

Release of Simian Virus 40 Virions from Epithelial Cells Is Polarized and Occurs without Cell Lysis

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Received 26 August 1988/Accepted 25 January 1989

We have investigated the process of release of simian virus 40 (SV40) virions from several monkey kidney cell lines. High levels of virus release were observed prior to any significantly cytopathic effects in all cell lines examined, indicating that SV40 utilizes a mechanism for escape from the host cell which does not involve cell lysis. We demonstrate that SV40 release was polarized in two epithelial cell types (Vero C1008 and primary African green monkey kidney cells) grown on permeable supports; release of virus occurs almost exclusively at apical surfaces. In contrast, equivalent amounts of SV40 virions were recovered from apical and basal culture fluids of nonpolarized CV-1 cells. SV40 virions were observed in large numbers on apical surfaces of epithelial cells and in cytoplasmic smooth membrane vesicles. The sodium ionophore monensin, an inhibitor of vesicular transport, was found to inhibit SV40 release without altering viral protein synthesis or infectious virus production.

Several distinct mechanisms are involved in the release of animal viruses from infected cells. Many enveloped viruses are released as they are assembled by budding at the plasma membrane of host cells. In polarized epithelial cells, maturation of these viruses is restricted to either the apical or basolateral membrane (18, 19, 24). Some families of enveloped viruses are assembled by budding at intracellular membranes and are transported to the cell surface by a vesicular transport process (23, 24). In contrast to enveloped viruses, nonenveloped viruses are generally believed to be cytopolytic and are thought to be released following cell lysis (20).

Simian virus 40 (SV40) is a nonenveloped virus which is assembled in the nuclei of infected cells. The precise mechanism of SV40 release from infected cells is not known, but it is generally believed that virions exit the cell after nuclear destruction and cell lysis (8, 13, 14, 17). In previous studies with polarized epithelial cells, we have used SV40 vectors to investigate the cell surface localization of influenza virus glycoproteins expressed from cloned viral genes (11, 21). During these studies, recombinant SV40 virions were found to accumulate on the apical surfaces of apparently intact cells (unpublished observations), suggesting that they were being transported to the cell surface in a polarized manner. This observation was of interest because the polarized release of a nonenveloped virus from epithelial cells had not been previously reported, and this pattern of release would be inconsistent with cell lysis as the release mechanism.

To investigate the process of SV40 release, we have examined polarized and nonpolarized cell types infected with wild-type SV40. Virus yields were determined from cells grown on permeable supports to quantitate SV40 release from apical and basolateral surfaces. The distribution of virions within the cytoplasm and on the cell surface was examined by electron microscopy. The effects of the sodium

ionophore monensin on SV40 release were also determined. The results indicate that SV40 virions are released by a novel process which does not involve cell lysis.

MATERIALS AND METHODS

Virus and cells. A stock of SV40 (small-plaque strain) was obtained from J. Lebowitz. Virus stocks were grown in monolayers of CV-1 cells and harvested at 60 h postinfection by freezing and thawing the crude lysate twice. The lysates were then clarified by centrifugation for 20 min at 1,000 × g and stored at -70°C. To infect cells, stock virus was allowed to adsorb to cell monolayers for 60 to 90 min at 37°C. The inoculum was then removed and replaced with Eagle minimal essential medium (EMEM) supplemented with 2% fetal bovine serum.

For plaque assays, dilutions of virus were adsorbed to duplicate monolayers of CV-1 cells in six-well plates (Becton Dickinson Labware, Oxnard, Calif.) for 90 min. The inoculum was then removed and replaced with an agar (0.6%) overlay containing Dulbecco modified Eagle medium without phenol red (Sigma Chemical Co., St. Louis, Mo.), 2% fetal bovine serum, and 0.2% dimethyl sulfoxide. The cells were fed every 4 to 5 days with an additional overlay. After 10 days, an additional agar (0.9%) overlay containing 0.01% neutral red was added. After an 8-h period, the plaques were scored by using transmitted light.

Primary African green monkey kidney (AGMK) cells were purchased from M. A. Bioproducts, Walkersville, Md. Vero C1008 cells were obtained from the American Type Culture Collection, Rockville, Md. These cells were previously shown to form tight epithelial monolayers in which maturation of enveloped viruses and expression of virus glycoproteins were found to be polarized (23). The CV-1 line of monkey kidney cells was obtained from E. Hunter. All cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum.

Light and electron microscopy. For light microscopy studies, infected AGMK and Vero C1008 cell monolayers were grown on 22-mm glass cover slips in six-well plates. At various intervals postinfection, the cells were washed with phosphate-buffered saline (PBS), fixed for 15 min at room temperature with 1% Formalin in PBS, mounted on micro-

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scope slides in a 1:1 solution of glycerol-1% bovine serum albumin in PBS, and photographed with a Nikon Optiphot microscope.

For electron microscopy, cell monolayers grown in plastic dishes were fixed and embedded as described previously (21). Monolayers grown in Millicell-HA filter chambers were also embedded as described previously (21), except that filters containing monolayers were cut away from the plastic chambers prior to embedding. For labeling endocytic vesicles with electron-dense markers, the culture fluids of SV40-infected AGMK and Vero C1008 cells were replaced at selected times with EMEM containing native ferritin (100 mg/ml), cationic ferritin (10 mg/ml), or a 1:10 dilution of Thoria sol (colloidal thorium oxide). After incubation with the markers for periods ranging between 20 min and 12 h, monolayers were rinsed and fixed for electron microscopy.

Antibodies and immunofluorescence labeling. Horse antiserum to SV40 was purchased from Flow Laboratories, Inc., McLean, Va. Fluorescein-conjugated rabbit anti-horse immunoglobulin G was purchased from Miles Laboratories, Inc., Elkhart, Ind. For indirect surface immunofluorescence staining, Vero C1008 cells were grown on glass tissue culture microscopic slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) and infected at low multiplicities of infection (MOI) with SV40 (MOI = 0.01). At 48, 54, and 60 h postinfection, duplicate cell monolayers were washed with EMEM and incubated with horse antiserum to SV40 (1:20 in PBS containing 0.1% bovine serum albumin) followed by incubation with fluorescein anti-horse immunoglobulin G (1:20). The cells were washed with PBS, and biotinylated SV40 was added. After a 20-min incubation at 4°C, the monolayers were washed with PBS and incubated with streptavidin-rhodamine isothiocyanate (1:20) (Southern Biotechnology, Birmingham, Ala.). After a 20-min incubation period, the monolayers were washed with PBS and fixed with 1% Formalin in PBS. Cover slips were then mounted, and the slides were observed for fluorescence and photographed with a Nikon Optiphot microscope equipped with a modified B2 cube.

Preparation of biotinylated SV40. Stock virus was pelleted by centrifugation for 2 h at 28,000 rpm in an SW28 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The virus pellet was then resuspended in EMEM and placed on top of a 10-ml CsCl solution (pH 7.2, 1.34 g/cm³) and centrifuged overnight at 35,000 rpm in an SW41 rotor (Beckman). The virus band was collected and dialyzed overnight at 4°C against three changes of TNE buffer (10 mM Tris hydrochloride, 100 mM NaCl, 1 mM EDTA). Virus samples containing 60 µg of viral protein were added to 60 µl of biotin succinimide ester dissolved in dimethyl sulfoxide (120 µg/ml) and were incubated for 4 h at room temperature. The biotinylated virus samples were then dialyzed overnight at 4°C against PBS. The virus samples were stored at -135°C.

