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Morphogenesis of Sindbis Virus in Three Subclones of *Aedes albopictus* (Mosquito) Cells

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The morphogenesis of Sindbis virus in three *Aedes albopictus* subcloned cell lines was examined. Each line was distinguishable with respect to morphology, cytopathic response to infection, and progeny yield. C7-10 cells, which produced the highest titers of virus and exhibited the most severe cytopathic response, were characterized ultrastructurally by the presence of budding particles at the cell surface and at the membranes of internal vesicles. C6/36 cells, which displayed a moderate cytotoxic response, manifested similar features in response to Sindbis virus infection. Both cell types also produced a structure composed of an electron-dense matrix in which nucleocapsids were embedded. Internally matured virions were released by exocytosis from these cells. In addition to a lack of cytopathic effect, u4.4 cells also failed to exhibit obvious morphogenetic changes upon infection. Virus particles were occasionally seen within vesicles, but budding at the cell surface was not detected. The mechanism of release of internally matured virions was not apparent. These studies provide further evidence that these three subcloned mosquito cell lines represent different tissues in the larval or adult insect.

Vectored viruses are successful in maintaining their life cycles in nature because they have acquired the ability to replicate in a variety of tissues within the arthropod host in addition to a broad range of vertebrate cell types. The abilities to overcome intrinsic barriers in the gut and salivary glands of the insect (8) and to multiply to titers sufficient to yield a productive infection upon inoculation into a vertebrate host distinguish the arthropod-borne viruses (arboviruses) and define their pathogenicity. Of particular interest is the phenomenon of how such pathogens infect invertebrate cells and mosquito vectors with no long-term deleterious effects while they induce cell destruction and disease in humans and animals.

The availability of continuous cell cultures derived from mosquito larvae has provided a significant opportunity for studying the interactions of arboviruses and their vector hosts at the cellular level. The original *Aedes albopictus* cell line generated by Singh (22) comprises a heterogeneous population of cell types cultivated from ground mosquito larvae. Early studies of alphavirus infection in these cultures revealed that the cells lapse into a persistently infected state after an acute phase of virus production without obvious cytopathic effect (5, 23).

Ultrastructural studies of alphavirus infection in these heterogeneous populations detailed a variety of mechanisms for virus maturation in these cells. Raghow et al. (14, 15) examined the morphogenesis of two alphaviruses, Ross River virus and Semliki Forest virus, and concluded that maturation of virus by budding through the plasma membrane is the primary source of infectious progeny. A study of Sindbis virus (SV) infection by Stollar et al. (24) provided evidence that virions can be released from infected *A. albopictus* cells both by budding through the plasma membrane and by budding into internal vesicles which expel their contents extracellularly.

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Two cell lines subcloned from the original *Aedes* cultures have been examined at the electron microscope level. Simizu and Maeda (21) briefly described the ultrastructural features of Western equine encephalitis virus infection in C6/36 cells (9) but did not elaborate on temporal morphogenesis or the mechanism of virus release from these cells. Gliedman et al. (7) detailed the time sequence of SV infection in mosquito cells, which they refer to only as *A. albopictus* cells but are actually a subcloned cell line later designated u4.4. These authors observed that virus maturation occurs almost exclusively within membrane-bound structures inside the cells and that infectious progeny are apparently released by reverse phagocytosis.

SV infection has been studied extensively in this laboratory at the biochemical level in three cell lines subcloned from Singh's original cultures: u4.4, derived in this laboratory in 1973; C6/36, isolated from the cloned C6 cell line (9); and C7-10, subcloned from the LT C-7 clone (18). Data from these studies have indicated that each of these three cell lines responds differently to such virus-associated phenomena as homologous interference (3), actinomycin D treatment (2), and production of and response to an antiviral factor (4). Such variations suggest that these cultures may represent partially differentiated larval cell types which may possess properties characteristic of various tissues in the whole insect. These observations have prompted us to undertake a comparative ultrastructural investigation of viral morphogenesis in these cells to document differences in this aspect of alphavirus infection. The data presented here show that these cell lines are distinguishable by their quantitative, cytopathic, and morphogenetic responses to SV infection.

MATERIALS AND METHODS

Cells, virus, and media. The three *A. albopictus* subclones used in this study were obtained from different sources but were all derived from Singh's original larval isolates (22). The u4.4 cells were cloned and cultivated in this laboratory from cells provided by Sonya Buckley (Yale Arbovirus Research Unit, New Haven, Conn.). The C6/36 cell line was

provided by Kenneth Ekels (Walter Reed Army Institute of Research, Washington, D.C.). The C7-10 cell line was provided by Victor Stollar (Rutgers Medical School, New Brunswick, N.J.). All cells were maintained in Eagle's minimal essential medium (7) supplemented with 10% fetal calf serum, 2 mM glutamine, and 5% tryptose phosphate broth, as described before (16).

The heat-resistant strain of SV, SVHR, obtained from the collection of Elmer Pfefferkorn (Dartmouth Medical School, Hanover, N.H.), was used to infect the mosquito cells. This SV variant has been described before (1).

A. albopictus cells were infected in 75-cm^2 tissue culture flasks with SV suspended in 1 ml of phosphate-buffered saline (PBS) containing 3% fetal calf serum, at a multiplicity of infection of approximately 100 PFU per cell. After a 1-h adsorption period at room temperature, the cells were washed three times in PBS, and 10 ml of fresh medium was added to the flasks. Cells were incubated at 28°C. Mock-infected cells were handled in an identical manner except for the omission of virus. At designated time points, aliquots of medium were collected from flasks of each cell line and assayed for virus by plaque production on BHK monolayers (16).

Scanning electron microscopy. Cells of each of the three mosquito subclones were allowed to adhere to glass coverslips and infected with SV. At 4 days postinfection (p.i.), cells on coverslips were washed once in PBS and fixed in 2.0% glutaraldehyde in PBS for 2 h at room temperature. Following three washes in PBS, cells were postfixed in 1.0% osmium tetroxide in PBS for 1 h at room temperature. The samples were dehydrated by passage through a graded ethanol series followed by passage through a graded amyl acetate-ethanol series. The amyl acetate-infiltrated cells were critical-point-dried with a Samdri-790 CPD apparatus (Tousimis Research Corp., Rockville, Md.) and coated with gold-palladium in a sputter coater (Ladd Industries, Inc., Burlington, Vt.). The specimens were photographed on a Phillips 515 scanning electron microscope operated at an accelerating voltage of 20 kV and a spot size of 20 nm. The images were recorded on Polaroid 55 P/N films and developed for 20 s.

Transmission electron microscopy. At various times after infection, cells were washed three times in PBS and fixed in 2.0% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.2) at 4°C for a minimum of 1 h. Fixed cells were washed three times in buffer and postfixed for 30 min in 1% osmium tetroxide in the same buffer. Cell pellets were embedded in 1% agarose and dehydrated through a graded ethanol-acetone series. Final embedding was in Mollenhauer's Epon-Araldite epoxy mixture no. 2 (13). Ultrathin sections were cut on a Sorvall MT5000 microtome and collected on copper grids. Sections were stained with uranyl acetate only or in addition to lead citrate and photographed at 60 kV in a JEOL 100CX transmission electron microscope.

