

## Vectorial Release of Poliovirus from Polarized Human Intestinal Epithelial Cells

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Polarized epithelial cells represent the primary barrier to virus infection of the host, which must also be traversed prior to virus dissemination from the infected organism. Although there is considerable information available concerning the release of enveloped viruses from such cells, relatively little is known about the processes involved in the dissemination of nonenveloped viruses. We have used two polarized epithelial cell lines, Vero C1008 (African green monkey kidney epithelial cells) and Caco-2 (human intestinal epithelial cells), infected with poliovirus and investigated the process of virus release. Release of poliovirus was observed to occur almost exclusively from the apical cell surface in Caco-2 cells, whereas infected Vero C1008 cells exhibited nondirectional release. Structures consistent with the vectorial transport of virus contained within vesicles or viral aggregates were observed by electron microscopy. Treatment with monensin or ammonium chloride partially inhibited virus release from Caco-2 cells. No significant cell lysis was observed at the times postinfection when extracellular virus was initially detected, and transepithelial resistance and vital dye uptake measurements showed only a moderate decrease. Brefeldin A was found to significantly and specifically inhibit poliovirus biosynthetic processes by an as yet uncharacterized mechanism. The vectorial release of poliovirus from the apical (or luminal) surface of human intestinal epithelial cells has significant implications for viral pathogenesis in the human gut.

Epithelial cells form highly organized contiguous sheets which line, and thereby serve to compartmentalize, the exterior and interior surfaces of higher organisms. Epithelial cells therefore fulfill an important role in viral pathogenesis since they represent the primary barrier to virus infection of the host, which must also be traversed prior to virus dissemination from the infected organism. The characteristics of the interaction of viruses or other pathogenic microorganisms with these cell types are therefore of interest. Epithelial cells are highly polarized and express distinct sets of lipids and proteins on their apical and basolateral surfaces (for recent reviews, see references 9, 15, 27, 47, and 48). The polarized phenotype is also exhibited by various continuous lines of epithelial cells which are similar in many respects to the tissues from which they originated (reviewed in reference 22). Thus, Caco-2 cells, which spontaneously form relatively well differentiated epithelial monolayers in vitro, exhibit several characteristics of human intestinal epithelial cells from which they are derived (19). These characteristics include the formation of apical microvilli and junctional complexes, the transport of water and ions to the basolateral surface, dome formation on impermeable substrates, and the expression of several enzyme activities typical of normal small intestine-absorptive enterocytes (40). A comparison of the properties of Caco-2 cells with the properties of human small intestine epithelial cells has suggested that Caco-2 cells most closely resemble the cells which line colonic crypts

(21). Caco-2 cell monolayers also resist the paracellular passage of macromolecules and develop a high transepithelial resistance when grown on porous supports (21, 26, 40). The growth of polarized epithelial cells on porous supports is advantageous since access to the basolateral cell surface is facilitated, thereby enabling an epithelial cell layer to be established under conditions which more closely resemble those prevalent in vivo (10, 35, 36). Epithelial cells grown on porous supports also show evidence of increased differentiation in comparison with cells grown on conventional solid surfaces (25, 45, 59).

In 1978, Rodriguez-Boulan and Sabatini reported that enveloped RNA viruses are released from the MDCK epithelial cell line in a polar fashion (41). They observed that vesicular stomatitis virus (VSV) release occurred predominantly from the basolateral surface, whereas Sendai and influenza virions budded exclusively from the apical domain. Subsequent studies have revealed that other enveloped viruses which form by budding at the cell surface also exhibit vectorial release from infected polarized epithelial cells both in vitro and in vivo (15, 55). The infection of polarized cells by nonenveloped viruses has not been investigated as extensively. However, studies using simian virus 40 (SV40) have indicated that nonenveloped viruses may also be targeted for release at a particular plasma membrane domain. Clayson et al. (13) observed an almost exclusive release of SV40 from a polarized epithelial cell line derived from the African green monkey kidney (Vero C1008) cells as well as from primary African green monkey kidney cells. Some evidence was presented that the vectorial release of SV40 was mediated by a vesicular transport mechanism and occurred prior to cell lysis (13).

In this study, we have investigated the release of poliovirus from polarized epithelial cells. Two epithelial cell lines,

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Caco-2 and Vero C1008, which differentiate in vitro to form highly polarized epithelial cell sheets, were used. We anticipated that the results obtained with Caco-2 cells, which have many of the characteristics of human gut epithelial cells, would be particularly relevant to virus infection in the human gut. Since the gastrointestinal tract provides both the portal of entry for poliovirus and the means for viral dissemination, such studies may provide new insight into the mechanisms involved in these processes.

