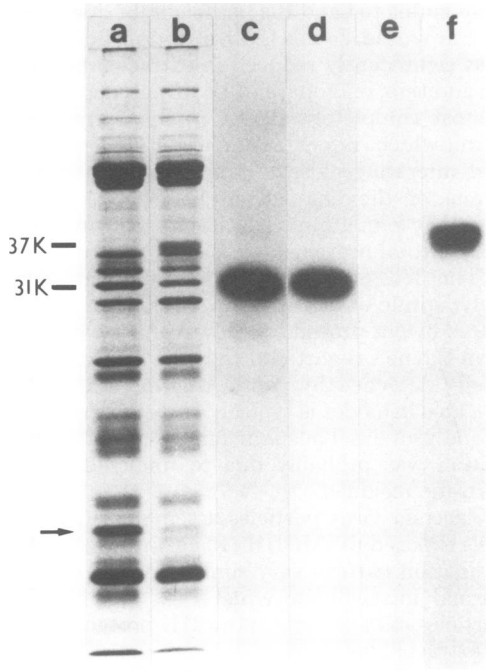

FIG. 2. *Polypeptide patterns of intra- and extracellular vaccinia virus with the characterization of two antisera raised against defined structural proteins. Forty micrograms of total virion protein per lane was separated on a 14% polyacrylamide gel in the presence of SDS. (a and b) Coomassie brilliant blue-stained polypeptide patterns of intracellular virus (a) and extracellular virus (b) (the arrow indicates a small polypeptide, most of which is lost on extracellular virus). (c through f) Immune replicas after using two specific antisera to mark corresponding antigens: anti-31K-protein serum on separated polypeptides from intracellular virus (c) and extracellular virus (d) and anti-37K-protein serum on separated polypeptides from intracellular virus (e) and extracellular virus (f) (note the presence of 31K protein in both virus forms and the presence of 37K protein only in extracellular virus).*

submitted for publication). This amount was equivalent to 1 mg of total virus protein. Extracellular virus contained nearly twice as much total phospholipid as did intracellular virus (Table 1). Both virus forms were characterized by a low ratio of neutral to negatively charged phospholipids, approximately 55:45. In comparison, cellular membranes show proportions around 80:20 (5). Interestingly, the relative loss of acyl bis(monoacylglycero)phosphate (Hiller et al., submitted for publication), which represented more than 55% of the negatively charged phospholipids in intracellular virions, was compensated in extracellular virions by the increase in phosphatidylserine and phosphatidylinositol content (Table 1). These results may indicate that vaccinia virus infection interferes with early steps of de novo phospholipid synthesis, that is, formation of phosphatidic acids. The cell-associated virus purified from IMCBH-treated cultures contained the same total amount of phospholipid as did normal intracellular virus. The distribution among various phospholipid classes seemed to be slightly different, showing an increase in phosphatidylserine and a decrease in acyl bis(monoacylglycero)phosphate for the IMCBH virus.

Virus dissemination. In an attempt to study the mechanism of IMCBH action, we followed the assembly and dissemination of progeny virus in drug-treated cells by immunofluorescence mi-