Virus release curves. Duplicate cell monolayers were grown to confluence on 30-mm-diameter, 0.45-µm-pore-size Millicell-HA filters (Millipore Corp., Bedford, Mass.) placed in six-well plates (Becton Dickinson Labware). SV40 (MOI = 20) was added to the apical chambers and allowed to adsorb for 1 h. The inoculum was removed, and the monolayers were rinsed twice with PBS prior to adding 2.0 ml of EMEM supplemented with 2% fetal bovine serum to both apical and basal chambers. At intervals postinfection, the apical and basal media were collected separately, clarified by centrifugation at 1,000 × g for 20 min, and frozen at -70°C. The virus titers in each sample were determined by plaque assay.

Trypan blue staining. Duplicate cell monolayers were grown to confluence on 35-mm-diameter tissue culture plates. One set of plates was infected with SV40 (MOI = 20), while the other set was mock infected. At intervals postinfection, attached cells were suspended with 0.25% trypsin and added to the original growth medium containing any detached cells. Trypan blue dissolved in water was added at a final concentration of 0.08%. After 5 min, a total cell count and a stained cell count were made by using a hemacytometer.

[³H]Jinulin transport. Duplicate cell monolayers were grown to confluence on Millicell-HA filter chambers placed in six-well plates. [³H]Jinulin (1 µCi per dish) was added to the growth medium in the basolateral chamber. Samples taken from apical and basolateral chambers were counted by liquid scintillation spectrometry.

Quantitation of cell-associated virus. Cell culture media from infected cell monolayers were collected, and the released virus titers were determined by plaque assay. The cell-associated virus was removed from cells by two cycles of freeze-thawing followed by sonication. The cell debris was pelleted by centrifugation at 2,000 rpm in an International Equipment Co. table top centrifuge, and virus titers present in the supernatant were determined by plaque assay.

Radiolabeling of viral polypeptides and immunoprecipitation. Confluent AGMK cells in 35-mm dishes (Nunc, Roskilde, Denmark) were infected with SV40 (MOI = 100). After a 90-min adsorption period at 37°C, the inoculum was removed and replaced with either fresh EMEM alone or fresh EMEM containing various concentrations of monensin. At 28 h postinfection, the cells were rinsed three times with methionine-free EMEM containing various concentrations of monensin and incubated for 1 h in the same medium. The cells were then incubated in labeling medium (80% methionine-deficient EMEM supplemented with 1% bovine serum albumin, 25 µCi of L-[³⁵S]methionine per dish, and various concentrations of monensin) for 7 to 8 h. At the end of the labeling period, cells were rinsed five times with cold PBS and then lysed with cold cell dissociation buffer (0.01 M Tris hydrochloride [pH 8.0], 0.25 M NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate). Radiolabeled polypeptides were immunoprecipitated with Formalin-fixed *Staphylococcus aureus* cells that had been preincubated with horse antiserum to SV40 for 1 h at 20°C. The precipitates were washed once with lysis buffer (0.5 M Tris hydrochloride [pH 7.4], 0.15 M NaCl, 0.02 M EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and twice with cell dissociation buffer. The final precipitates were suspended in sample reducing buffer (0.067 M Tris hydrochloride [pH 6.7], 6.3% glycerol, 1% SDS, 1% β-mercaptoethanol, 0.006% bromophenol blue) and boiled for 2 min just prior to loading onto a gel. Electrophoresis was performed on an SDS-10% polyacrylamide gel system as described by Laemmli (12) and modified for use with slab gels (1). The protein bands on the dried gel were visualized by standard fluorography techniques (5).

Chemicals and isotopes. Monensin was obtained from Calbiochem-Behring, La Jolla, Calif. L-[³⁵S]methionine (1,440 Ci/mmol) and [³H]Jinulin (4 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, Ill. Thoria sol (colloidal thorium oxide) was purchased from Polysciences Inc., Warrington, Pa. Native and cationic ferritin were purchased from Sigma.

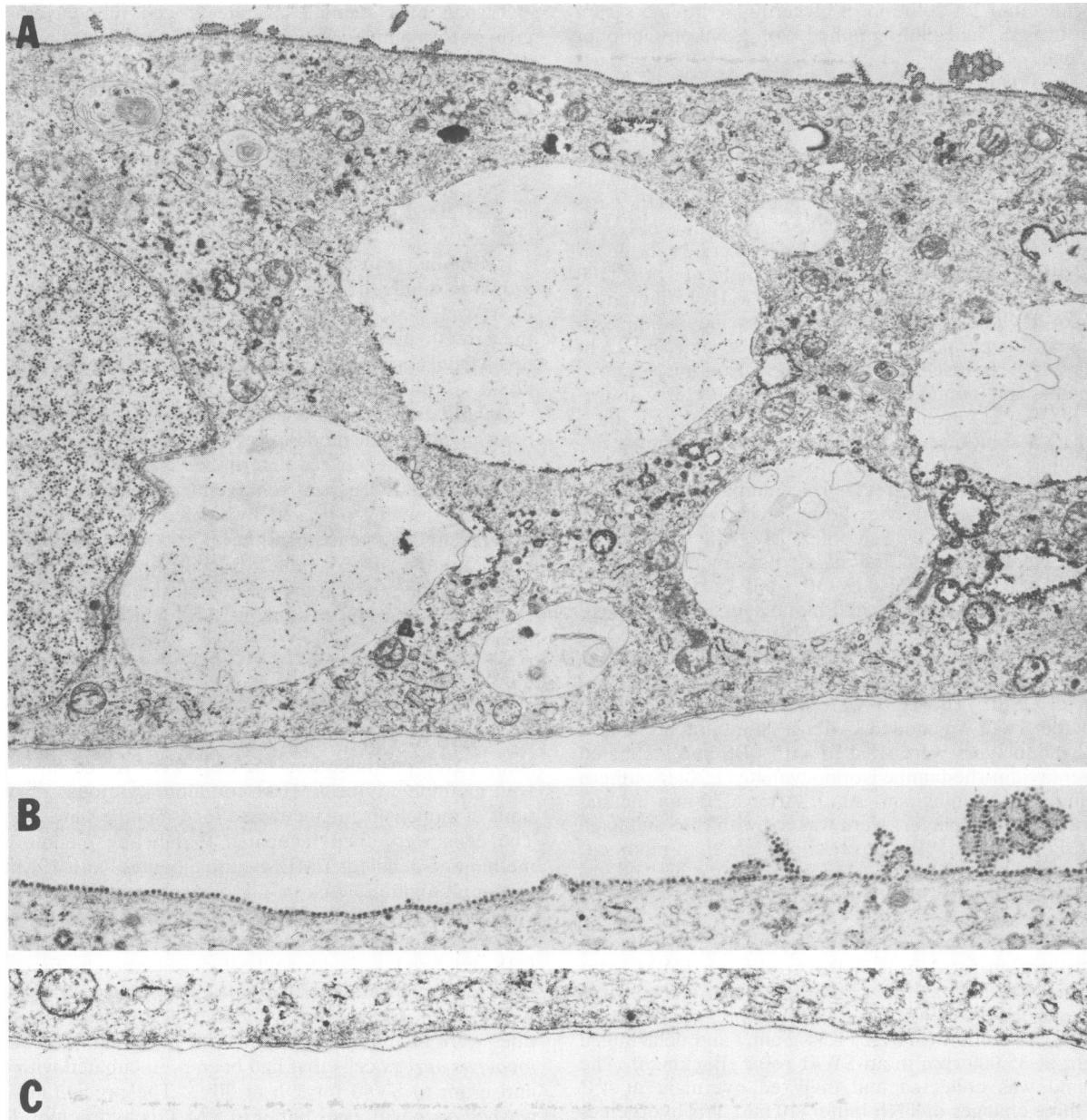


FIG. 1. Association of SV40 virions with apical surfaces of AGMK cells. Monolayers of AGMK cells were infected with SV40 (MOI = 100) and prepared for electron microscopy at 48 h postinfection. (A) Low magnification of an infected AGMK cell showing both the apical and the basal aspects of the cell and large cytoplasmic vacuoles (magnification, $\times 8,500$). (B) Higher magnification of the apical surface of the cell in panel A, showing virions covering the entire surface (magnification, $\times 17,000$). (C) Higher magnification of the basal surface of the cell in panel A; it is devoid of virus particles (magnification, $\times 17,000$).