## MATERIALS AND METHODS

**Reagents.** Brefeldin A (BFA) was obtained from Epicenter Technologies Corp. (Madison, Wis.). Monensin and ammonium chloride were purchased from Sigma Chemical Co. (St. Louis, Mo.). Mouse polyclonal antiserum to poliovirus type 1 was purchased from Whittaker Bioproducts (Walkersville, Md.).

**Cells and virus.** Hep-2, Caco-2, Vero C1008, BHK-21, and CV1 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modification of minimal essential medium supplemented with 10% (Hep-2, CV1, BHK-21, and Vero C1008) or 20% fetal bovine serum (Caco-2). Caco-2 cells were used between passages 13 and 27, and Vero C1008 cells were used between passages 22 and 31. Transwell tissue culture inserts (4.5 cm<sup>2</sup>; Costar Corp., Cambridge, Mass.) were seeded with  $2 \times 10^6$  epithelial cells and incubated for at least 7 days prior to analysis. Apical and basal media were replaced at 2-day intervals during this period. Transwell inserts were similarly seeded with CV1 cells 2 days before analysis. The integrity of the epithelial cell sheet formed was assayed (see below), and only intact cell monolayers were used. Tissue culture inserts containing pores of 0.4- $\mu$ m diameter were used for all studies involving poliovirus, since our previous studies have shown that epithelial cells are able to migrate through 3.0- $\mu$ m-diameter pores (56), and tissue culture inserts with membranes containing intermediate pore sizes are not currently available from commercial sources. For VSV studies involving cells grown on filters containing 3.0- $\mu$ m-diameter pores, virus infection was performed at 6 days postseeding (the time at which cells were found to have sloughed off the lower filter surface [56]), and the lower filter surface was vigorously washed with phosphate-buffered saline (PBS) to remove any cells which may have remained on this surface. Poliovirus type 1 Mahoney was kindly supplied by Casey Morrow and was grown and titered by plaque assay in Hep-2 cells. For infection of cells grown on Transwell tissue culture inserts, poliovirus was adsorbed for 2 h to either the apical or basolateral surface. VSV (Indiana strain) was grown and titered by plaque assay in BHK-21 cells.

**Measurements of monolayer integrity.** Resistance measurements were made by using a Millicell ERS apparatus (Millipore, Bedford, Mass.) according to the manufacturer's instructions. Measurements were made by using cells grown on Transwell tissue culture inserts at room temperature in Dulbecco's modified minimal essential medium in the absence of serum. Monolayer permeability measurements of cells grown under the same conditions were made by using <sup>3</sup>H-inulin according to previously described procedures (7). A rate of inulin diffusion of less than 1%/h was considered to indicate the presence of intact junctional complexes.

**Immunoprecipitation studies.** Vero C1008 and Caco-2 cells grown on Transwell tissue culture inserts were infected with poliovirus at a multiplicity of infection (MOI) of approximately 10 PFU per cell and labeled with 50  $\mu$ Ci of

Express<sup>35</sup>S<sup>35</sup>S (New England Nuclear, Boston, Mass.) per ml in both apical and basal chambers for 1 h at 16 h postinfection (hpi). Lysates (lysis buffer: 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, and 1% Triton X-100 in PBS) were incubated overnight with a 1:500 dilution of poliovirus-specific polyclonal mouse serum. Immune complexes were collected by using protein A-agarose beads. After a 2-h incubation (4°C), the beads were washed three times with wash buffer and once with 25 mM Tris (pH 6.8) and resuspended in sample buffer prior to analysis by SDS-polyacrylamide gel electrophoresis (PAGE) (30).

## RESULTS

**Poliovirus is released almost exclusively from the apical domain of polarized Caco-2 cells.** To determine whether poliovirus is released in a vectorial fashion from two types of polarized epithelial cells, Caco-2 and Vero C1008 cells were grown on Transwell tissue culture inserts. These cells were infected via either the apical or basolateral surface, and the rate of virus release was measured by plaque assay. A nonpolarized cell type, CV1, was used for comparison. To ensure that the epithelial cell types were polarized, cells were infected with VSV, which characteristically buds from the basolateral plasma membrane. An almost exclusive release of VSV from the basolateral surface of both cell types was observed (Fig. 1B), indicating that these cells were polarized. In each cell type, extracellular poliovirus was initially detected between 12 and 16 hpi (see below). A comparison of the amounts of virus released into the apical and basolateral media at this time revealed a marked difference in the pattern of virus release exhibited by each cell type. Polarized Vero C1008 cells exhibited a slightly increased amount of virus released into the apical compartment, but the distribution of released virus was essentially identical to that of the nonpolarized control CV1 cells (Fig. 1A). In contrast, extracellular poliovirus was almost exclusively detected in the apical media overlying monolayers of infected Caco-2 cells, irrespective of the membrane domain inoculated (Fig. 1A). The latter result, which was found to be highly reproducible, suggests that poliovirus release may be mediated by a vectorial transport mechanism in polarized human intestinal epithelial cells. Interestingly, comparatively high titers of poliovirus were detected in culture media prior to significant visually apparent cytopathology (Fig. 2). In addition, although reductions in both transepithelial resistance and vital dye uptake were observed in infected Caco-2 cell monolayers, these values remained comparatively high during the time postinfection at which extracellular virus was initially detected (Fig. 2). Although it is difficult to exclude the possibility of individual cell lysis, these results are at least consistent with the possibility of a nonlytic release mechanism at early times postinfection.