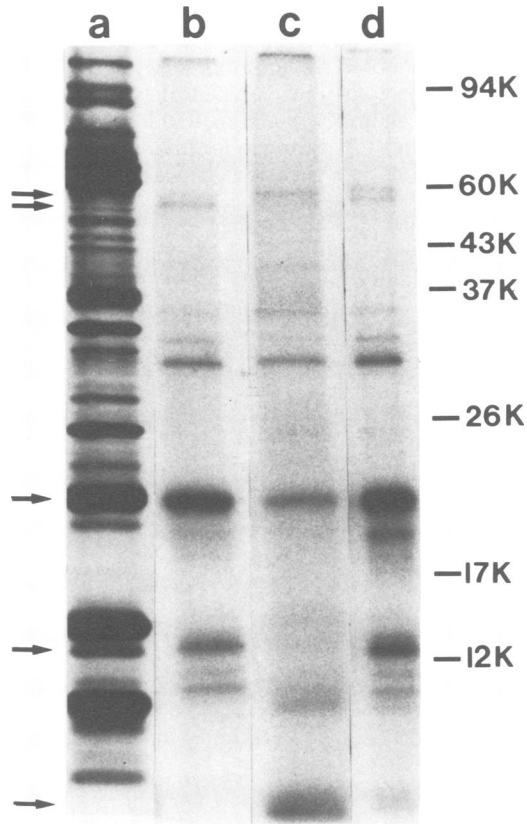

FIG. 3. *Surface labeling of purified vaccinia virus particles by lactoperoxidase-catalyzed iodination. For details of iodination, see the text. Labeled polypeptides were analyzed on 14% polyacrylamide gels in the presence of 0.1% SDS. For reference, some molecular weights are indicated. (a) [^{35}S]methionine-labeled intracellular virus, showing the complex total polypeptide pattern. (b through d) Iodinated surface polypeptides from intracellular virus (b), extracellular virus (c), and intracellular particles assembled in the presence of IMCBH (d). The polypeptides which clearly differ either in size or amount are indicated by arrows.*

croscopy (8, 9), using antisera to defined virion proteins. The first polypeptide was chosen on the ground of its relatively early appearance on assembled particles. It was characterized by a molecular weight of approximately 31,000 in SDS-polyacrylamide gel electrophoresis. Corresponding to band 7a in the nomenclature of Sarov and Joklik (20) (see Fig. 2), it should have been representative of the intracellular particle population. A second protein, 37K, was chosen because it seemed to be specific for the extracellular virus form (see Fig. 2). Both virion polypeptides were isolated by preparative SDS-polyacrylamide gel electrophoresis and used for immunization of guinea pigs (31K) and rabbits (37K) (for details, see Materials and Methods). The specificities of the resulting antibodies are illustrated in Fig. 2 using the immunoreplica technique (4). Antiserum to 31K protein recognized only one 31K polypeptide in both intracellular and extracellular particles. Antiserum to 37K protein showed no reaction with structural proteins of intracellular vaccinia virus particles, but clearly recognized specifically the 37K protein of extracellular virions (Fig. 2).

Figure 4 shows the distinct effect of IMCBH on progeny particle distribution in chicken embryo fibroblasts infected with vaccinia virus WR detected by immunofluorescence microscopy with anti-31K protein. Typical of drug-treated cells were large fluorescently labeled cytoplasmic areas and very few single particles distrib-

TABLE 1. *Phospholipid contents and compositions of three vaccinia virus variants*[a]

Variant	Phospholipid content (nmol/mg of virion protein)			
	Acyl bis(monoacylglycero)-phosphate	Phosphatidylethanolamine	Phosphatidylcholine	Phosphatidylserine, phosphatidylinositol, sphingomyelin
Intracellular	17 ± 3 (25%)	6 ± 2 (9%)	30 ± 6 (45%)	14 ± 2 (21%)
Extracellular	8 ± 2 (6%)	24 ± 5 (18%)	52 ± 6 (39%)	49 ± 7 (37%)
IMCBH	11 ± 2 (15%)	5 ± 2 (7%)	30 ± 1 (41%)	27 ± 3 (37%)

[a] Thin-layer plates (Silica Gel 60, Merck) were developed in chloroform–methanol–25% ammonium (65:30:3, by volume). In this solvent system phosphatidylserine, phosphatidylinositol, and sphingomyelin are not separated from each other. The quantitative data represent phosphate estimations and are averaged from four different preparations. Numbers in parentheses are moles percent of total phospholipids.