RESULTS

Production of SV and cytopathic response in three mosquito cell lines. Figure 1 shows the kinetics of SV growth in the three mosquito cell lines at 28°C during the acute phase of infection. Extracellular virus titers remained at high levels for approximately 7 days in u4.4 and C6/36 cells, after which the titers fell to levels characteristic of persistently infected cells. C7-10 cells maintained high titers of extracellular virus through 10 days after infection, which tapered to values

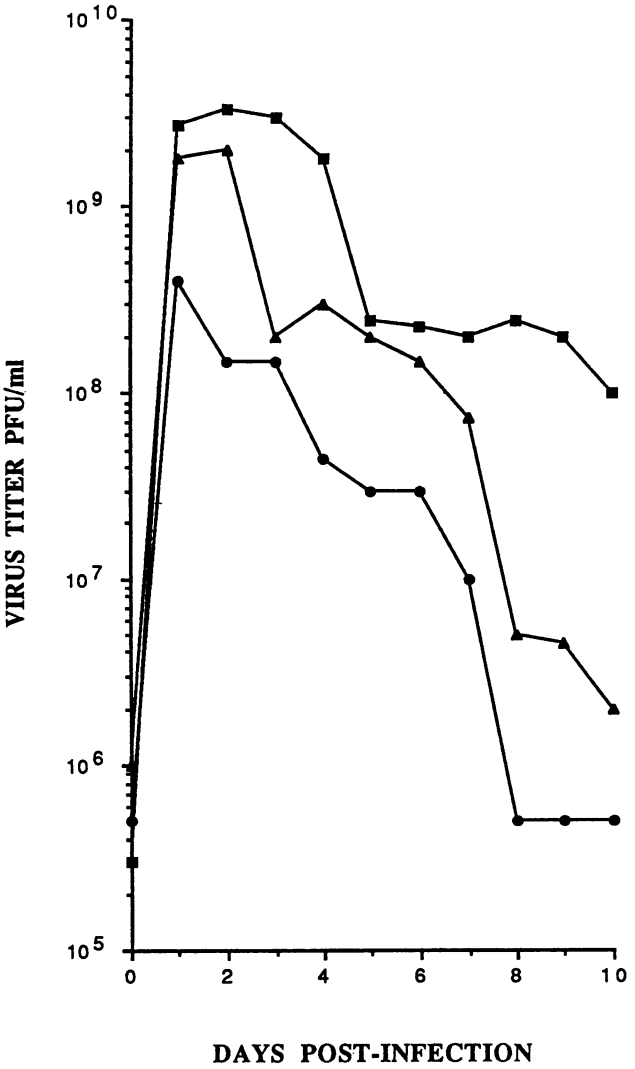

FIG. 1. Growth curves for SV in the three mosquito cell lines. Monolayers of C7-10 (■), C6/36 (▲), and u4.4 (●) cells were infected with SV at a multiplicity of infection of 100 PFU per cell for 1 h at room temperature and then incubated in full medium volume at 28°C. At the indicated time points, aliquots of medium were assayed for plaque production on BHK monolayers.

indicative of the persistent state at approximately 12 days p.i. (data not shown). This last subcloned line consistently yielded the highest titers of SV, with C6/36 cells producing slightly lower amounts and u4.4 cells generating the lowest levels of virus. Virus titers were determined by measuring the amount of virus accumulated in the medium up until the time of processing for electron microscopy. The decrease in titer during this phase of the infection is attributed to the natural instability of the virus over time at 28°C, readsorption of virions to the monolayer, and a reduction in the amount of virus released as the cells shift from an acutely infected condition to a persistently infected state.

Following infection with SV, these cell lines exhibited various degrees of cytopathic effect at the light-microscope level, which correlated with the amounts of virus released. The onset of cytopathology was most rapid in C7-10 cells, with obvious changes detectable in the monolayer at 24 h p.i.

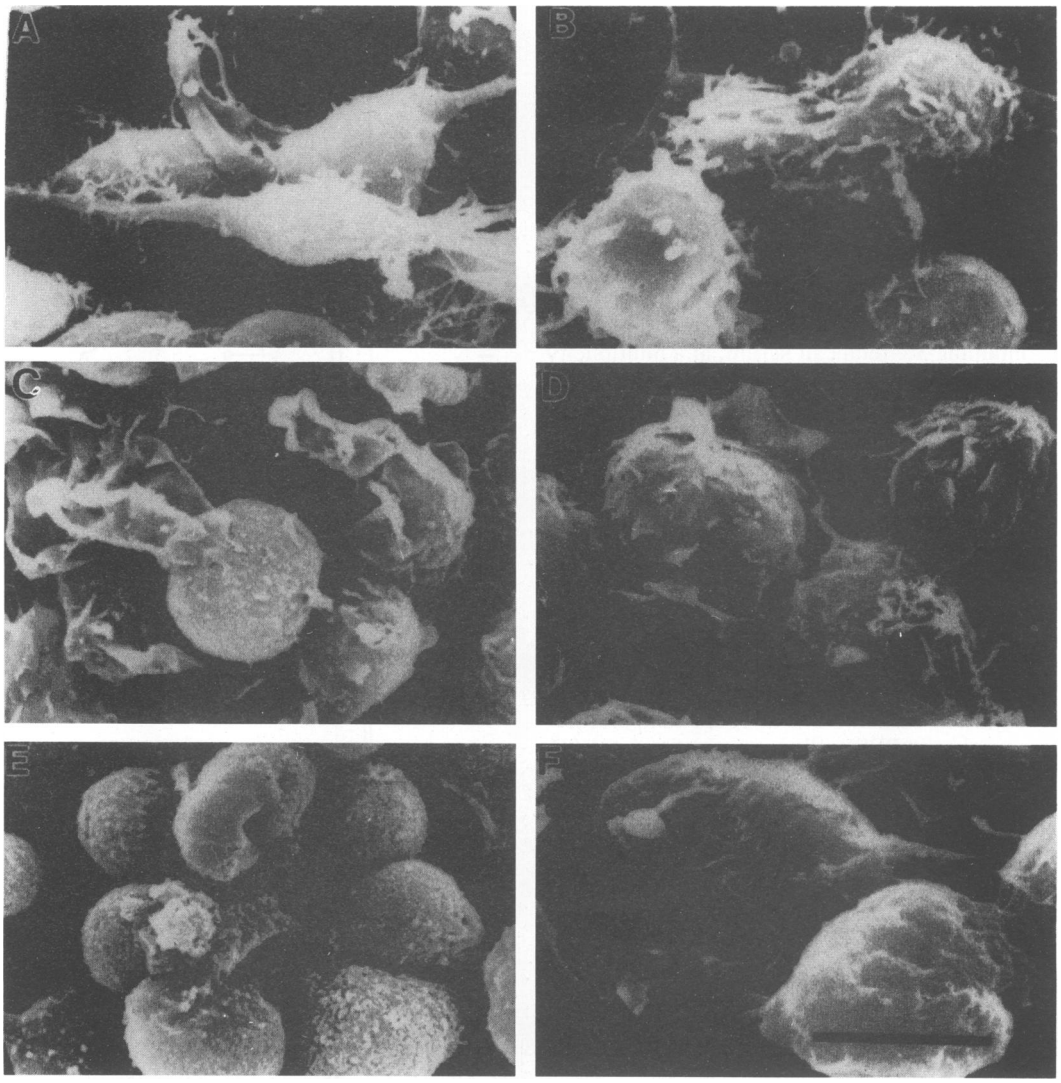

FIG. 2. Scanning electron microscopy of three mosquito cell lines, revealing surface morphology in the absence or presence of SV. Mock-infected (A, C, E) and infected (B, D, F) monolayers of the three subcloned cell lines were processed for microscopy as described in the text at day 4 p.i. (A and B) u4.4 cells; (C and D) C6/36 cells; (E and F) C7-10 cells. Bar, 10 μm.

and significant cell destruction apparent at 48 h after infection. This response was characterized by clustering of cells and dispersion of the monolayer. Cells appeared more fibroblastlike, exhibited an increase in the number of internal vesicles, and varied in diameter. By 5 days into the infection, these cells appeared to spread out and rearrange into a uniform layer and resumed the appearance of uninfected monolayers by 7 days p.i. C6/36 cells showed minimal cytopathology at 48 h p.i., which became more dramatic over the next 24 h. These cells exhibited the same cytopathic features as C7-10 cells but to a lesser degree and recovered within the same time period. No cytopathic response was detected in u4.4 cells at any time throughout the acute infection period (data not shown).