The potential mechanisms involved in the vectorial release of a nonenveloped virus which replicates in the cytoplasm may not necessarily be related to those involved in the transport of membrane-bound or secretory molecules. Initially we considered four possible processes, which are shown schematically in Fig. 3. The first of these assumes a vectorial transport of virus particles contained within vesicles (Fig. 3A). Such a mechanism is consistent with the current hypothesis for the polarized transport of membrane-bound and secretory materials which are packaged into distinct vesicular structures in the late Golgi complex, although there is no evidence that release of poliovirus involves such a transport process. The second model involves

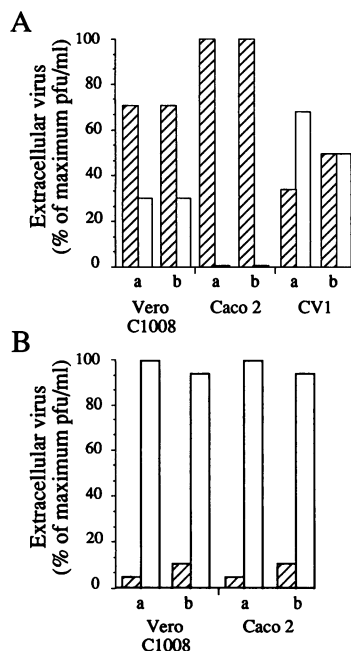


FIG. 1. Release of poliovirus and VSV from polarized and non-polarized cells. Monolayers of polarized Caco-2 and Vero C1008 cells and nonpolarized CV1 cells grown on 4.5-cm<sup>2</sup> Transwell filters with 0.4- $\mu$ m-diameter pores (A) or 3.0- $\mu$ m-diameter pores (B) were infected with poliovirus (A) or VSV (B) (MOI of 1 PFU per cell) via either the basolateral (b) or apical (a) surface. Transwell filters containing 3.0- $\mu$ m-diameter pores were used for experiments involving VSV because this virus was found to be unable to efficiently traverse membranes containing 0.4- $\mu$ m-diameter pores. At 16 hpi (A) or 8 hpi (B), media were collected from the apical (hatched bars) and basal (open bars) chambers and the titer of extracellular virus was determined by plaque assay. The titer of residual inoculum virus was determined by plaque assay of media collected at 2 hpi and was found to be less than 10 PFU/ml in each case. This value was deducted from the titer obtained at 16 hpi. Virus titers are expressed as a percentage of the maximal virus titer measured in the apical and basolateral media for each cell line and route of inoculum.

the targeted transport of viral aggregates, or viroplasm, to the apical plasma membrane (Fig. 3B). It is possible that this process is similar to that employed by specialized secretory epithelial cells which package their products into secretory granules prior to export (4). The other two possibilities do not involve the vectorial transport of virions within an intact cell. Vectorial cell lysis (Fig. 3C) may occur if the disrupted cells maintain comparatively intact junctional contacts with neighboring cells and only the apical plasma membrane is ruptured. Finally, extrusion of an infected cell into the apical compartment may occur prior to its lysis (Fig. 3D). The latter scenario necessitates the establishment of junctional contacts between the cells remaining in the monolayer to ensure integrity of the cell sheet. To further investigate the processes operating during apical release of poliovirions from Caco-2 cells, infected cells were examined by transmission electron microscopy at 16 hpi. The cytoplasm was seen to be highly vesiculated, with virions apparently present both within the vesicles and free in the cytoplasm (Fig. 4A). Electron-dense bodies which likely represent aggregates of viral particles were also observed (Fig. 4B). Although these bodies appeared to be distinct from the surrounding cytoplasm, they were evidently not confined by