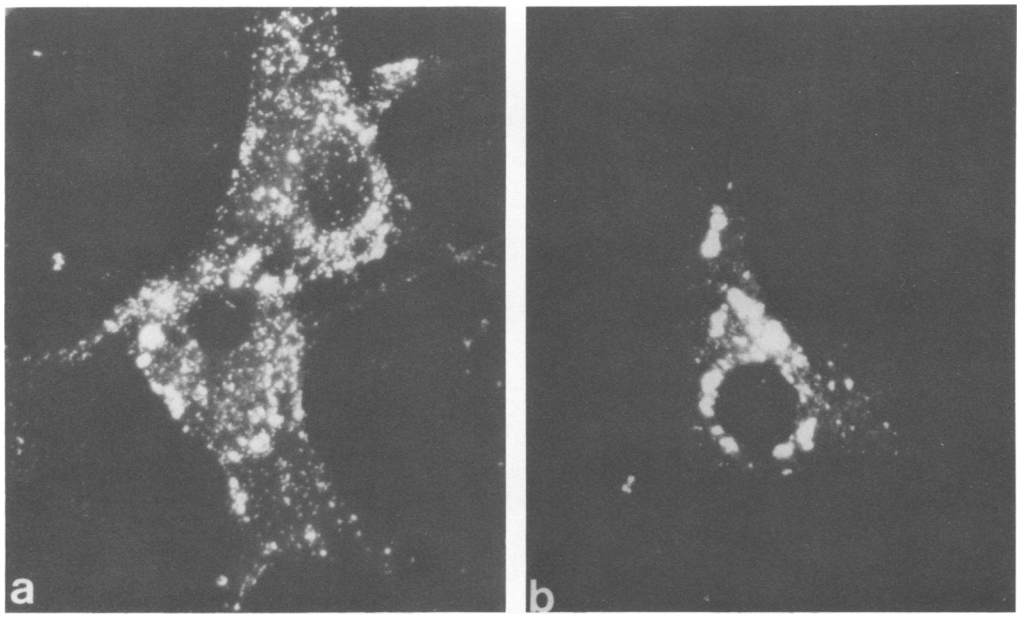

FIG. 4. *Block in vaccinia virus dissemination upon treatment of infected cultures with IMCBH. Vaccinia virus WR-infected chicken embryo fibroblasts, either treated with 10 μg of IMCBH per ml from the end of inoculum adsorption until fixation (18 h postinfection) (b) or untreated (a), were stained with anti-31K-protein serum for intracellular particle distribution. Note the complete dissemination of single virions throughout the whole cytoplasm in untreated cells and the larger labeled areas near the cytocentrum in drug-treated cells.* ×800.

uted beyond these organizations. In addition, neither the large fluorescent areas nor the single particles were very close to the cell periphery (Fig. 4b). This was in clear contrast to vaccinia virus WR infection in the absence of the drug, where many single particles could be found along the cellular margins (Fig. 4a). Electron microscopical analysis of thin-sectioned cells identified the large cytoplasmic areas labeled with 31K protein antiserum as heaps of tightly packed but morphologically mature vaccinia virus particles (Fig. 5) (see also reference 18). Comparison of IMCBH-treated and untreated vaccinia-infected cells by both immunofluorescence and electron microscopy proved, in addition, that 31K protein antiserum did not stain nonparticulate viroplasm, representing the typical viral factories.

Further support for a model in which IMCBH interferes with virus dissemination to the plasma membrane came from scanning electron microscopy. The surface of vaccinia virus WR-infected chicken fibroblasts was relatively rough, showing typical virus-specific microvilli (9, 22) and plasma membrane-associated virus particles (Fig. 6a). In contrast, the surface of drug-treated infected cells appeared relatively smooth and did not reveal the typical virus-induced alterations (Fig. 6b).