Scanning electron microscopy. Scanning electron micrographs revealed differences in the morphology as well as the cytopathic response of the three mosquito cell lines. Figure 2 shows the arrangement of cells in the monolayer at 4 days p.i. or after mock infection. Cells of the u4.4 line failed to change in size or appearance in response to SV infection. The other two subcloned lines showed an obvious change in the appearance of the monolayer and in cell size and surface morphology. Whereas u4.4 cells retained the spiny projections and cellular processes in the presence and absence of infection (Fig. 2A and B), C6/36 cells assumed a rougher surface appearance, with wispy, flaplike projections, which appeared more fragile than those of their mock-infected counterparts (Fig. 2C and D). Many cells appeared larger in diameter and were spread into a less dense distribution and flattened onto the substrate. SV-infected C7-10 cells showed the least resemblance to mock-infected cells, also appearing larger and less rounded, and showing smooth surfaces interspersed with wrinklelike ridges. These figures also demonstrated the differences in morphology among the three cell lines in the absence of virus infection (Fig. 2A, C, and E).

Electron microscopy of SV-infected C7-10 cells. Mock-infected C7-10 cells were round and contained a variety of vesicles which varied in diameter and contents. Fusion of

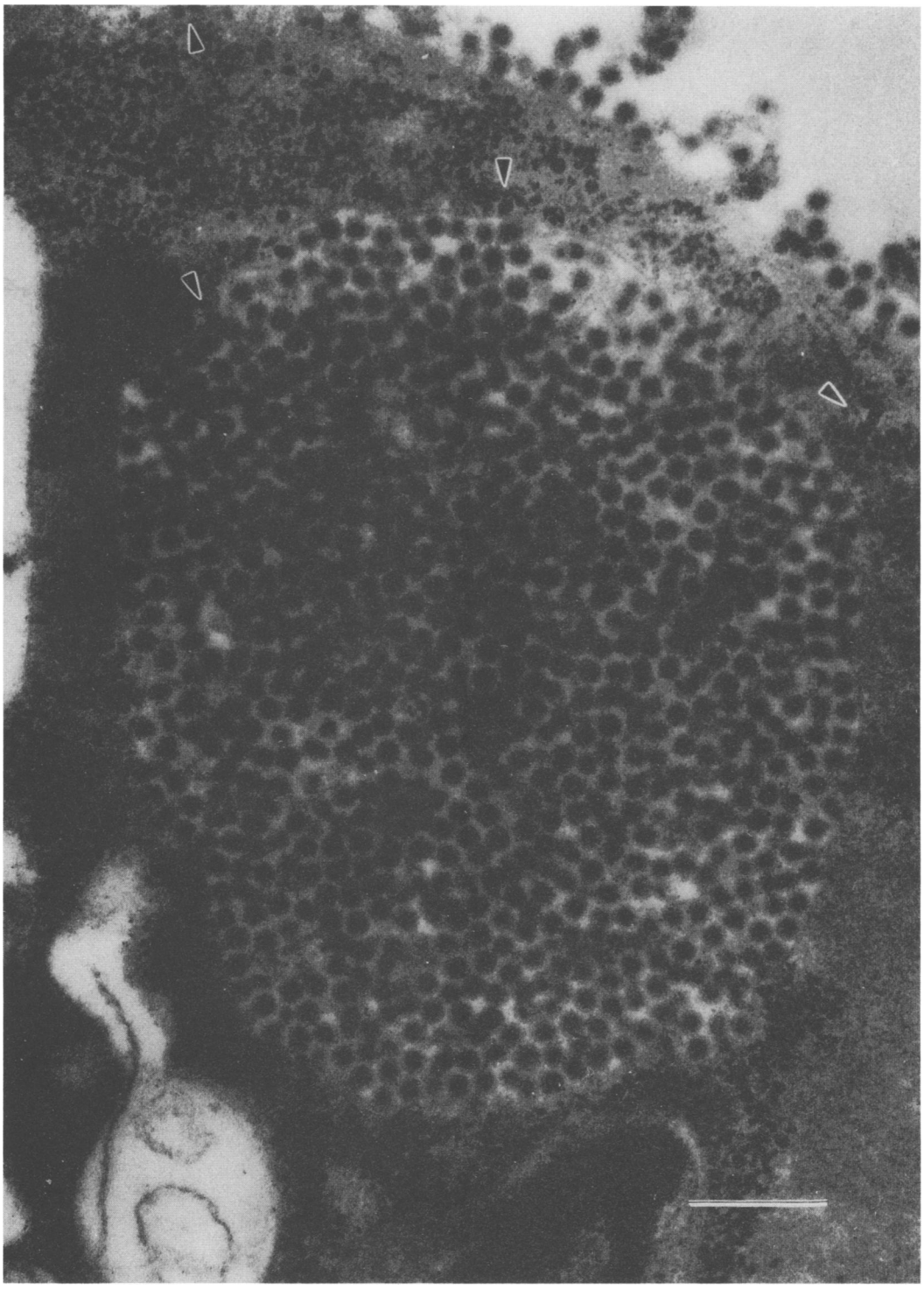

FIG. 3. C7-10 cell containing a vesicle packed with virus particles which have acquired membranes by budding through the limiting membrane of this structure. Nucleocapsids are detectable in the surrounding cytosol and at the plasma membrane (arrowheads). Particles in various stages of maturation line the cell surface (50 h p.i.). Bar, 0.5 μm.

these vesicles, best described as the "pouring" of the contents of one vesicle into another, was apparent. After 25 to 30 h under mock-infected conditions, an apparent increase in the number of vesicles was the only detectable change. There was no evidence of endogenous virus (data not shown).

Morphological structures characteristic of SV infection in C7-10 cells were detected at 8 h p.i. Virus particles in various