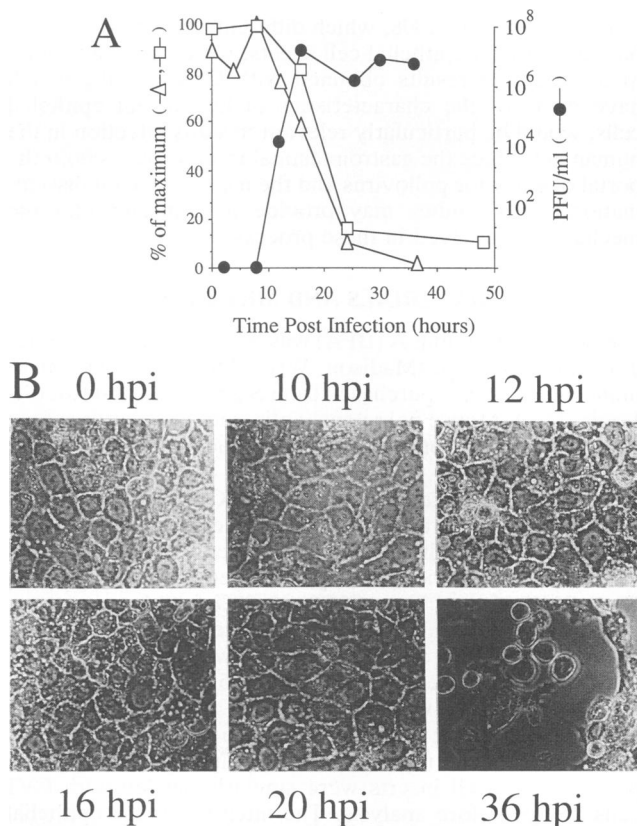


FIG. 2. Time course of poliovirus release, cytopathology, and monolayer integrity during poliovirus infection of Caco-2 cells. Monolayers of Caco-2 cells grown on 4.5-cm<sup>2</sup> Transwell filters (A) or tissue culture plastic (B) were infected with poliovirus (MOI of 1 PFU per cell) via the apical surface. At the indicated times postinfection, media were collected and the extracellular virus titer was determined by plaque assay (A, closed circles). Cytopathic effects were monitored by phase-contrast microscopy (B) and by the method of Finter (18), which measures the accumulation of the vital dye neutral red in viable cells (A, open triangles). Cell viability results are expressed as a percentage of the maximal absorbance due to neutral red uptake (A, open triangles). Transepithelial resistance measurements (A, open squares) were made at the times indicated and are expressed as a percentage of the maximal resistance recorded.

a limiting membrane. The surfaces of infected cells revealed vesiculated virions which appeared to be in the process of being expelled by fusion of the vesicle with the plasma membrane (Fig. 4C). Structures corresponding to viral aggregates in the process of extrusion were also observed (Fig. 4D). The processes shown in Fig. 4C and D were observed only at the apical membrane and were observed in several separate sections. Although these electron microscopic observations are open to varied interpretation, they are at least consistent with either or both of the models described in Fig. 3A and B.

We also considered the possibility that the targeted secretion of an inhibitory substance into the basolateral chamber could result in a reduction in the titer of virus and produce a result similar to that described above. To test this hypothesis, different amounts of poliovirus were incubated at several different temperatures and for various periods with media collected from either the basolateral or apical surfaces of Caco-2 and Vero C1008 cell monolayers. None of the media



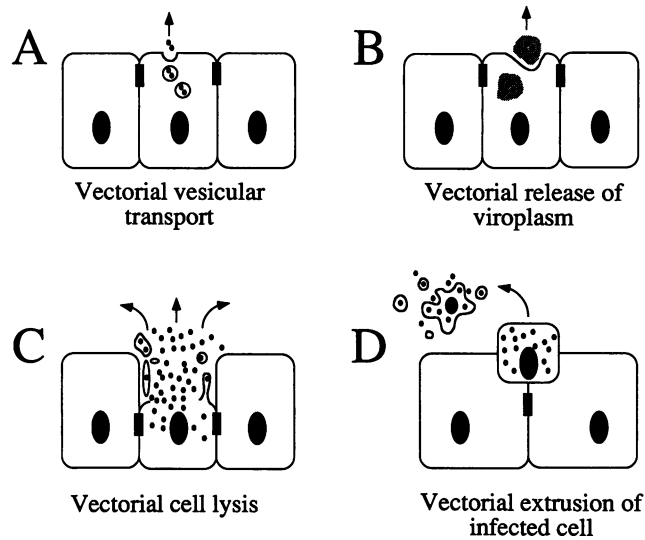


FIG. 3. Models for the polarized release of poliovirus, showing schematic representation of possible mechanisms involved in the polarized release of poliovirus from infected Caco-2 cells. Tight junctions are represented by solid bars at the apical periphery, nuclei are indicated by shading, and the small solid circles represent poliovirions.

tested appeared to have any effect on the titer of virus recovered (not shown). This result suggests that the apparent asymmetry of poliovirus release from Caco-2 cells is likely a phenomenon related to the mechanism of release and not a consequence of selective virus inactivation.