When cells late in normal infection were studied by immunofluorescence microscopy with anti-37K-protein serum, single virus particles disseminated through the whole cytoplasm were seen (Fig. 7a). In IMCBH-treated cells, no particulate virion staining was observed, and the large regions of heaps of immobilized virions (see above) were not visualized (Fig. 7b). Double fluorescent labeling experiments with 31K protein and 37K protein antisera on treated and untreated infected cultures (Fig. 8) confirmed these results and excluded the possibility of a lower degree of infection upon drug treatment. Anti-31K protein staining clearly demonstrated numerous virus particles and heaps of particles (Fig. 8a; see also Fig. 4b), whereas anti-37K protein revealed no particles in the same cells (Fig. 8b). Comparison of Fig. 8c and d proves the sequential mode of addition of 31K and 37K proteins to virus particles in untreated cells. The immature particles present around the factories situated deep in the cytoplasm apparently carried 31K protein, but did not yet reveal 37K protein. In addition, some more peripherally localized virus particles were positive for 31K and not for 37K protein. Whether these particles still represented immature forms or corresponded to morphologically mature and infectious virions of typical intracellular appearance could not be

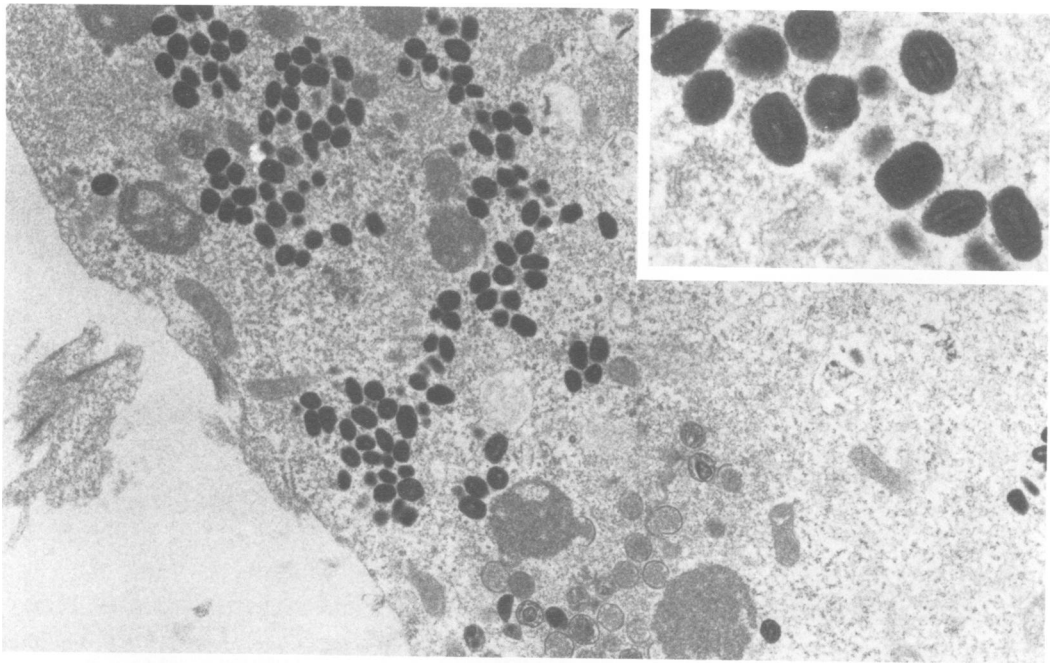

FIG. 5. *Undistributed heaps of morphologically mature virions characteristic of IMCBH treatment. Besides poxvirus factories and some immature particles, drug-treated fibroblasts showed characteristic heaps of tightly packed morphologically mature virions (18 h postinfection). Magnifications: ×12,500; inset, ×35,800.*