RESULTS

SV40 virions accumulate on apical surfaces of infected cells.

In previous studies using SV40 late expression vectors to investigate the directional transport of viral glycoproteins (11, 21), we observed that primary AGMK cell cultures consisted predominantly of polarized epithelial cells in which influenza virus was released at apical surfaces and vesicular stomatitis virus was observed to bud at basolateral membranes. During these studies, progeny SV40 virions were observed to be associated with the apical, but not the basolateral, membranes of infected AGMK cells (unpub-

lished observations), suggesting that SV40 virions were also being transported to the cell surface in a polarized manner in these cells. To investigate these observations further, we initially examined the distribution of wild-type SV40 virions in infected AGMK cells. In cells examined at 48 h postinfection, numerous SV40 virions were found to be associated with apical surfaces (Fig. 1 and 2). In contrast, the basal surfaces were devoid of virions (Fig. 1C). Virions on apical surfaces were found in closely packed arrays (Fig. 2), essentially saturating the available surface area, including microvilli. Virions were also found to occur in the nucleus

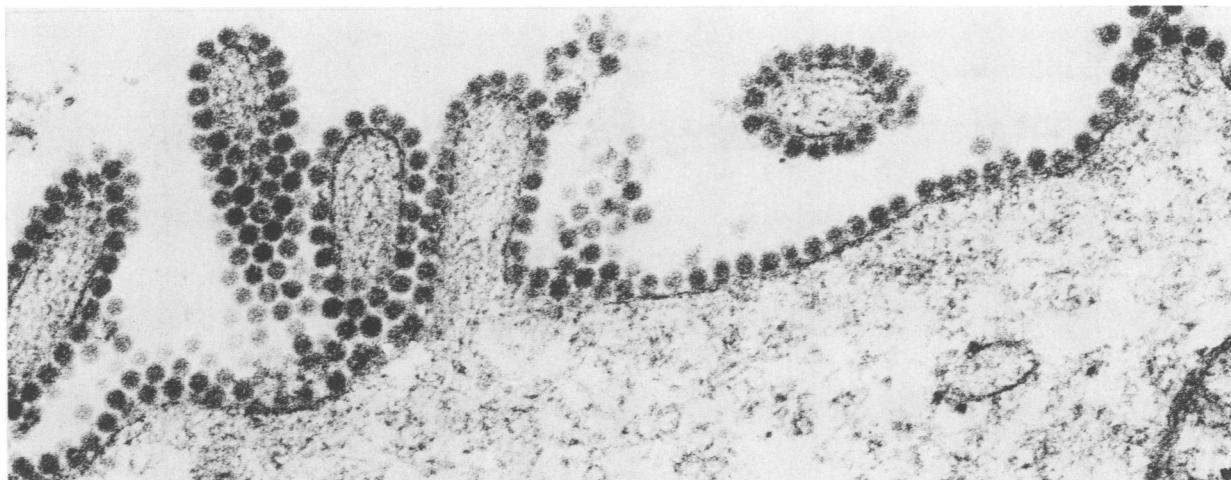


FIG. 2. Densely packed virions on the apical surface of an AGMK cell. Part of the apical surface of an AGMK cell at 48 h postinfection at a high magnification showing SV40 virions completely covering microvilli and the adjacent portion of the apical surface (magnification. $\times 76,000$).

and in the cytoplasm; the latter were always enclosed in small vesicles or in large smooth membrane vacuoles (Fig. 3A and B) and appeared to adhere closely to the vesicle surface (Fig. 1 and 3B). Virions were never observed free in the cytoplasm or within cisternae of the rough endoplasmic reticulum or the Golgi complex. Infected cells remained apparently intact, although they contained large numbers of cytoplasmic and surface virions.

We considered that the virions on surfaces of infected cells may be the result of transport of virus particles to the cell surface in cells actively producing SV40 virions, or alternatively, readorption of virions that have been released into the culture medium. To distinguish between these two possibilities, cell monolayers infected at an MOI of 0.01 PFU per cell were examined by electron microscopy and by surface immunofluorescence. Under these conditions of low MOI, virions were observed lining the apical surfaces of infected cells only (i.e., cells containing nuclear virions) and were virtually absent from surfaces of adjacent, apparently uninfected cells (Fig. 4). The presence of virions on infected cells could also be recognized when intact monolayers were examined by surface immunofluorescence using an antiserum which recognizes viral structural proteins and a fluorescein isothiocyanate-labeled conjugate. Antigen-positive (fluorescent) cells were observed in the midst of antigen-negative cells, indicating that surface virions were restricted to very few cells in monolayers infected at a low MOI (Fig. 5A). However, the restriction of surface virions to selected cells within the monolayer was not due to the lack of receptors on neighboring cells. This was proven by adding biotinylated, purified SV40 virions to the same infected monolayers and by localization of these virions with rhodamine-labeled streptavidin. The results demonstrate that almost all of the cells in the monolayer are capable of binding SV40 virions (Fig. 5B), indicating that the restriction of surface virions to infected cells is not due to the lack of receptors on neighboring cells. Taken together, these results indicate that surface virions originate from within the infected cells and that readorption of released virus is not a major factor in the appearance of SV40 virions on surfaces of infected cells.

SV40 is released from apical cell surfaces. The finding of virions exclusively on apical surfaces of polarized epithelial

cells suggested that virus release might also be occurring preferentially from apical surfaces. To quantitate the release of SV40 from apical and basolateral surfaces of polarized cells and to establish the time course of virus release, virus growth and release curves were determined by using confluent monolayers of cells grown on nitrocellulose filters. Titers of media in apical and basolateral chambers were determined separately by plaque assay to determine the amount of infectious virus present in each sample. Virus release from CV-1 cells was detected as early as 24 h postinfection and was found to be essentially complete by 54 to 60 h postinfection (Fig. 6A). Similar amounts of virus were recovered from both apical and basolateral chambers, confirming the nonpolarized nature of the CV-1 cell line. SV40 release from AGMK cells was detected as early as 30 h postinfection and was essentially complete by 48 h postinfection (Fig. 6B). Between 42 and 72 h postinfection, over 99% of the virus released was recovered from the apical chamber, indicating that virus release from AGMK cells is highly polarized. SV40 release from polarized Vero C1008 cells was first observed at 48 h postinfection and was complete by 60 h postinfection (Fig. 6C). Between 54 and 72 h postinfection, over 99% of the released virus was recovered from the apical chamber, indicating that virus release is also highly polarized in this cell line. Cells grown on plastic were found to release virus with the same kinetics as cells grown on filters (data not shown). To compare the yields of released and cell-associated virus, plaque assays were performed on AGMK and Vero C1008 cells at 54 and 66 h postinfection, respectively. Cell-associated virus accounted for 31% of the total virus yield obtained from AGMK cells and for 27% of the yield from Vero C1008 cells, indicating that in both cell types most of the virus is released rather than remaining cell associated.