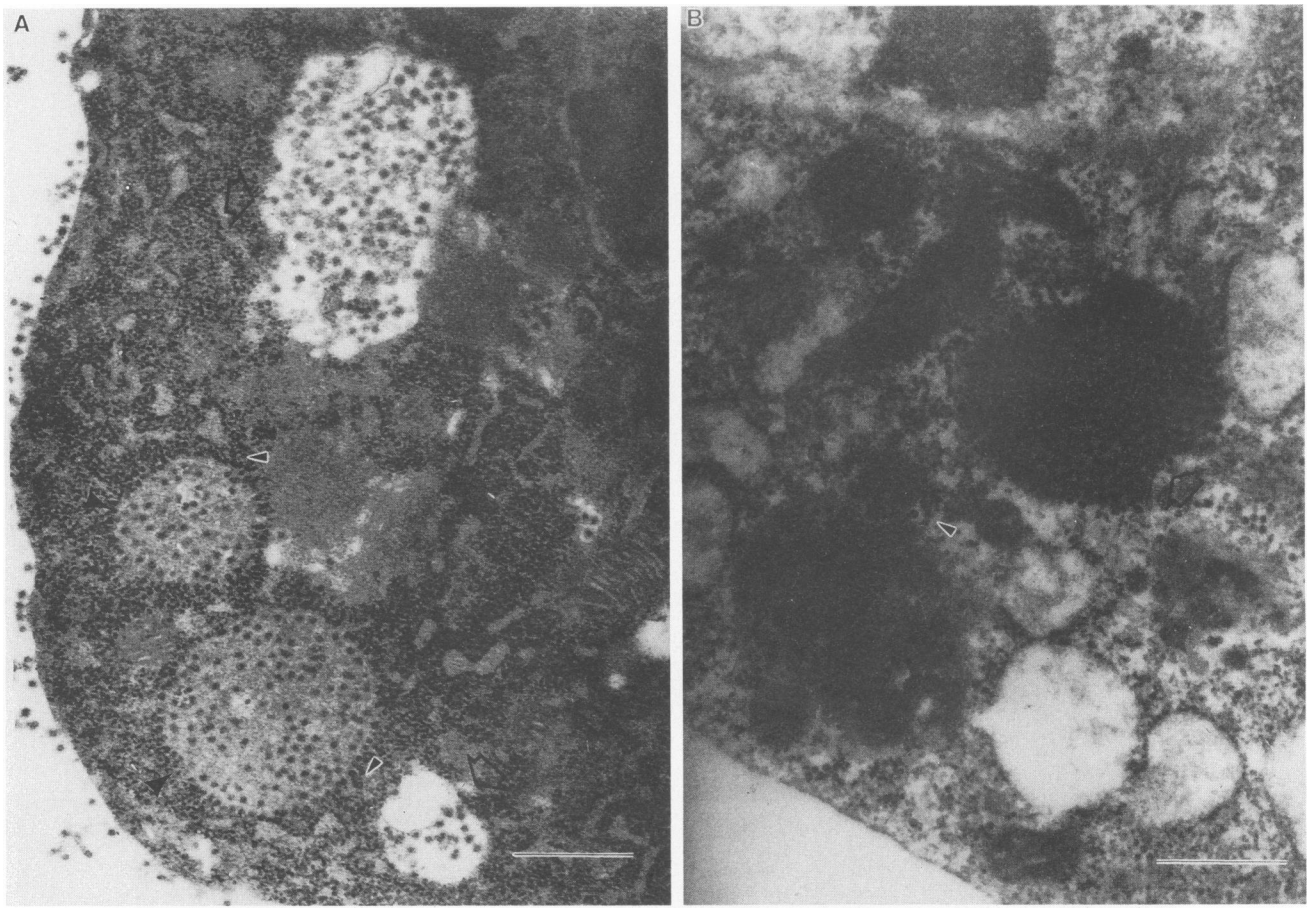

FIG. 4. C7-10 cells displaying different types of amorphous matrices in which nucleocapsids are embedded. (A) Structures with no apparent limiting membrane (solid arrowheads) in proximity to vesicles in which virions have collected (large, open arrows); (B) a vesicle containing nucleocapsids embedded in an electron-dense matrix, also in proximity to a vesicle packed with virions arranged in a paracrystalline array (open arrow). Nucleocapsids are detectable in the surrounding cytoplasm in both figures (white-outlined arrowheads) (30 h p.i.). Bar, 0.5 μm. Panel A is reprinted from reference 12 with permission of Springer-Verlag, New York.

stages of budding were present at the plasma membrane of a few cells. At 10 h p.i., vesicles containing enveloped particles were seen in the cytoplasm (Fig. 3). Nucleocapsids were observed proximal to the membranes of these structures, apparently in various stages of budding into these vesicles. Within the next 2 h, cytoplasmic nucleocapsids and budding viral structures at the plasma membrane and at vesicular membranes were observed in most cells. Membrane-bound regions, containing nucleocapsids embedded in an electron-dense, amorphous material, were detected in the cytosol of some cells. Similar structures, containing amorphous material having the same appearance, were never detected in mock-infected cells. These regions did not appear to extend or originate from existing organelles, and ribosomes were not seen in association with these structures. In many cases, these structures were obviously membrane bound but were also observed with no apparent limiting bilayer. These areas varied in the degree of electron density that they imparted and were often found in proximity to vesicles into which nucleocapsids appeared to be budding (Fig. 4A and B).

At 25 h p.i., when the highest titers of extracellular virus were obtained, most cells appeared intact. Evidence that the vesicles containing virions fused within the cells was demonstrated by the presence of "channels" filled with virus particles and other materials normally seen within vesicles. These channels were occasionally observed to have opened to the exterior of the cell, indicating that the membranes of the vesicles fused not only internally, but ultimately with the plasma membrane, allowing release of virions from the cells (Fig. 5). As demonstrated in this figure, virus release was often observed to be localized to a specific region of the cell.

By 30 to 35 h p.i., large amounts of virus were seen in the extracellular medium and adhered in large clusters to the cell surfaces. Most cells possessed several amorphous, electron-dense regions which contained nucleocapsids in addition to numerous vesicles filled with enveloped particles. There were also many nucleocapsids free in the cytoplasm, and budding figures were apparent at the cell surface.

As the infection progressed, signs of cytopathic effect became obvious at the ultrastructural level. Many cells contained osmophilic bodies and vesicles filled with densely stained, membranous strands. Cell fragments and debris were frequently observed, and many cells revealed obvious signs of degeneration. During the next 48 h, cells continued to manifest a marked cytopathic response while retaining the structures characteristic of SV infection. By 4 days p.i., a greater percentage of cells appeared intact and still showed

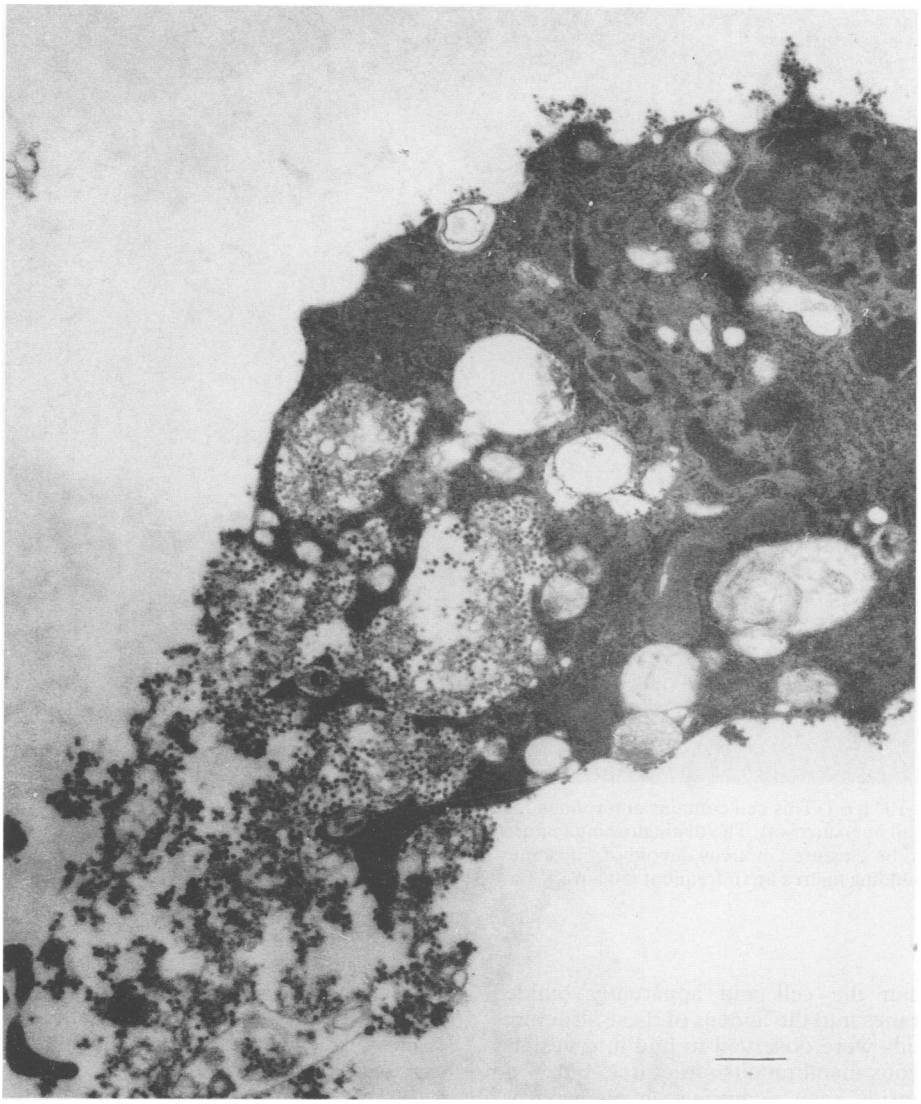

FIG. 5. C7-10 cell releasing SV. Vesicles have fused with one another and ultimately with the plasma membrane at one end of the cell, allowing the release of their internal contents from the cell (50 h p.i.). Bar, 1.0 μm. Reprinted from reference 12 with permission of Springer-Verlag, New York.

evidence of internal virus maturation and budding at the plasma membrane.