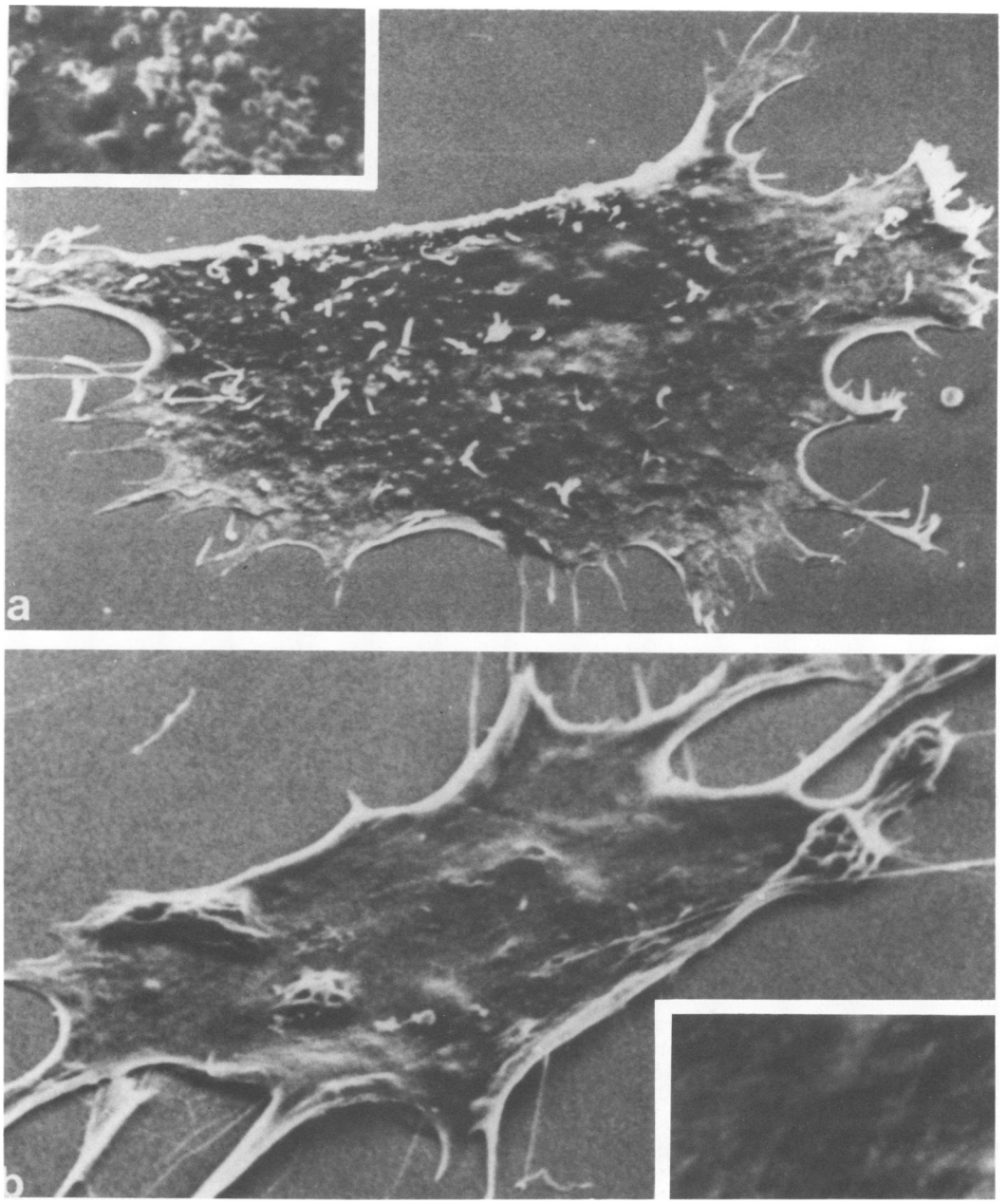

FIG. 6. *Surface of cells infected in the presence or absence of IMCBH. Vaccinia virus WR-infected chicken embryo fibroblasts were fixed at 20 h postinfection for examination by scanning electron microscopy. Untreated cells showed some virus-induced thick microvilli and numerous progeny virus particles associated with the plasma membrane (a), whereas drug-treated cells displayed a relatively smooth surface, lacking similar structures (b). The insets emphasize the lack of plasma membrane-associated virions in drug-treated cells. Magnifications: ×3,000; insets, ×10,000.*

decided by our experiments. However, the comparison with IMCBH-treated cells and the ratio of positive to negative particles seen by using the anti-37K-protein serum argue for the latter possibility (see Discussion). Thus, a striking feature of IMCBH treatment was the relative lack of virus dissemination and the absence of 37K protein incorporation.