Release occurs without cell lysis. When observed by light microscopy, infected AGMK and Vero C1008 cell monolayers were found to remain morphologically intact as late as 60 to 72 h postinfection (data not shown), which is 12 to 24 h after the completion of virus release, indicating that virus release occurs prior to cell lysis in these two cell lines. To demonstrate the integrity of cell membranes during virus release, infected cells were exposed to trypan blue at selected time points. In all cell lines examined, at times when

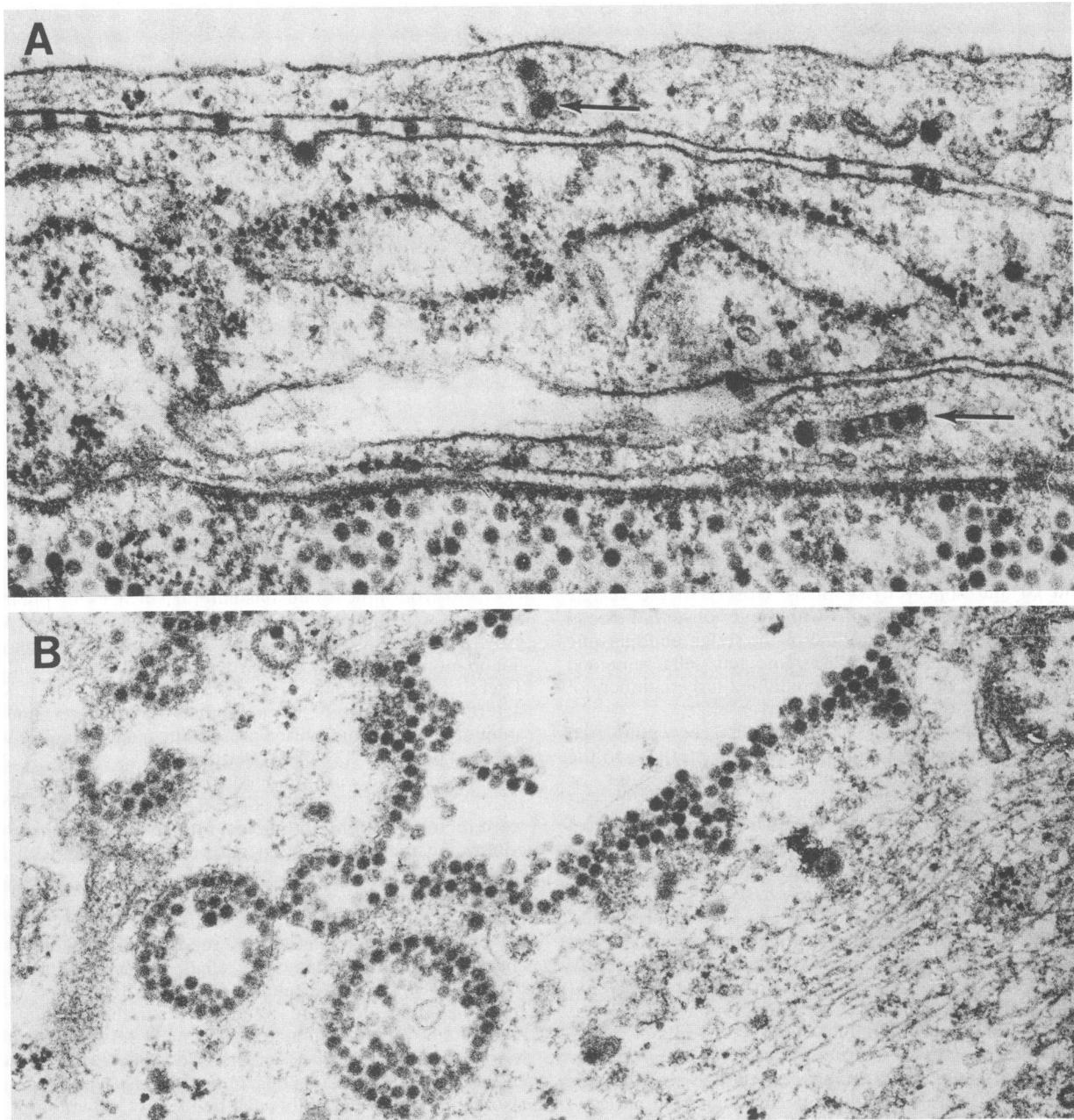


FIG. 3. Cytoplasmic virions enclosed in vesicles. (A) Virions in the cytoplasm of an AGMK cell at 48 h postinfection are enclosed in smooth cytoplasmic vesicles (arrows). Cisternae of the rough endoplasmic reticulum are devoid of virus particles (magnification, $\times 57,000$). (B) Cytoplasmic virions are contained in smooth membrane vesicles and vacuoles. Luminal membrane surfaces are completely covered with virus particles (magnification, $\times 53,000$).

maximum virus yields were obtained in culture media, less than 10% of the cells in the monolayers were stainable with trypan blue (Fig. 6D to F), indicating that infected cells remain viable during virus release.

The integrity of Vero C1008 cell monolayers during viral infection was also examined by determining the transepithelial permeability of [3 H]inulin at selected time points. The basolateral surfaces of monolayers grown on nitrocellulose filters were exposed to medium containing [3 H]inulin. At 60 h postinfection, when maximum virus yields are obtained in this cell line (Fig. 6C), [3 H]inulin diffused into the apical chambers of infected monolayers at background levels (Fig.

7B), demonstrating that the integrity of the epithelial monolayer had not been disrupted during virus release. These results indicate that virus release from polarized epithelial cells occurs without cell lysis or destruction of the impermeability of the epithelial cell monolayer.

Intracytoplasmic virions are restricted to vesicular cisternae. To investigate the possible intracellular pathway of SV40 release, infected Vero C1008 cell monolayers were examined by electron microscopy at intervals during SV40 infection. At 24 h postinfection (prior to virus release), virions were occasionally found in the nucleus in small numbers but were rarely observed in the cytoplasm or on the

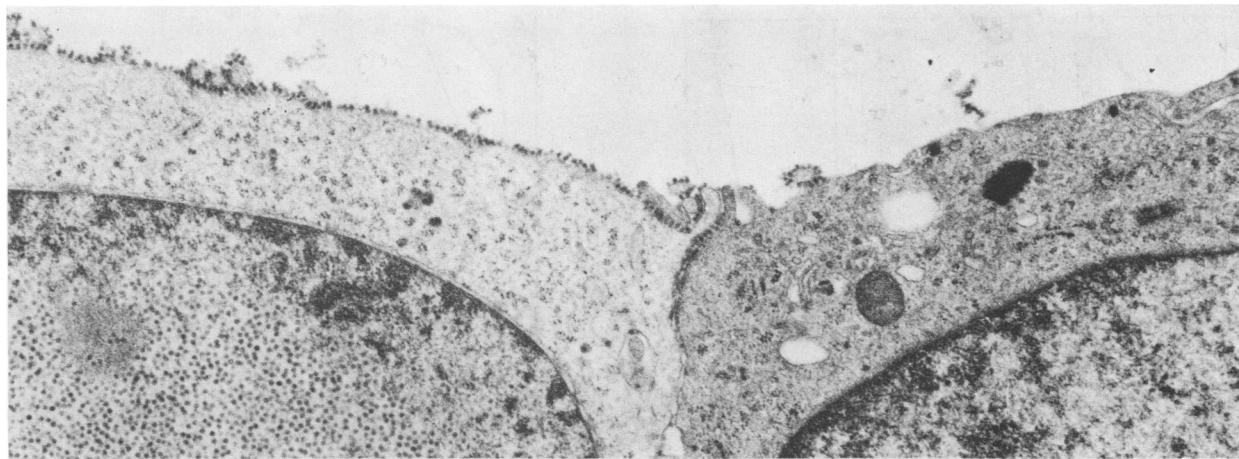


FIG. 4. Distribution of SV40 virions on cell surfaces after low MOI. A monolayer of Vero C1008 cells was infected with SV40 (MOI = 0.01) and prepared for electron microscopy at 54 h postinfection. The cell on the left is infected; SV40 virions are present in the nucleus and on the cell surface. In contrast, the cell on the right exhibits no nuclear virions and is essentially free of surface virions (magnification, $\times 17,000$).

cell surface (Table 1). By 36 h postinfection, virions were observed in some sections of nuclei. At 48 h postinfection, progeny virions were present in large numbers in most nuclei and were occasionally observed in the cytoplasm enclosed in an interconnecting network of smooth membrane reticular structures (Fig. 8A and B). In some sections, the membranes

of these structures appeared to be continuous with the outer nuclear membrane or with the rough endoplasmic reticulum. At this time point, no virions were observed on the cell surface or in cytoplasmic vesicles. Between 54 and 66 h postinfection (during the release period), nuclear, cytoplasmic, and surface virions were frequently observed (Table 1).