The only notable change at 4 to 5 days p.i. was an apparent increase in the number of nucleocapsids free in the cytoplasm and an apparent decrease in the number of nucleocapsids seen to be in the stages of budding, either internally or at the plasma membrane. Many regions in the cytoplasm, especially near the cell periphery, appeared devoid of ribosomes and other cytoplasmic ground substance, yet nucleocapsids were clustered in these areas (Fig. 6).

Throughout the course of the infection, there were no apparent changes in the nucleus or in the appearance of other organelles. By approximately 25 h p.i., structures of every type described were found in cells, even up to 5 days p.i. Both budding into internal vesicles and budding at the plasma membrane were observed in the same cells, indicating that neither maturation route predominated in a given cell.

Electron microscopy of SV-infected C6/36 cells. Mock-infected C6/36 cells had a dense cytoplasm interspersed with ribosomes. These cells contained an assortment of vesicles, some empty and others with contents varying from fine particulate material to membranous structures and electron-dense, amorphous matter. Vesicles also varied in diameter and number among cells. At 25 h under mock-infection conditions, these cells exhibited a slight increase in the number of vesicles. At approximately 40 to 50 h under conditions of mock infection, the increase in vesicles was more apparent, and osmophilic bodies, which in our experience represent a stress response in these cells, were seen more frequently. There was no evidence of endogenous virus (data not shown).

In SV-infected C6/36 cells, the first signs of infection were the presence of nucleocapsids in the cytoplasm and budding of viruses at the plasma membrane in a small number of cells. These structures appeared at approximately 6 h p.i. and were more numerous at 8 h p.i. Within the next 2 h, nucleocapsids lined the cytoplasmic faces of membrane-

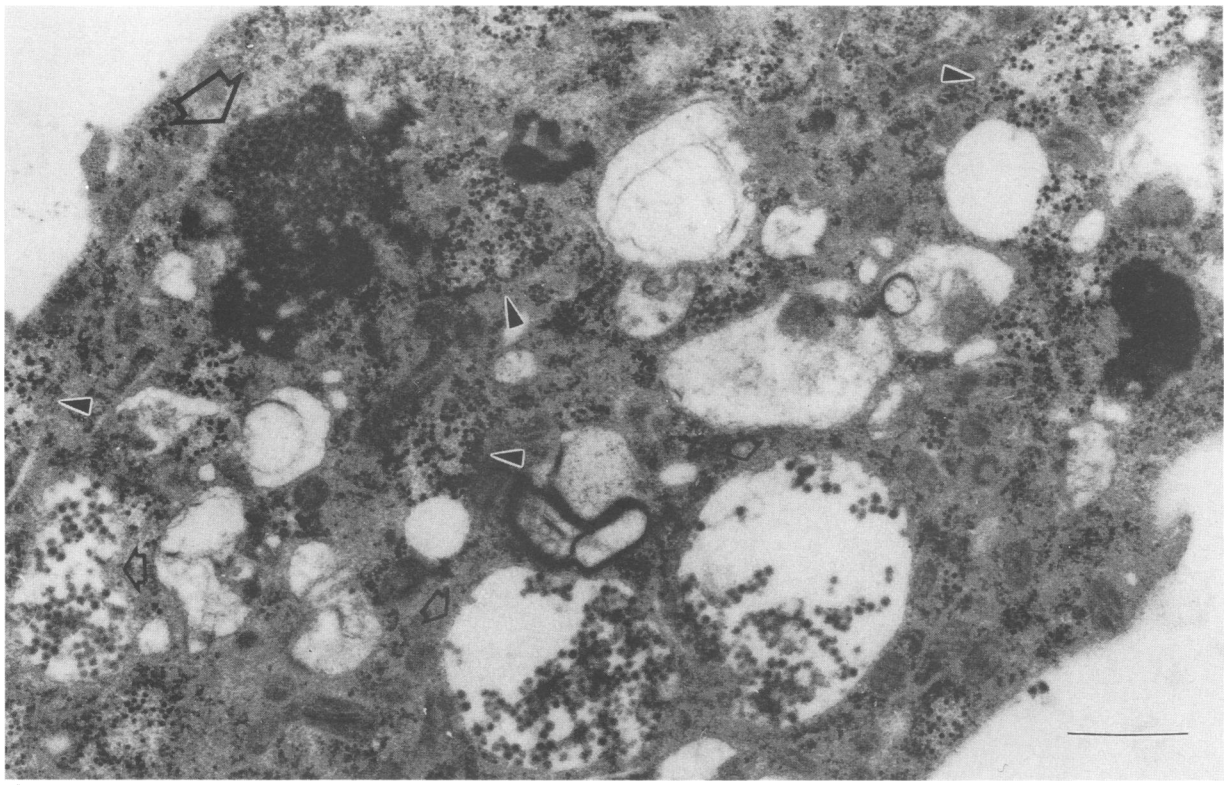

FIG. 6. C7-10 cell at 100 h p.i. This cell contains amorphous regions containing nucleocapsids (large open arrow) and vesicles packed with enveloped particles (small open arrows). The distinguishing feature of infection at this point is the large number of cytoplasmic nucleocapsids, most of which appear to be clustered in areas devoid of ribosomes and cytoplasmic ground substance (arrowheads). Virions are seen at the cell exterior, but viral budding figures are infrequent (50 h p.i.). Bar, 1.0 μm. Reprinted from reference 12 with permission of Springer-Verlag, New York.

bound vesicles within the cell and apparently budded through these membranes into the lumens of these structures (Fig. 7). Nucleocapsids were observed to bud into vesicles which contained various membranous structures, but at no time were nucleocapsids seen acquiring envelopes from membranes located within the vesicle interior.

By 12 to 15 h p.i., evidence of internal maturation and external budding was more apparent, in addition to cytoplasmic accumulations of nucleocapsids with no obvious association with any membranous structures. Structures containing nucleocapsids embedded in an amorphous, electron-dense matrix were also demonstrated at this time, in addition to vesicles tightly packed with enveloped particles. These virions were clustered randomly or as partially ordered arrangements within the vesicles but were also occasionally assembled in paracrystalline arrays similar to those seen in C7-10 cells.

As the infection progressed and more virion-filled vesicles were detected, the membranes of the internal vesicles were often found in proximity with one another, facilitating fusion. That such fusion was occurring was evident by the presence of channels within these cells, which in many cases had opened to the cell exterior, allowing release of the virions and other contents (Fig. 8). In addition to release of mature particles, these cells were also observed to release nucleocapsids without obvious membranes and other components of the infection, including the electron-dense matrices shown in Fig. 4 (Fig. 9). As was observed in C7-10 cells, at 4 days p.i., the number of budding structures at the surfaces of C6/36 cells decreased and regions in the cytoplasm were observed which appeared to be devoid of ribosomes and cytoplasmic ground substance yet contained small clusters of nucleocapsids.