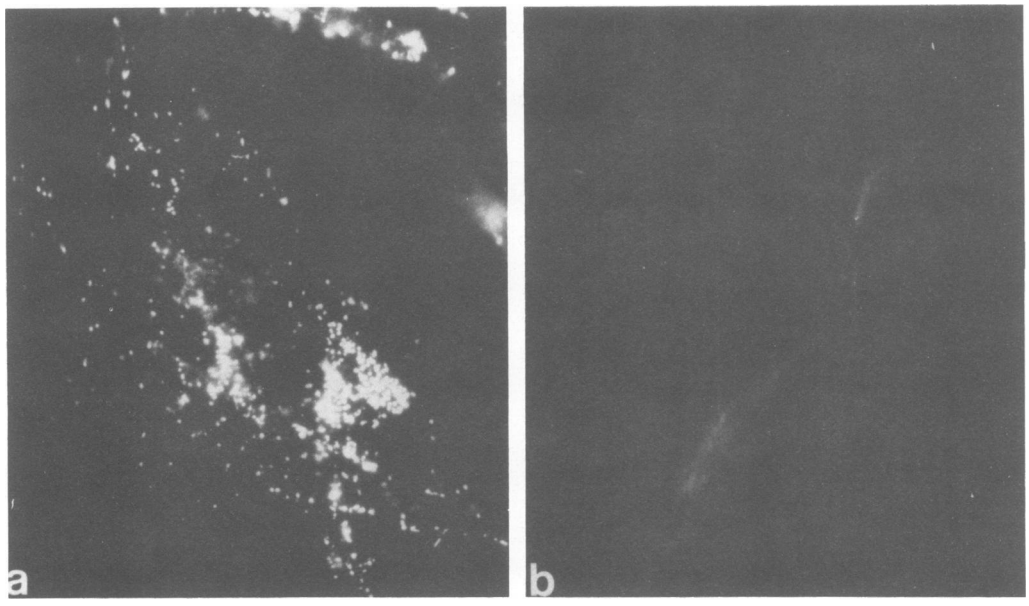

FIG. 7. *Distributions of 37K viral protein in normal and IMCBH-treated cells. In normal infection, numerous vaccinia particles were revealed (18 h postinfection) by staining with anti-37K-protein serum (a). Treatment of cultures with 10 μg of IMCBH per ml prevented particle staining and resulted in very weak general cytoplasmic fluorescence (b). ×1,500.*

DISCUSSION

Vaccinia virions isolated from the growth medium of infected tissue cultures appear lighter in buoyant density than standard virions obtained from experimentally lysed cells (19). We confirm this finding and extend the biochemical characterization of the two virion types by a comparison of their major structural proteins, an analysis of their peripheral particle proteins, and a determination of their phospholipid contents and compositions.

Density gradient-purified extracellular virus contains about twice as much phospholipid as does intracellular virus (see Table 1) and reveals a major new 37K polypeptide. Probably due to a peripheral location, the 37K protein can rather specifically be extracted by Brij 58 (see also reference 16). The results on the total phospholipid content per unit weight of viral protein or viral DNA are in line with the observed lower buoyant density of extracellular virus. The amount of total phospholipid found in purified intracellular vaccinia virus by us and others (21, 26) is generally assumed to be sufficient to provide a lipid bilayer-related structure around the particle (6). Among the phospholipids specific for the intracellular vaccinia virus variant is a bisphosphatidic acid analog carrying three fatty acid residues (Hiller et al., submitted for publication). A similar triacylated compound and a diacylated derivative, bis(monoacylglycero)-phosphate, are thought to represent phospholipid markers for cellular lysosomes (23). Whether this reflects a functional role for lysosomal or related lipid structures in poxvirus membrane assembly is currently unknown.

The increased phospholipid content of extracellular virus may indicate an additional membrane structure acquired by this virus form (see below). Previous electron microscopical studies point to the possibility that poxvirus particles may be enwrapped by Golgi-derived membranes (10, 15, 18).