**Effects of BFA, monensin, and ammonium chloride on poliovirus replication in epithelial cells.** To further define the mechanism involved in the vectorial release of poliovirus from Caco-2 cells, we investigated the effects of several compounds which inhibit vesicular transport processes. BFA is a fungal metabolite which causes a marked disruption of the Golgi complex in many cell types (reviewed in references 29 and 39). Since the vesicles involved in the vectorial transport to the cell surface of membrane-bound and soluble materials originate in the Golgi complex, BFA would be expected to interfere with these processes. Indeed, there is evidence that BFA treatment of epithelial cells perturbs polarized sorting and transport (28, 31). Monensin is a sodium ionophore which is reported to block the release of secretory vesicles from trans-Golgi membranes (53). The effects of monensin upon poliovirus release were of particular interest because earlier studies with another nonenveloped virus, SV40, revealed that this compound inhibited virion release from the apical surface of polarized monkey kidney epithelial cells (13). The weak base ammonium chloride is also known to inhibit vectorial transport processes (8).

Poliovirus-infected Caco-2 cells were incubated with increasing concentrations of BFA, monensin, or ammonium chloride from 2 to 16 hpi. Media containing BFA were replenished every 4 to 6 h during this period to maintain the concentration of this compound, which is reported to be subject to metabolic inactivation (11). Although none of the compounds selectively abolished the apical release of poliovirus (Fig. 5A), some interesting effects were observed. The addition of  $10^{-8}$  M monensin caused an increase in the amount of virus shed into the basal medium (Fig. 5Bi). Since

no corresponding decrease in the virus titer associated with the apical medium or cell extract was apparent, treatment with  $10^{-8}$  M monensin appeared to result in an increased virus yield. Some compounds which raise the pH of intracellular compartments are reported to enhance poliovirus-mediated host cell shutoff (32), and it is possible that such an effect increases virus yield. It is not clear whether the general increase in virus titer and the increase in basolateral release observed in the presence of  $10^{-8}$  M monensin are related. At  $10^{-7}$  and  $10^{-6}$  M monensin, the amount of virus detected in both the extracellular domains was reduced, whereas monensin treatment at concentration in excess of  $10^{-5}$  M lacked a selective effect on virus release and resulted in a reduction in both cell-associated and extracellular virus titers (Fig. 5Bi). Ammonium chloride appeared to inhibit basolateral virus release only at concentrations of 1 and 5 mM (Fig. 5Bii), whereas at 10 mM, release in both directions was impaired. It is noteworthy that low concentrations of both monensin and ammonium chloride had a more significant effect on the titer of virus in the basal media than on the titer in the apical media. BFA had a particularly striking effect, resulting in a marked reduction in the titer of both extracellular and intracellular virus (Fig. 5Biii). Poliovirus replication in Vero C1008 cells was similarly inhibited by these compounds (not shown).

Since low concentrations of monensin and ammonium chloride appeared to selectively inhibit virus release from Caco-2 cells, it is conceivable that vesicular transport mechanisms contribute to the release of this nonenveloped virus. However, both compounds had a more marked influence on basolateral release than apical release, which was significantly inhibited only at concentrations which reduced intracellular virus titers. It is likely, therefore, that mechanisms which are not inhibited by monensin or ammonium chloride are involved in the vectorial release of poliovirus from the apical surface of Caco-2 cells. Infected cells treated with  $10^{-5}$  M monensin or 50 to 100 mM ammonium chloride exhibited a reduction in cell-associated as well as extracellular virus titers. Monensin is reported to have cytotoxic effects (44, 53), and it is possible that the reductions in virus titers which were observed at the comparatively high concentrations used above are a consequence of cytotoxicity. In contrast, the inhibitory effect of BFA was apparent at concentrations which are considered to be extremely low and unlikely to be cytotoxic.

**Mechanism of BFA inhibition of poliovirus replication.** To determine whether BFA has an effect on virus release at later times postinfection, 10  $\mu$ g of BFA per ml was added to poliovirus-infected Caco-2 cells at 4, 8, or 12 hpi. The titers of virus associated with cell extracts and with the apical and basal media were measured at 16 hpi. BFA treatment at 8 and 12 hpi had no significant effect on cell-associated virus or the release of virus from either membrane domain (not shown). In contrast, addition of BFA at 4 hpi resulted in a decrease in intracellular and apical extracellular virus titers (not shown).