Electron microscopy of SV-infected u4.4 cells. The u4.4 subcloned line exhibited an ultrastructural morphology distinct from those of the other cell lines studied. Mock-infected cells were rectangular, with many small vesicles which contained a variety of materials. Cytoplasmic ground substance was uniformly dense and studded with ribosomes. At 15 h under mock infection conditions, vesicles appeared which contained membranous strands and resembled the structures described by Gliedman et al. (7). There was no consistent temporal increase in the size or number of these vesicles. These structures were seen merging with one another within the cell. There was no indication of endogenous virus (data not shown).

Evidence of SV production was observed very infrequently in these cells, and extracellular release of internally matured virus was never detected. At approximately 10 h p.i., vesicles containing membranous strands, similar to those seen in mock-infected cells, were evident, but at no time were these structures observed to contain nucleocapsids or mature particles. Some extracellular viruses were detected at the cell surface, but there were no obvious budding structures, and cytoplasmic nucleocapsids were seen only rarely. Over the next several hours, during the acute phase of infection, mature virus particles were infrequently observed within membrane-bound structures (Fig.

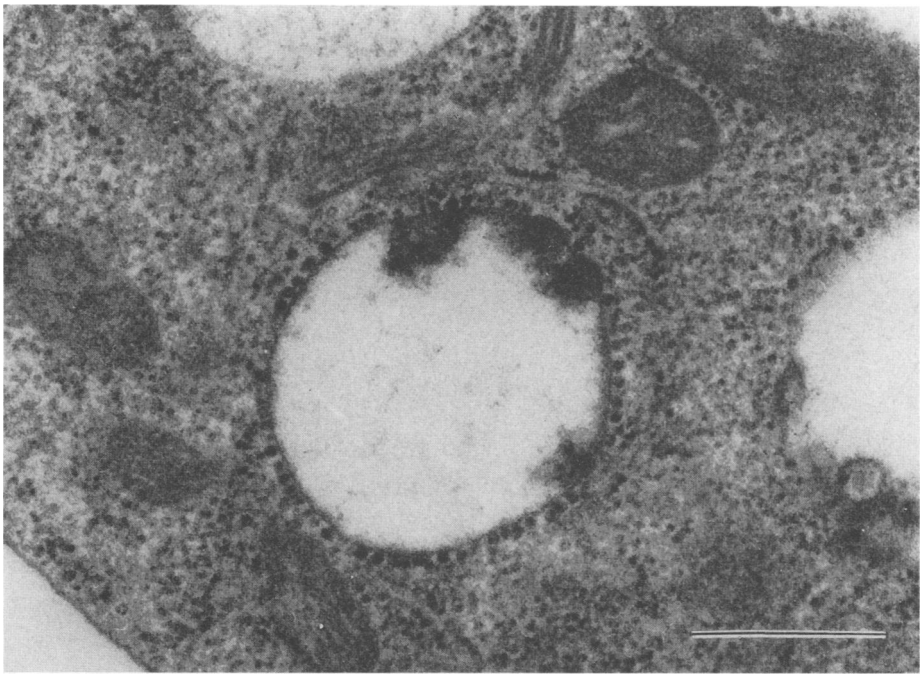

FIG. 7. Nucleocapsids lining vesicular membranes in SV-infected C6/36 cell. Nucleocapsids outline the exterior surfaces of membrane-bound vesicles in which mature particles are starting to accumulate (30 h p.i.). Bar, 0.5 μm.

10). During the time points coinciding with peak virus production, virions were seen more frequently within vesicles, but there was no evidence of exocytosis of internally matured particles at these or subsequent time points.

Determination of infectivity of internally matured virions. To determine whether virus matured internally was infectious, cells of each line, infected with SV for 25 h, were washed repeatedly in PBS to remove external virus and disrupted in a Dounce homogenizer as described before (17, 19). Virus titers in the resulting cell extracts were determined by the plaque assay. The values obtained represent a 20, 13, and 4% increase in overall virus yield from C7-10,

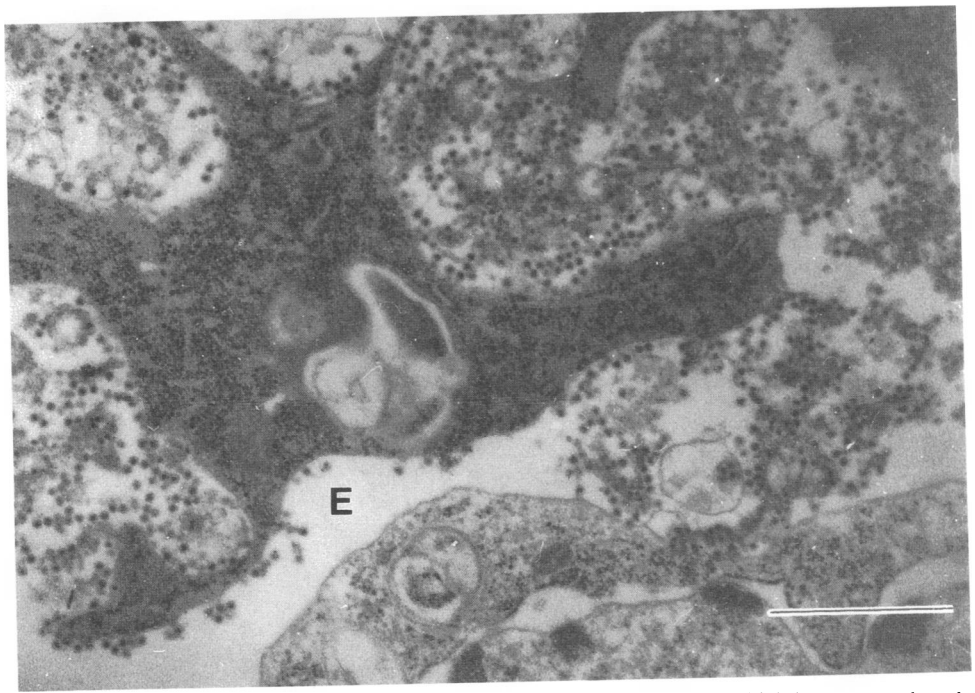

FIG. 8. Release of SV from C6/36 cell. Vesicles filled with virions have fused into channels which have opened to the cell exterior (E), allowing release of the contents (50 h p.i.). Bar, 1.0 μm.

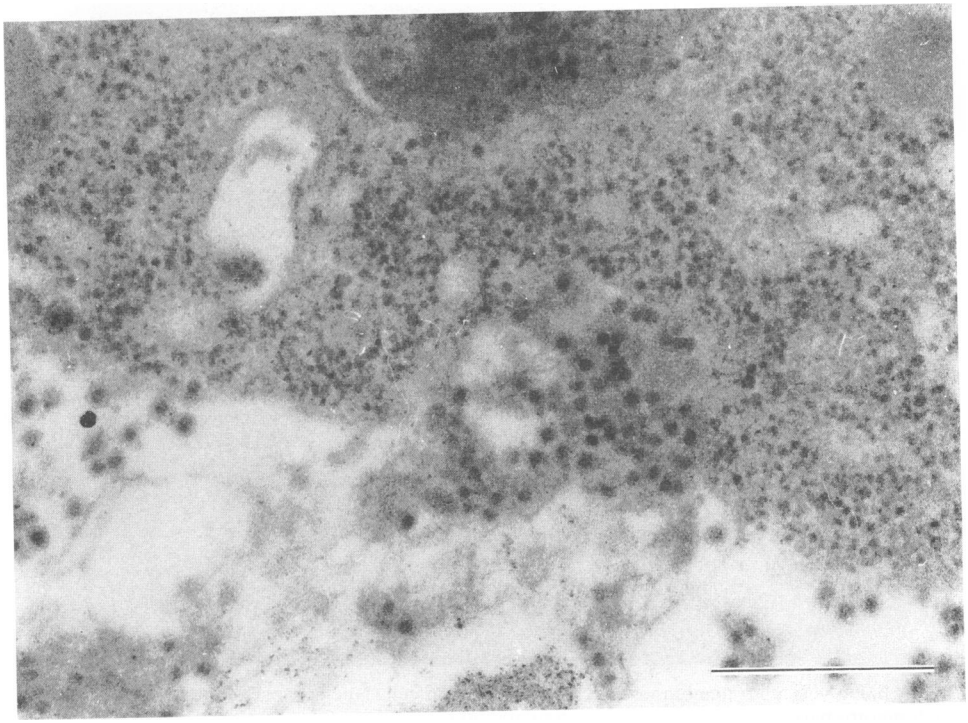

FIG. 9. Release of amorphous structure from C6/36 cell. A vesicle containing nucleocapsids embedded in an amorphous matrix is exocytosed from the cell (50 h p.i.). Bar, 0.5 μm.