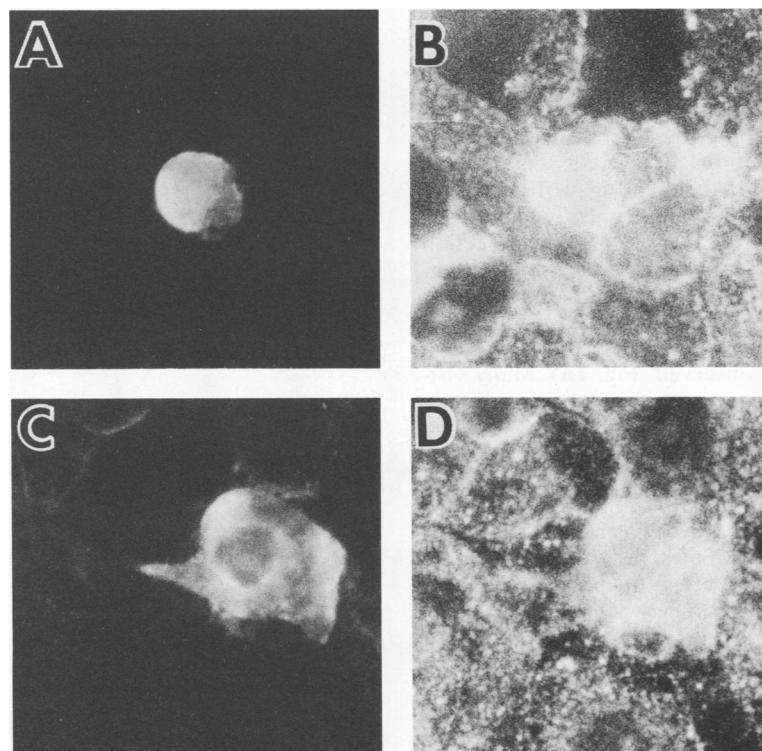


FIG. 5. SV40 virions observed by surface immunofluorescence. Monolayers of Vero C1008 cells were infected with SV40 (MOI = 0.01). At 54 h postinfection, cell monolayers were treated with horse anti-SV40 serum and a fluorescein conjugate. Biotinylated purified SV40 was then added, followed by the addition of streptavidin-rhodamine. (A and C) Antigen-positive cells (fluorescein isothiocyanate) are shown to be isolated among antigen-negative cells. (B and D) Cells essentially negative for surface virions in panels A and C are shown to be capable of binding exogenously added virus, as shown by rhodamine fluorescence.

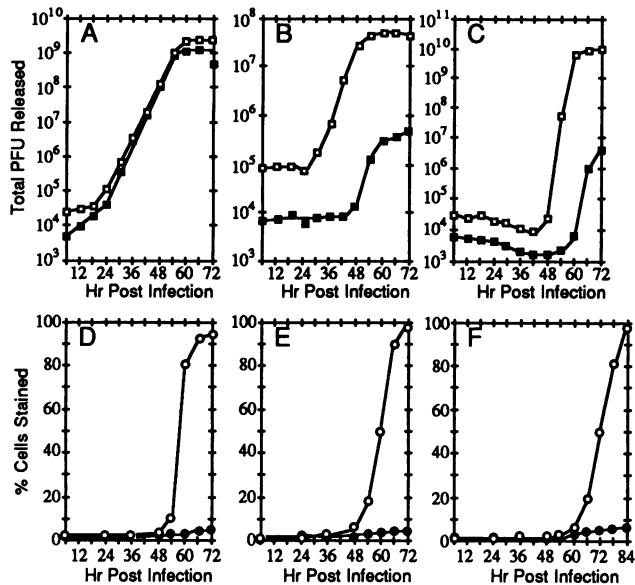


FIG. 6. Time course of virus release and cell lysis. (A to C) Duplicate monolayers were grown on nitrocellulose filters and infected with SV40 (MOI = 20). At intervals postinfection, the culture medium from both the apical and basal chambers was collected separately and virus release was measured by plaque assay. Symbols: □, infectious virus recovered in the apical growth medium; ■, infectious virus recovered in the basal growth medium. (A) Virus release from nonpolarized CV-1 cells. (B) Virus release from primary AGMK cells. (C) Virus release from polarized Vero C1008 cells. (D to E) Infected and mock-infected cells were exposed to trypan blue and counted by using a hemacytometer. Symbols: ○, infected cell monolayers; ●, mock-infected cell monolayers. (D) CV-1 cell viability. (E) AGMK cell viability. (F) Vero C1008 cell viability.

Numerous virions were found to be associated with free apical surfaces, while the basal surfaces were essentially free of virions, as shown for AGMK cells in Fig. 1. In contrast, virions were observed on both the upper and lower surfaces of nonpolarized CV-1 cells examined at 48 h postinfection (data not shown). Cytoplasmic virions were primarily enclosed individually and, in some instances, in groups within smooth membrane vesicles. By 72 h postinfection (after virus release), large cytoplasmic vacuoles typical of SV40 infections were occasionally observed (Fig. 1A). Many virions were observed in the nucleus, in small cytoplasmic vesicles, and in the large vacuoles. At this time, plasma membranes, as well as nuclear membranes, appeared to remain intact. After 78 h postinfection, nuclear membranes were disrupted in many of the cells examined. Virions were still observed in the nucleus and cytoplasm and on the cell surface at this time, indicating that many virions remained cell associated after the completion of active release of SV40. Because the nuclear membrane was no longer intact, virions were observed in the cytoplasm associated with the cytoplasmic surfaces of membrane organelles as well as enclosed within vesicles.

Since the virion-containing vesicles were most prevalent during the period of virus release, we considered that they may play a role in virus release. To determine whether virion-containing vesicles originate from the cell surface, infected cells were incubated in medium containing thorium oxide, cationic ferritin, or native ferritin and examined by electron microscopy. None of the markers were observed in

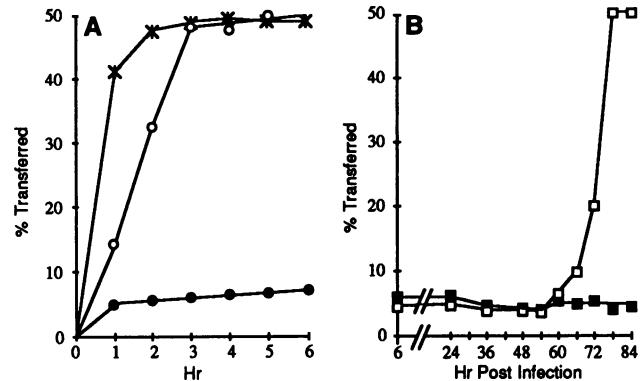


FIG. 7. Diffusion of [³H]inulin across cell monolayers. Cells were grown on nitrocellulose filter chambers as described in Materials and Methods. To determine the time needed for [³H]inulin diffusion to reach equilibrium, 1 µCi of [³H]inulin was added to the basal chamber at time zero, and at the times indicated, the presence of [³H]inulin in the apical and basal chambers was determined by liquid scintillation spectrometry. Plotted is the percent of total [³H]inulin present in the apical chamber. (A) Diffusion of [³H]inulin to the apical chamber in monolayers of nonpolarized CV-1 cells (X) nearly reached equilibrium within 2 h, while only 7% of the [³H]inulin was found in the apical chamber when monolayers of polarized Vero C1008 cells (●) were examined after a 24-h incubation period. This restriction of transport across the monolayer was shown to require the integrity of tight junctional complexes; when Vero C1008 cells were treated with 30 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid] (○), [³H]inulin passed through the filter and cell layer, nearly reaching equilibrium within 3 h. (B) For analysis of SV40-infected Vero C1008 cell monolayers, 1 µCi of [³H]inulin was added to the basal chamber 6 h prior to the time indicated. □, Infected Vero C1008 cell monolayers; ■, noninfected Vero C1008 cell monolayers.

the smaller SV40-containing vesicles, even though some vesicles were in very close proximity to the cell surface. However, some of the large SV40-containing vacuoles similar to those shown in Fig. 1A were found to be labeled (data not shown). These results, as well as the late appearance of the large vacuoles, suggest that the large virion-containing vacuoles may have originated by endocytosis of particles at the cell surface, whereas small virus-containing vesicles do not appear to result from endocytosis and may be involved in virus release.