The effects of BFA are known to be readily reversible following removal of the inhibitor. If the BFA-specific reduction in virus titer was a consequence of a block in virus assembly or a similar postsynthetic step, we reasoned that removal of the inhibitor might result in a burst of progeny virus. Infected Caco-2 cells were incubated in the presence or absence of BFA from 2 to 16 hpi. The cell sheets were then washed and incubated for an additional 8 h in the absence of BFA. Poliovirus titers in cell lysates and in apical and basal media were measured at 16 hpi (immediately after

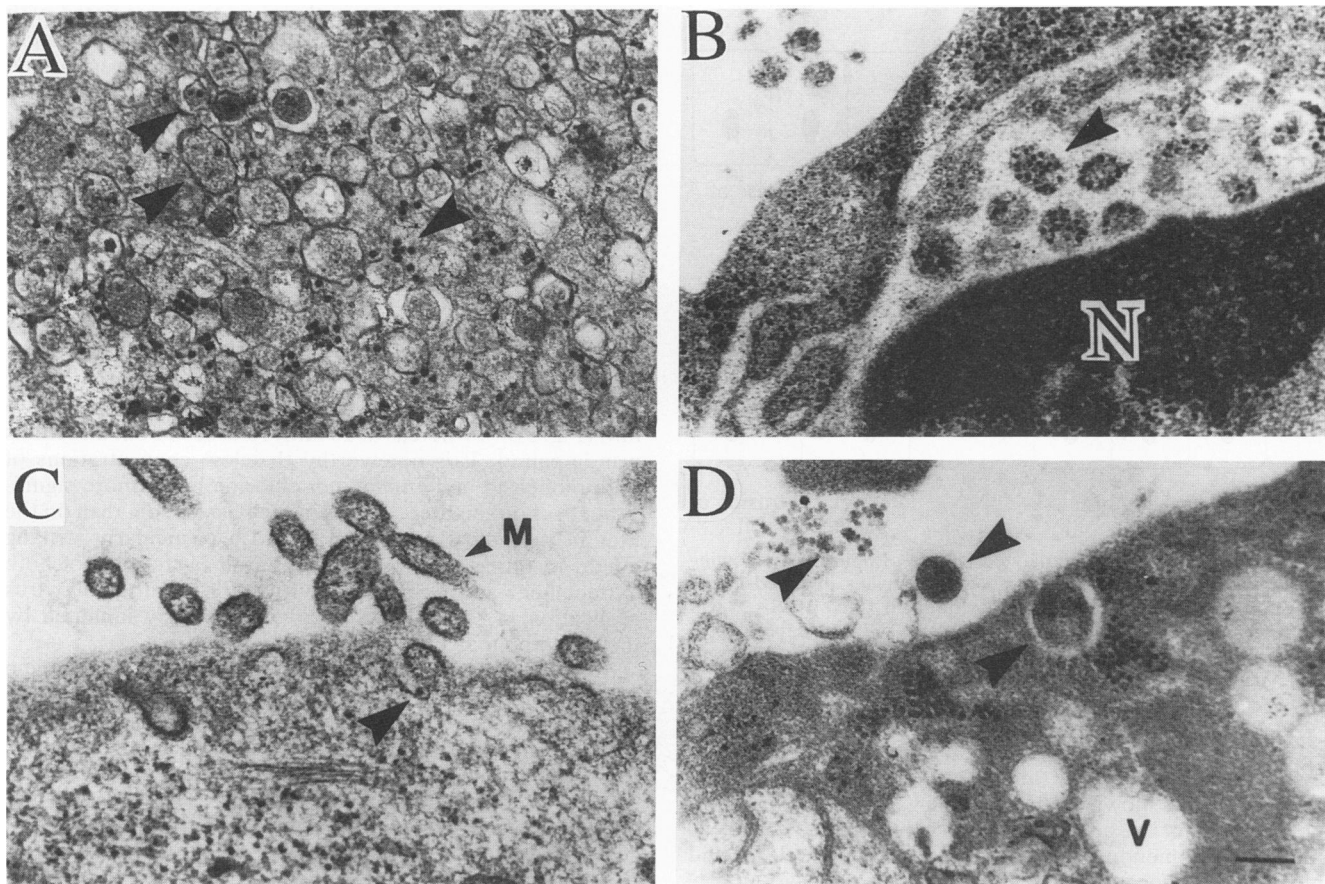


FIG. 4. Analysis of poliovirus-infected Caco-2 cells by transmission electron microscopy. Monolayers of Caco-2 cells grown on 4.5-cm<sup>2</sup> Transwell filters were infected with poliovirus (MOI of 1 PFU per cell) at the apical surface. At 16 hpi, samples were processed for transmission electron microscopy as described previously (56). (A) Micrograph depicting the extensive vesiculation of the cytoplasm evident in poliovirus-infected cells. Arrowheads highlight virions apparently contained within vesicles and free in the cytoplasm. (B) The arrowhead highlights an area of electron-dense viral aggregates. These structures did not exhibit a boundary membrane and were not observed in mock-infected cells. N, nucleus. (C) The large arrowhead highlights a poliovirion associated with a vesicle apparently in the process of fusing with the plasma membrane. M, microvillus. (D) Arrowheads highlight viral aggregates apparently being ejected from the surface of infected cells and becoming disrupted in the extracellular medium. V, vacuole. Bar, 0.2  $\mu$ m.