C6/36, and u4.4 cells, respectively (data not shown), in comparison to titers of extracellular virus. These data indicate that at 25 h p.i., during the peak period of virus release, these percentages of total infectious virions were inside the cells. A similar experiment, conducted with BHK cells, showed that during the period of virus release, less than 0.01% of the total infectious virus was cell associated (19).

Immunofluorescence of three mosquito cell lines. Because

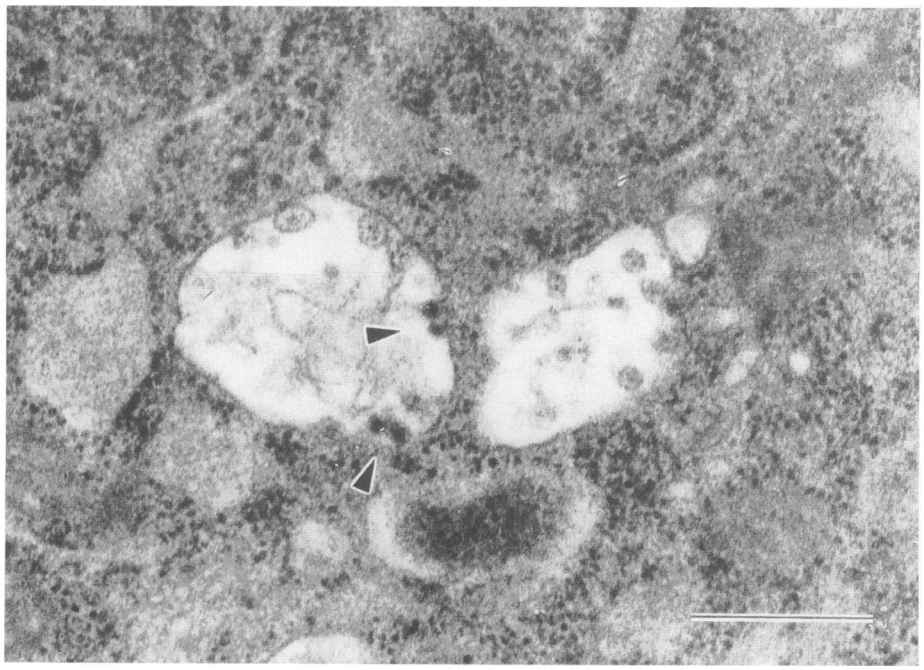

FIG. 10. SV particles in u4.4 cells. Virions are contained within a vesicle (arrowheads) containing other membranous structures and amorphous materials. There are no nucleocapsids visible in the cytoplasm (30 h p.i.). Bar, 0.5 μm.

so few u4.4 cells demonstrated evidence of SV production at the ultrastructural level, these cells were examined by immunofluorescence to determine the number of u4.4 cells which were actually infected by SV. As indicated by the response to treatment with rabbit polyclonal anti-SV serum, 100% of the u4.4 cells appeared to be positive for the production of viral antigens. Also, 100% of the C6/36 and C7-10 cells demonstrated the presence of viral proteins by immunofluorescence (data not shown).

DISCUSSION

The morphogenesis of alphaviruses in vertebrate cells in culture is well documented. Hallmarks of the infection are the production of nucleocapsids in the cytoplasm and maturation of virions by budding through the plasma membrane, with subsequent cell destruction and death (for review, see references 12 and 20). In comparison, far less is understood about this process in cultured invertebrate cells.

It is evident from the observations at the light and scanning electron microscope levels that the three *A. albopictus* subcloned lines examined in this study are morphologically distinct, which is in support of the suggestion that these three cell lines represent different cell types which may exist in the whole mosquito (2–4). However, since previous studies with whole insects have revealed that cytopathic effect in tissues of alphavirus-infected adult mosquitoes is minimal or nonexistent (10, 26–30), no obvious correlations can be made between cell lines such as C6/36 and C7-10 and specific insect tissues. Since the original *A. albopictus* culture was generated from mosquito larvae, the subcloned lines which manifest significant cytopathic response may represent larval tissue which is degenerated by histolysis as the insect undergoes a variety of developmental forms (13) and are therefore not present in the adult mosquito. On the basis of studies of alphavirus-infected mosquitoes, the u4.4 cell line is the most suitable candidate for representation in the adult insect.

These data show a direct correlation between the amount of virus produced by the three *A. albopictus* subclones and the degree of cytopathology manifested during the acute phase of SV infection, similar to observations made regarding Semliki Forest virus infection in *A. albopictus* clones (25). In the cell lines which exhibit cytopathology, recovery of the culture coincides with a reduction in release of virus from the cells. That u4.4 cells also show a reduction in virus release over a similar time course implies that the host response which regulates cytopathology in these subclones is different or that the same modulating host response is manifested much later in cells which establish cytopathic effect. The u4.4 cells may possess this modulating function at the onset of infection and produce it continuously, presumably for another function in the cell. In comparison, vertebrate cells apparently never produce this modulating factor, or at least not rapidly enough to affect recovery from the infection. The response of C7-10 cells, therefore, may represent an intermediate example of this modulating function, as these mosquito cells are most like vertebrate cells in their response to alphavirus infection yet are able to bring the infection under control in time to establish persistence.

The observations on the morphogenesis of SV in u4.4 cells correlate with those made by Gliedman et al. (7). Only rarely were nucleocapsids seen within the cytoplasm of these cells, similar to what Gliedman et al. reported. The mechanism of maturation in u4.4 cells remains obscure. That 100% of u4.4 cells exhibited the presence of viral antigen as determined by immunofluorescence is somewhat paradoxical, considering that nucleocapsids and virus particles were detected within a very small number of cells. This phenomenon may be explained by the possibility that all of the cells are extremely efficient at releasing virus or that only a small subpopulation of cells is capable of generating viral proteins and RNA species which can be successfully assembled into infectious particles.

In C6/36 and C7-10 cells infected with SV, no new vesicle types present only in virus-infected cells were observed. Viruses appeared to bud through the membranes of vesicles which were already present for another purpose. Also, the particles budded only through the outer membranes of these vesicles and were not seen budding through the membranous strands seen within some vesicles. These structures were frequently observed to fuse with one another in both infected and mock-infected cells, although the phenomenon of channel formation was only detected in cells producing virus. This fusion may represent a mechanism for movement of materials within and from the cell.