TABLE 1. Subcellular distribution of SV40 virions in infected Vero C1008 cells

Subcellular location	% of cell sections with ≤ 1 virion at ^a :						
	24 h	36 h	48 h	54 h	60 h	72 h	78 h
Nucleus	10	26	82	98	98	100	98
Networks ^b	0	0	6	8	17	20	3
Vesicles	2	1	0	1	32	86	85
Cell surface	0	0	0	0	32	86	85
Vacuoles ^c	0	0	0	0	0	14	49

^a Vero C1008 cell monolayers were infected with 20 PFU per cell and were prepared for electron microscopy at the postinfection times indicated. Values represent the percent of cell sections exhibiting at least one virion in each of the specified locations. For each time period indicated, over 100 cell sections were examined. Only cells with a nucleus in the plane of the section were counted; this counting method may result in underrepresentation of cytoplasmic structures.

^b Structures such as those depicted in Fig. 8 are designated as networks.

^c Smooth membrane structures with a diameter of $\geq 2.5 \mu\text{m}$ are designated as vacuoles.

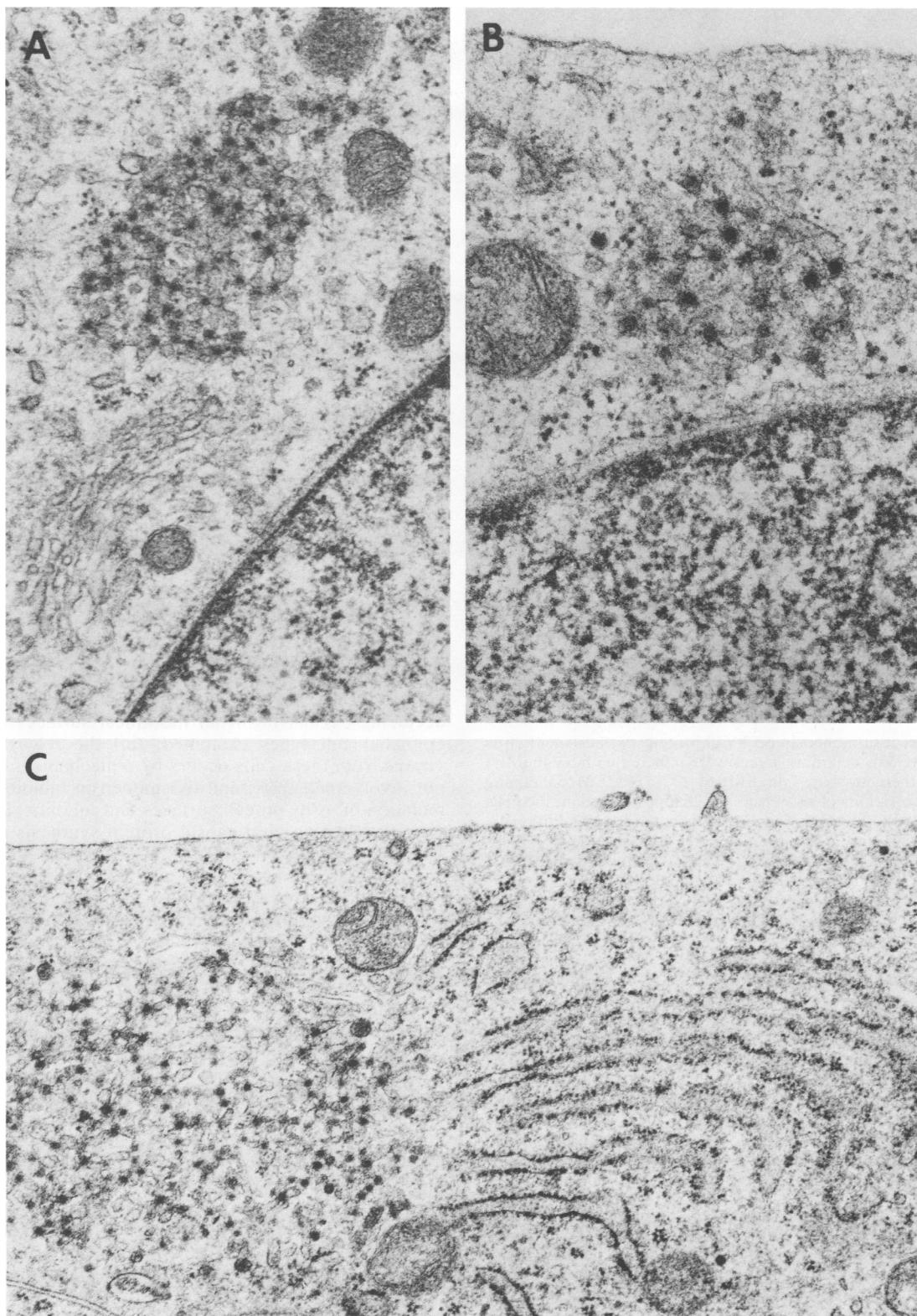


FIG. 8. Cytoplasmic particles in smooth membrane reticular structures prior to virus-release. (A and B) Vero C1008 cells were infected with SV40 (MOI = 20) and prepared for electron microscopy at 48 h postinfection. (A) SV40 virions are enclosed within smooth membrane reticular structures (magnification, $\times 40,000$). (B) Similar reticular structure in close association with the outer nuclear membrane (magnification, $\times 78,000$). (C) AGMK cells were infected with SV40 (MOI = 20) and incubated for 48 h in EMEM containing 10^{-6} M monensin. Virions are present in smooth membrane reticular structures but are absent from the cell surface (magnification, $\times 34,000$).

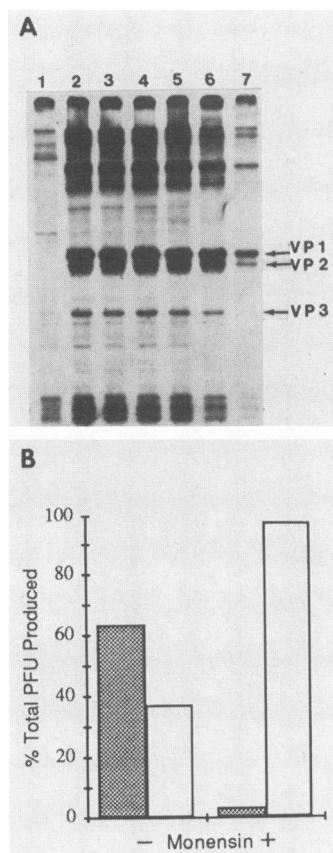


FIG. 9. Effect of monensin on SV40 protein synthesis and virus release. (A) AGMK cell monolayers were infected with SV40 (MOI = 100) and then incubated in EMEM or in EMEM containing various concentrations of monensin. At 28 h postinfection, the cells were labeled with [³⁵S]methionine and incubated for an additional 8 h. Viral proteins were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. Lanes: 1, uninfected AGMK cell lysates; 2, untreated SV40-infected AGMK cells; 3 to 7, monensin-treated, SV40-infected AGMK cells: lane 3, 10⁻⁸ M monensin; lane 4, 10⁻⁷ M monensin; lane 5, 10⁻⁶ M monensin; lane 6, 10⁻⁵ M monensin; and lane 7, 10⁻⁴ M monensin. (B) AGMK cell monolayers were infected with SV40 (MOI = 100) and incubated in EMEM with or without 10⁻⁶ M monensin. At 48 h postinfection, the titers of the released virus and the cell-associated virus were determined separately by plaque assay. Results are presented as the percent of total infectious virus recovered in the cell supernatant (shaded bars) and the percent of total infectious virus remaining cell associated (unshaded bars).