Immunofluorescence microscopy shows that progeny virus particles near the cytoplasmic poxvirus factories already have the viral 31K protein common to both purified virus variants but do not reveal the 37K protein. More peripherally located particles, found close to the focal planes of the plasma membrane, are characterized by both viral proteins (Fig. 8c and d). Thus, 37K protein is somehow acquired during the cytoplasmic dissemination phase. To explore the differential behavior of the two proteins, we made use of the influence of IMCBH on infected cells. This drug is known to inhibit the release of virions from infected cells and, consequently, virus spread in culture (12, 18). In IMCBH-treated cells vaccinia virions are readily visualized by antiserum to 31K protein in large "aggregated" areas, which are shown by electron microscopy to be close to the viral factories. Even very late in infection, cellular extensions

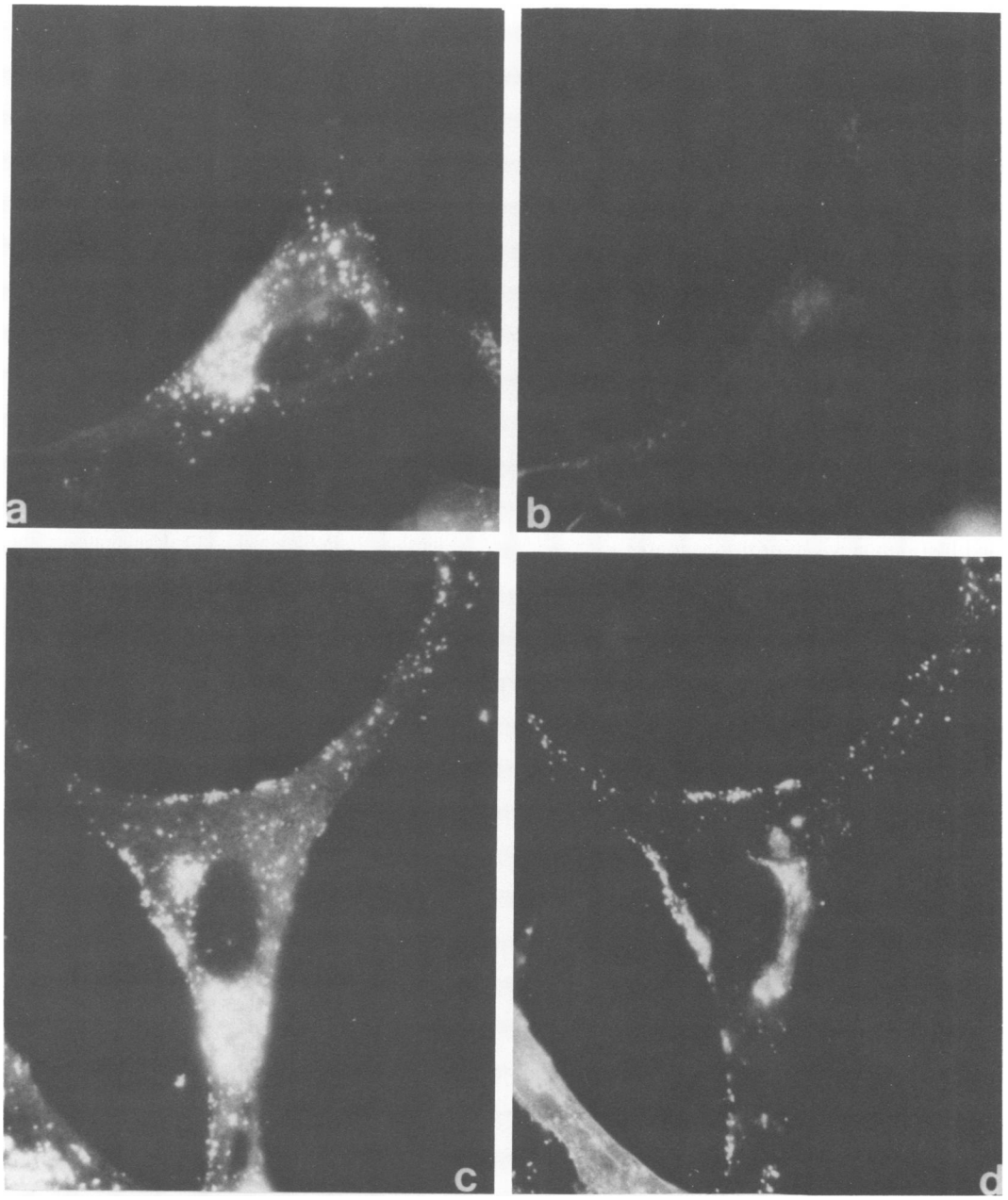

FIG. 8. *Double fluorescence microscopy of cells infected with vaccinia virus in the absence or presence of IMCBH, using anti-31K-protein and anti-37K-protein sera simultaneously. Drug-treated or untreated cells were fixed 18 h after infection and processed for immunofluorescence microscopy. IMCBH-treated cells showed large aggregate areas and few single particles when stained by 31K antiserum (a), whereas these structures were not stained by 37K antiserum (b). In untreated cells, 31K protein antiserum revealed numerous virions disseminated in the cytoplasm and heaps of particles in the neighborhood of the nucleus (c). In the same cells, 37K protein antiserum revealed virions only close to the cellular margins (d). The perinuclear heaps of particles remained conspicuously unlabeled. ×1,200.*

remain free of virions, whereas in untreated cells numerous particles are typically found in these areas. Thus, IMCBH interferes in a yet unknown manner with the process of virus dissemination through the cytoplasm. Alterations on the cell surface, such as the induction of specialized microvilli (9, 22), which are thought to require mature virions near the plasma membrane, are

not observed in the presence of IMCBH. This is taken as an additional indication of the failure of mature virions to reach the cell periphery.