The amorphous regions which contained nucleocapsids (Fig. 4, 6, and 9) were the only structures produced in C6/36 and C7-10 cells as an apparent direct response to SV infection. These regions may be the sites of synthesis of nucleocapsids, which are then transferred into the cytoplasm, or these regions may represent nucleocapsids which have been sequestered from the cytoplasm because they were recognized as foreign substances which will ultimately be exported from the cell. The observation that these regions often stained densely in the presence of uranyl acetate, a stain specific for nucleic acid, might argue in favor of the first possibility. However, if this is the case, there was no apparent mechanism for the release of nucleocapsids from these structures, which were frequently observed to be membrane bound. There is evidence that these structures are expelled from C6/36 cells, as demonstrated in Fig. 9, which would support the idea that these structures represent collection centers for foreign materials destined for release from the cell.

This study also demonstrates that the viruses matured internally are infectious, as indicated by an increase in titer following mechanical disruption of these cells. This substantiates similar observations made by Scheefers-Borchel et al. (19). These data also provide direct evidence that virus is released from mosquito cells by fusion of internal vesicles with the plasma membrane.

Our studies indicate that among cell clones derived from a single parental *A. albopictus* population, the host response varies significantly, and some cell types are better adapted to restrict the process of virus replication and thus avoid the deleterious effects of a cytopathic response. Such differences may reflect similar variations among certain tissues in the larval or adult form of the insect with respect to whether they are resistant or susceptible to alphavirus infection. The demonstration that specific cell types show cytopathology upon infection with alphaviruses in both in vitro and in vivo systems may provide insight into the mechanisms which restrict the host range of many viruses to one or, at most, a very few insect species.

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REFERENCES

1. **Burge, B. W., and E. R. Pfefferkorn.** 1966. Isolation and characterization of conditional-lethal mutants of Sindbis virus. Virology **30**:204–213.
2. **Condreay, L. D., R. H. Adams, J. Edwards, and D. T. Brown.** 1988. Effect of actinomycin D and cycloheximide on replication of Sindbis virus in *Aedes albopictus* (mosquito) cells. J. Virol. **62**:2629–2635.
3. **Condreay, L. D., and D. T. Brown.** 1986. Exclusion of superinfecting homologous virus by Sindbis virus-infected *Aedes albopictus* (mosquito) cells. J. Virol. **58**:81–86.
4. **Condreay, L. D., and D. T. Brown.** 1988. Suppression of RNA synthesis by a specific antiviral activity in Sindbis virus-infected *Aedes albopictus* cells. J. Virol. **62**:346–348.
5. **Davey, M. W., D. P. Dennett, and L. Dalgarno.** 1973. The growth of two togaviruses in cultured mosquito and vertebrate cells. J. Gen. Virol. **20**:225–232.
6. **Eagle, H.** 1959. Amino acid metabolism in mammalian cell cultures. Science **130**:432–437.
7. **Gliedman, J. B., J. F. Smith, and D. T. Brown.** 1975. Morphogenesis of Sindbis virus in cultured *Aedes albopictus* cells. J. Virol. **16**:913–926.
8. **Hardy, J. L., E. J. Houk, L. D. Kramer, and W. C. Reeves.** 1983. Intrinsic factors affecting vector competence of mosquitoes for arboviruses. Annu. Rev. Entomol. **28**:229–262.
9. **Igarashi, A.** 1978. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to Dengue and Chikungunya viruses. J. Gen. Virol. **40**:531–544.
10. **Larsen, J. R., and R. F. Ashley.** 1971. Demonstration of Venezuelan equine encephalitis virus in tissues of *Aedes aegypti*. Am. J. Trop. Med. Hyg. **20**:754–760.
11. **Lockshin, R. A.** 1985. Programmed cell death, p. 301–317. *In* G. A. Kerkut and L. I. Gilbert (ed.), Comprehensive insect physiology, biochemistry and pharmacology. Pergamon Press, Inc., New York.
12. **Miller, M. L., and D. T. Brown.** 1991. Alphavirus infection in cultured tissue cells. Adv. Dis. Vector Res. **8**:107–142.
13. **Mollenhauer, H. H.** 1964. Plastic embedding mixtures for use in electron microscopy. Stain Technol. **49**:305–308.
14. **Raghow, R. S., M. W. Davey, and L. Dalgarno.** 1973. The growth of Semliki Forest virus in cultured mosquito cells: ultrastructural observations. Arch. Gesamte Virusforsch. **43**: 165–168.
15. **Raghow, R. S., T. D. C. Grace, B. K. Filshie, W. Bartley, and L. Dalgarno.** 1973. Ross River virus replication in cultured mosquito and mammalian cells: virus growth and correlated ultrastructural changes. J. Gen. Virol. **21**:109–122.
16. **Renz, D., and D. T. Brown.** 1976. Characteristics of Sindbis virus temperature-sensitive mutants in cultured BHK-21 and *Aedes albopictus* (mosquito) cells. J. Virol. **19**:775–781.
17. **Riedel, B., and D. T. Brown.** 1977. Role of extracellular virus in the maintenance of the persistent infection induced in *Aedes albopictus* (mosquito) cells by Sindbis virus. J. Virol. **23**:554–561.
18. **Sarver, N., and V. Stollar.** 1977. Sindbis virus-induced cytopathic effect in clones of *Aedes albopictus* (Singh) cells. Virology **80**:390–400.
19. **Scheefers-Borchel, U., H. Scheefers, J. Edwards, and D. T. Brown.** 1981. Sindbis virus maturation in cultured mosquito cells is sensitive to actinomycin D. Virology **110**:292–301.
20. **Schlesinger, S., and M. J. Schlesinger.** 1990. Replication of Togaviridae and Flaviviridae, p. 697–711. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, Ltd., New York.
21. **Simizu, B., and S. Maeda.** 1981. Growth patterns of temperature-sensitive mutants of Western Equine Encephalitis virus in cultured *Aedes albopictus* (mosquito) cells. J. Gen. Virol. **56**:349–361.
22. **Singh, K. R. P.** 1967. Cell cultures derived from larvae of *Aedes albopictus* (Skuse) and *Aedes aegypti* (L.). Curr. Sci. **36**:506–508.
23. **Stevens, T. M.** 1970. Arbovirus replication in mosquito cell lines (Singh) grown in monolayer or suspension culture. Proc. Soc. Exp. Biol. Med. **134**:356–361.
24. **Stollar, V., K. Harrap, V. Thomas, and N. Sarver.** 1979. Observations related to cytopathic effect in *Aedes albopictus* cells infected with Sindbis virus, p. 277–296. *In* E. Kurstak (ed.), Arctic and tropical arboviruses. Academic Press, Inc., New York.
25. **Tooker, P., and S. I. T. Kennedy.** 1981. Semliki Forest virus multiplication in clones of *Aedes albopictus* cells. J. Virol. **37**:589–600.
26. **Weaver, S. C.** 1986. Electron microscopic analysis of infection patterns for Venezuelan equine encephalomyelitis virus in the vector mosquito, *Culex* (*Melanoconian*) *taeniopus*. Am. J. Trop. Med. Hyg. **35**:624–631.
27. **Weaver, S. C., T. W. Scott, L. H. Lorenz, K. Lerdthusnee, and W. S. Romoser.** 1988. Togavirus-associated pathologic changes in the midgut of a natural mosquito vector. J. Virol. **62**:2083–2090.
28. **Whitfield, S. G., F. A. Murphy, and W. D. Sudia.** 1971. Eastern equine encephalomyelitis virus: an electron microscopic study of *Aedes triseriatus* (Say) salivary gland infection. Virology **43**:110–122.
29. **Whitfield, S. G., F. A. Murphy, and W. D. Sudia.** 1973. St. Louis encephalitis virus: an ultrastructural study of infection in a mosquito vector. Virology **56**:70–87.
30. **Wiederhold, A. H., P. G. Jupp, and J. J. Alexander.** 1990. Sindbis and West Nile viruses: an electron microscope study of salivary gland infection in the vector mosquito *Culex univittatus*. J. Entomol. Soc. South Afr. **53**:141–149.