removal of the BFA block) and at 20 and 24 hpi. No significant increase in the amount of poliovirus was observed within 8 h of removal of the inhibitor (not shown). This result indicates that BFA-treated poliovirus-infected cells probably do not contain a pool of noninfectious virus particles or their constituents, which can form infectious particles after the BFA block is removed. A reduction in the ratio of apical to basal virus titers was apparent in the untreated control samples from 16 to 24 hpi, which presumably corresponds to an increased cytopathic rupturing of the epithelial cell sheet allowing virus in the apical chamber to diffuse through the cell sheet to the opposing chamber. Prolonged incubation resulted in a further reduction in the apical/basal ratio to approximately 2:1 to 4:1 by 48 hpi. Interestingly a ratio of 1:1 was not achieved in these experiments even though the cytopathology observed in parallel samples grown on optically transparent substrates was virtually complete by 48 hpi and the level of media had equilibrated between the two chambers of the filter grown cells (not shown). It is possible that the higher titer of virus in the apical chamber was maintained by an impaired rate of diffusion through filter pores blocked by the remains of epithelial cells and extra-

cellular membrane components. Taken together, the results described above indicate that BFA does not inhibit the processes involved in virus assembly or release at late times postinfection.

The effect of BFA on poliovirus-specific polypeptide synthesis was determined by immunoprecipitation of radiolabeled cell extracts prepared from infected Caco-2 cells incubated in the presence of increasing concentrations of BFA. A significant inhibitory effect was observed (Fig. 6), indicating that the BFA-mediated reduction in virus titer is likely a consequence of inhibition of a poliovirus biosynthetic process and is probably unrelated to the process of vectorial release. Interestingly, at the concentrations which were observed to be effective at inhibiting poliovirus-specific polypeptide synthesis, BFA had little or no effect on the synthesis of host cell polypeptides (not shown). Therefore, the effect of BFA appears highly specific for poliovirus. One polypeptide of approximately 80 kDa appeared to be induced in BFA-treated infected and uninfected cells (not shown). The identity of this molecule and whether it is involved in a BFA-induced antiviral response remain to be established. Interestingly, the inhibitory effects of BFA on protein secre-

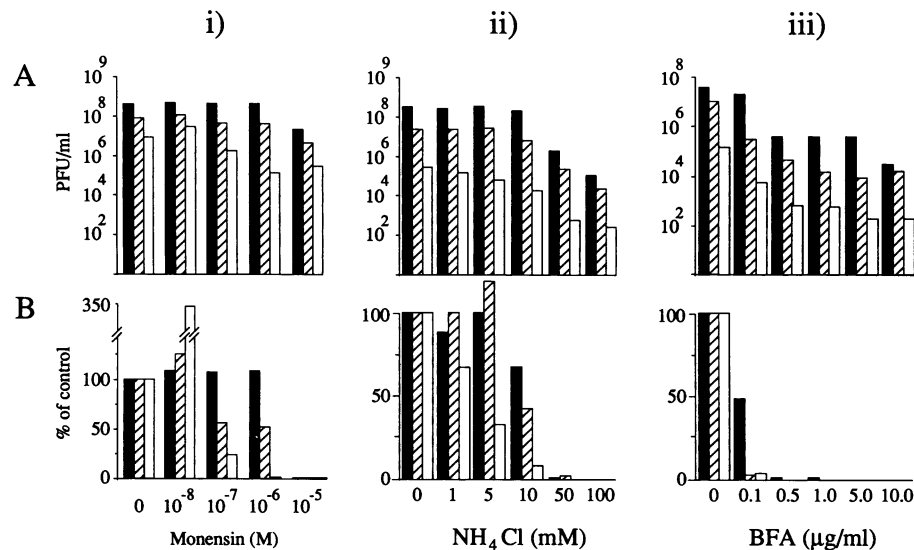


FIG. 5. Effects of monensin, ammonium chloride, and BFA on poliovirus release from Caco-2 cells. Monolayers of Caco-2 cells grown on 4.5-cm<sup>2</sup> Transwell filters were infected with poliovirus (MOI of 1 PFU per cell) at the apical surface. At 2 hpi, media were exchanged for media containing the indicated concentrations of monensin (i), ammonium chloride (ii), or BFA (iii). At 16 hpi, the apical (hatched bars) or basolateral (open bars) media were collected. The titer of cell-associated virus (solid bars) was determined after the cell monolayers were subjected to three rounds of freeze-thaw. Virus titers are expressed both on logarithmic scales (A) and as a percentage of the titer obtained in the absence of inhibitor (B).