Particles and particle aggregates present in drug-treated cells are clearly not visualized by antiserum to 37K protein. That at least some 37K protein is synthesized under these conditions is indicated by a weak general cytoplasmic fluorescence and the fact that antibodies to 37K protein precipitate some of this protein in lysates of infected cells labeled with radioactive leucine (our preliminary results). Vaccinia virus particles isolated from drug-treated cells show a polypeptide pattern, either in fluorography after labeling with [^{35}S]methionine or after staining with Coomassie brilliant blue, similar to that of intracellular virus purified from untreated cells. They also possess the same infectivity (18; data not shown) and differ only slightly in phospholipid composition [see the relative increase in phosphatidylserine and the relative decrease in acyl bis(monoacylglycero)phosphate].

Our combined biochemical, immunofluorescence, and electron microscopical results, together with previous electron micrographs of infected cells (10, 15) and the arguments already made by Payne and Kristenson (18), support the following model. In untreated cells those vaccinia virus particles which reach the cell periphery have been disseminated through large parts of the cytoplasm in a double-membranate vesicular structure. Fusion of its outer membrane with the plasma membrane releases a "classical" poxvirus virion now situated in a single membranated vesicle (see, for instance, the micrographs in reference 10). This enwrapped virus, when purified by density gradient centrifugation (buoyant density, 1.237 g/cm^3), reveals a higher phospholipid content and a different phospholipid composition than those of so-called intracellular virus. The latter type is generally isolated by lysis of cells late in infection, when electron micrographs reveal that there are relatively few enwrapped virions but that the whole cell is filled with morphologically mature virions free of enwrapping structures (18). Purification by density gradient centrifugation leads to a major product showing a buoyant density of 1.272 g/cm^3. The 37K viral protein seems to be associated only with those particles in the cytoplasm which are vesicularized, thus allowing a discrimination between free and enwrapped virions by immunofluorescence microscopy. IMCBH seems to prevent poxvirus particle vesicularization and thereby may prevent further intracytoplasmic virus dissemination. Lack of vesicularization is paralleled by a lack of incorporation of the viral 37K protein. Further experiments are necessary to explore which viral, or perhaps cellular, function is inhibited by IMCBH and which roles 37K and other viral proteins play during the dissemination process.

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