Monensin blocks SV40 release. We investigated the effects of the sodium ionophore monensin on virus release. Monensin is reported to affect intracellular transport by blocking the release of secretory vesicles from trans-Golgi membranes (25, 26) and by blocking the recycling of plasma membrane receptors to the cell surface (3, 27). The ionophore had no significant effect on viral protein synthesis at concentrations up to 10⁻⁵ M. The three capsid proteins of SV40, VP-1, VP-2, and VP-3, were clearly visible in [³⁵S]methionine-labeled- and SV40-infected-cell lysates (Fig. 9A), as were three cellular histones typically observed in SV40 immunoprecipitates. Only at monensin concentrations of 10⁻⁴ M (Fig. 9A, lane 7) was viral protein synthesis significantly decreased. Therefore, the effect of monensin on SV40 release was investigated by incubating infected

AGMK cells in the presence or absence of 10⁻⁶ M monensin (Fig. 9B). In control cells, 63% of the total virus (PFU) was recovered in the cell culture medium while 37% remained cell associated. However, in monensin-treated cells, only 3% of the total virus was recovered from the cell culture medium while 97% remained cell associated. The total yields of virus from treated and untreated cells were approximately equal. These results indicate that monensin inhibits the release of SV40 from infected cells without affecting virus replication.

To investigate the effects of monensin on the intracellular distribution of SV40 virions, we examined SV40-infected, monensin-treated AGMK cells by electron microscopy. Virions were absent from apical cell surfaces of monensin-treated cells between 36 and 60 h postinfection (Fig. 8C) but were present on the surfaces of control cells. In untreated cells at 48 h postinfection, most cytoplasmic virions were enclosed in vesicles. By contrast, in monensin-treated cells at 48 h postinfection, cytoplasmic virions were mostly restricted to an interconnecting network of smooth membrane reticular structures similar to (but much larger than) those seen in untreated cells (Fig. 8). The accumulation of virus particles in these smooth membrane reticular structures, under conditions in which their appearance on the cell surface is prevented, suggests that these structures may be intermediates in the transport and release of SV40.

DISCUSSION

The present study reveals that (i) SV40 virions accumulate on the apical surfaces of productively infected polarized epithelial cells; (ii) the release of SV40 virions is polarized, occurring exclusively from the apical surfaces of the two epithelial cell types examined; (iii) the release of SV40 virions from these cells occurs by a mechanism which does not involve cell lysis; and (iv) monensin inhibits the accumulation of virus on cell surfaces and inhibits virus release without affecting viral capsid protein synthesis or progeny virus production.

The initial finding of large numbers of virions on apical surfaces of infected cells, prior to significant cytopathology, stimulated us to investigate the process of SV40 release. We considered that the accumulation of virions on cell surfaces could be the result of one or more of three processes: the accumulation of parental virus that had not been internalized, the readsorption of virions that had been released into the medium, or the transport of virions to the cell surface by cells actively producing virus. The possibility that the accumulation of surface virions was due to parental virus was excluded since surface virions were not observed between 2 h postinfection and the onset of virus release in all cells examined (Table 1). In cells infected at a low MOI, virions were primarily restricted to surfaces of infected cells, although uninfected cells were shown to be capable of binding virions, indicating that readsorption of released virions is not responsible for the large numbers of virions found on infected cell surfaces. Our results are consistent with the idea that surface virions result from the transport of virions to the cell surface; however, the relationship between surface virions and released virus has not been established. The nature of the binding of virions to cell surfaces remains to be determined. Although we demonstrated that almost all cells in the cultures are capable of binding SV40 virions, the number of virions which bind to uninfected cells at saturation is lower than that estimated on surfaces of infected cells (6). Thus, if surface virions are bound to SV40 receptors it

would appear that receptors are present at much higher levels on surfaces of infected cells than on surfaces of uninfected cells. Alternatively, virions may be bound to surfaces of infected cells by another mechanism, possibly involving a virus-encoded protein.

The release of infectious virus was found to occur only from the apical surfaces of polarized cells. It is unlikely that virus release from the basal surface into the basal chamber is blocked by a basement membrane or by the filter itself because virus was not observed on basolateral surfaces by electron microscopy and because SV40 virions can freely diffuse through polarized cell monolayers grown on filters if tight junctional complexes between cells are disrupted (6). Although not all SV40 virions produced were released from cells, the fraction (approximately one-third) of the virus yields which remained cell associated was not released even after the appearance of extensive cytopathology. The cell-associated virus was found to be infectious when recovered from cells; however, it seems unlikely that such virus plays a significant role in the spread of viral infection *in vivo*. The finding that a nonenveloped virus is released preferentially from the apical surfaces of polarized epithelial cells may have implications for the pathogenesis of viral diseases. Apical release from epithelial cells may limit the spread of viral infection to the epithelial lining of infected tissues, while nondirectional release from lysed cells, or basolateral release from epithelial cells, may facilitate spread of the infection to underlying tissues and lead to systemic infection, provided that cells of other tissues can support virus replication. SV40 infection in the rhesus monkey (its natural host) is characterized by a persistent infection of the kidneys and by the presence of virus in the urine in the absence of a viremia or any other clinical signs of infection (2, 15). These observations suggest that viral infection in the kidney is restricted to the epithelium and are consistent with our results. Several nonenveloped viruses are known to infect the epithelial surfaces of various tissues but fail to infect cells underlying the epithelial lining. The rhinoviruses infect the epithelial surfaces of the nasopharynx with little or no spread to underlying tissues (9). Certain parvoviruses, as well as most rotaviruses, primarily infect epithelial cells lining the small intestine (4, 7, 10). These viruses also infect nonepithelial cells *in vivo* and *in vitro*, indicating that the restriction of infection to epithelial surfaces is not merely a result of an inability of other cell types to support virus replication. It will be of interest to determine whether polarized release from epithelial cells plays a role in limiting the spread of infection by these viruses.

It is generally believed that nonenveloped viruses are released from cells following cell lysis (9). Our results demonstrate that SV40 virions are released into the culture medium well before the destruction of individual cells within intact monolayers. Similar observations have been reported by other workers (16). The appearance of virus in vesicles, as well as the inhibition of virus release by monensin, suggests that a vesicular transport process may be involved in the release of SV40 virions. Although the larger virus-containing cytoplasmic vacuoles may have resulted from endocytosis, smaller vesicles were not labeled by endocytic markers and could play a role in delivery of virus to the cell surface. The directional movement of virus-containing vesicles to the apical surface would provide a plausible mechanism for the delivery of surface virions, as well as released virus, to the apical surface. However, assuming that the assembly of such virions takes place in the nucleus, the process by which these particles are transported from the

nucleoplasm to the lumina of cytoplasmic vesicles remains to be understood. Since the virions must traverse two membranes to exit the nucleus, a simple vesiculation process at the nuclear envelope would not suffice. Several possibilities may be considered, including transport through nuclear pores into smooth cytoplasmic vesicles, although this would represent a novel process not described previously. Alternatively, although not observed in the present study, several investigators have observed SV40 virions within the perinuclear cisternae late in infection (8, 14, 17). Once virions are in the perinuclear space, vesicles or reticular structures may develop from the outer nuclear membrane or the contiguous portion of the rough endoplasmic reticulum. Finally, the nuclear virions could enter vesicles during dissolution of the nuclear envelope during mitosis, as has been suggested for the SV40 T antigen, a protein which is expressed in the nucleus as well as on the cell surface (22).

Monensin was found to inhibit the release of SV40 and to cause accumulation of SV40 virions within smooth membrane reticular structures. In general, these reticular structures appeared to be larger and to occur more frequently in monensin-treated cells than in untreated cells; this may be due to the inhibition of virus release, resulting in the accumulation of virions within these structures. The accumulation of virions in these structures in monensin-treated cells, as well as the time course of appearance of these structures, is consistent with a role for the reticular structures in virus release; however, this remains to be demonstrated. SV40 virions were not found to be associated with Golgi membranes; therefore, it is unlikely that blockage of Golgi traffic by monensin is responsible for the inhibition of SV40 release. It is possible that monensin blocks SV40 release and the accumulation of surface virions by inhibiting the recycling of SV40 receptors to the cell surface, as has been reported for low-density lipoprotein receptors and receptors for mannose-containing glycoproteins (3, 27). Our results show that progeny SV40 virions adhere to the luminal surfaces of intracellular vesicles and the extracellular surfaces of infected cells; therefore, SV40 virions may be bound to receptors on intracellular membranes while within smooth membrane vesicles. Recently, we obtained evidence that SV40 receptors are expressed exclusively on apical surfaces of polarized Vero cells (6). The transport of virions to apical cell surfaces may therefore be a result of the directional transport of virus-receptor complexes to apical plasma membranes, and the release of virions may occur by dissociation of virions from their receptors following their directional transport to the apical surface.

ACKNOWLEDGMENTS

We thank L. R. Melsen and E. Arms for assistance with electron microscopy and B. Jeffrey for secretarial assistance.

This study was supported by Public Health Service grants AI 12680 from the National Institute of Allergy and Infectious Diseases and CA 13148 and CA 18611 from the National Cancer Institute and by Public Health Service Research Service Awards CA 09128 and AI 07150.

LITERATURE CITED

- Anderson, C. W., P. R. Baum, and R. F. Gesteland. 1973. Processing of adenovirus-2-induced proteins. *J. Virol.* 12:241-252.
- Ashkenazi, A., and J. L. Melnick. 1962. Induced latent infection of monkeys with vacuolating SV40 papovavirus. Virus in kidneys and urine. *Proc. Soc. Exp. Biol. Med.* 111:367-372.
- Basu, S. K., J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1981. Monensin interrupts the recycling of low density

- lipoprotein receptors in human fibroblasts. *Cell* **24**:493-502.
4. Carlson, J. H., and F. W. Scott. 1977. Feline panleukopenia. II. The relationship of intestinal mucosal cell proliferation rates to viral infection and development of lesions. *Vet. Pathol.* **14**: 173-181.
 5. Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* **98**:132-135.
 6. Clayson, E. T., and R. W. Compans. 1988. Entry of SV40 is restricted to apical surfaces of polarized epithelial cells. *Mol. Cell. Biol.* **8**:3391-3396.
 7. Cooper, B. J., L. E. Carmichael, M. J. Appel, and H. Greisen. 1979. Canine viral enteritis. II. Morphologic lesions in naturally occurring parvovirus infection. *Cornell Vet.* **69**:134-144.
 8. Granboulan, N., P. Tournier, R. Wicker, and W. Bernhard. 1963. An electron microscope study of the development of SV40 virus. *J. Cell Biol.* **17**:423-441.
 9. Gwaltney, J. M., Jr. 1975. Rhinoviruses. *Yale J. Biol. Med.* **48**:17-45.
 10. Holmes, I. H. 1979. Viral gastroenteritis. *Prog. Med. Virol.* **25**:1-36.
 11. Jones, L. V., R. W. Compans; A. R. Davis, T. J. Bos, and D. P. Nayak. 1985. Surface expression of influenza virus neuraminidase, an amino-terminally anchored viral membrane glycoprotein, in polarized epithelial cells. *Mol. Cell. Biol.* **5**:2181-2189.
 12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
 13. Maul, G. G. 1976. Fibrils attached to the nuclear pore prevent egress of SV40 particles from the infected nucleus. *J. Cell Biol.* **70**:714-719.
 14. Maul, G. G., G. Rovera, A. Vorbrodt, and J. Abramczuk. 1978. Membrane fusion as a mechanism of simian virus 40 entry into different cellular compartments. *J. Virol.* **28**:936-944.
 15. Meyer, H. M., H. E. Happs, N. G. Rogers, B. E. Brooks, B. C. Bernheim, W. P. Jones, A. Nisdalak, and R. D. Douglass. 1962. Studies on simian virus 40. *J. Immunol.* **88**:796-806.
 16. Norkin, L. C., and J. Ouellette. 1976. Cell killing by simian virus 40: variation in the pattern of lysosomal enzyme release, cellular enzyme release, and cell death during productive infection of normal and simian virus 40-transformed simian cell lines. *J. Virol.* **18**:48-57.
 17. Oshiro, L. S., H. M. Rose, C. Morgan, and K. C. Hsu. 1967. Electron microscopic study of the development of simian virus 40 by use of ferritin-labeled antibodies. *J. Virol.* **1**:384-399.
 18. Rodriguez Boulan, E., and M. Pendergast. 1980. Polarized distribution of viral envelope proteins in the plasma membrane of infected epithelial cells. *Cell* **20**:45-54.
 19. Rodriguez Boulan, E., and D. D. Sabatini. 1978. Asymmetric budding of viruses in epithelial monolayers: a model system for the study of epithelial polarity. *Proc. Natl. Acad. Sci. USA* **75**:5071-5075.
 20. Roizman, B. 1985. Multiplication of viruses: an overview, p. 69-75. In B. N. Fields and D. M. Knipe (ed.), *Fundamental virology*. Raven Press, N.Y.
 21. Roth, M. G., R. W. Compans, L. Giusti, A. R. Davis, D. P. Nayak, M. J. Gething, and J. Sambrook. 1983. Influenza virus hemagglutinin expression is polarized in cells infected with recombinant SV40 viruses carrying cloned hemagglutinin DNA. *Cell* **33**:435-443.
 22. Sharma, S., L. Rodgers, J. Brandsma, M.-J. Gething, and J. Sambrook. 1985. SV40-T antigen and the exocytic pathway. *EMBO J.* **4**:1479-1489.
 23. Srinivas, R. V., N. Balachandran, F. V. Alonso-Caplen, and R. W. Compans. 1986. Expression of herpes simplex virus glycoproteins in polarized epithelial cells. *J. Virol.* **58**:689-693.
 24. Stephens, E. B., and R. W. Compans. 1988. Assembly of animal viruses at cellular membranes, p. 489-516. In L. Ornston (ed.), *Annual review of microbiology*, vol. 42. Annual Reviews Inc., Palo Alto, Calif.
 25. Strous, G. J. A. M., R. Willemsen, P. van Kerckhof, J. W. Slot, H. J. Geuze, and H. F. Lodish. 1983. Vesicular stomatitis virus glycoprotein, albumin, and transferrin are transported to the cell surface via the same Golgi vesicles. *J. Cell Biol.* **97**:1815-1822.
 26. Tartakoff, A. M., and P. Vassalli. 1977. Plasma cell immunoglobulin secretion: arrest is accompanied by alterations of the Golgi complex. *J. Exp. Med.* **146**:1332-1345.
 27. Wileman, T., R. L. Boshans, P. Schlesinger, and P. Stahl. 1984. Monensin inhibits recycling of macrophage mannose-glycoprotein receptors and ligand delivery to lysosomes. *Biochem. J.* **220**:665-675.