tion from Caco-2 cells appeared to be selective. The quantity of some polypeptides present in the media remained unaltered after treatment with up to 10  $\mu$ g of BFA per ml, whereas others exhibited a marked decrease at BFA concentrations in excess of 0.1  $\mu$ g/ml (not shown). These effects on polypeptide secretion were evident in both poliovirus-infected and mock-infected Caco-2 cells.

## DISCUSSION

The results presented in this report provide evidence that poliovirus is released from Caco-2 cells in a vectorial fashion. The vectorial release of poliovirus may be restricted to epithelial cells of intestinal origin, since another polarized epithelial cell line derived from the African green monkey kidney (Vero C1008 cells) released virus in an essentially nondirectional manner. However, it is noteworthy that the transepithelial resistance values reported for Vero C1008 cells are approximately an order of magnitude lower than those achieved by Caco-2 cell monolayers (57), suggesting that Vero C1008 cell monolayers may be more intrinsically

leaky, a property which is likely to be exacerbated by poliovirus induced cytopathology.

We have considered several possibilities for the mechanism involved in the vectorial release of poliovirus from polarized epithelial cells: (i) the vectorial transport of virus particles contained within vesicles which fuse with the intact apical plasma membrane (Fig. 3A); (ii) the vectorial transport of viral aggregates which are expelled through the apical plasma membrane (Fig. 3B); (iii) the directional lysis of virus infected cells (Fig. 3C); and (iv) the extrusion of infected cells from the monolayer followed by lysis in the apical chamber (Fig. 3D). We observed structures consistent with the first two possibilities, vectorial vesicle and aggregate transport, by transmission electron microscopy. In addition, our studies with monensin and ammonium chloride revealed that both of these inhibitors of vesicular transport mechanisms had some selective effect on poliovirus release from Caco-2 cells. However, the effects of both compounds were more manifest on the levels of virus detected in the basolateral media than on the levels detected in the apical media. The latter observation suggests that although monensin- and ammonium chloride-sensitive processes may be involved in the release of a proportion of progeny virus (particularly at the basolateral surface), the mechanisms involved in the polarized release of the large majority of poliovirus from the apical domain of Caco-2 cells do not involve a monensin- or ammonium chloride-sensitive step. In contrast, monensin was reported to significantly inhibit the polarized release of SV40 from the apical domain of African green monkey kidney epithelial cells (13), suggesting that different processes are involved in the vectorial targeting of these non-enveloped viruses. SV40 is assembled in the nucleus, and it has been proposed that virus released into the perinuclear space (20, 33, 37) may become enclosed in vesicles derived from the contiguous rough endoplasmic reticulum (RER) (13). Vectorial transport may then be mediated by the

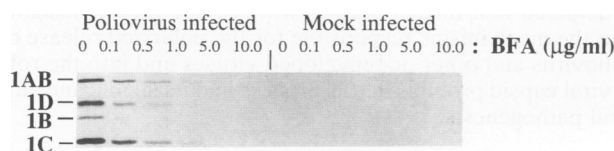


FIG. 6. Effect of BFA on poliovirus protein synthesis. Monolayers of Caco-2 cells grown on 4.5-cm<sup>2</sup> Transwell filters were infected with poliovirus (MOI of 1 pfu per cell) at the apical surface. Control monolayers were mock infected with PBS. At 2 hpi, media were exchanged for media containing the indicated concentrations of BFA. At 16 hpi, cells were metabolically labeled in the presence of BFA and lysates were analyzed by immunoprecipitation and SDS-PAGE using 12% polyacrylamide gels.



association and subsequent transport of SV40 virions with the virus receptor, which is targeted almost exclusively to the apical plasma membrane (13, 14). This comparatively simple model accounting for the vectorial release of SV40 is not applicable to poliovirus, since poliovirus replication is confined to the cytoplasm and our recent observations indicate that its receptor is present on both the apical and basolateral surfaces of Caco-2 cells (57).

Nonenveloped viruses, such as poliovirus and SV40, are generally considered to be released by lysis of the infected cell. However, several apparently nonlytic release processes, which likely involve some form of vesicular transport in order to circumvent the barrier presented by the plasma membrane in a non-disruptive manner, have also been described (2, 13, 17). Such a vesicular transport mechanism would account for the directional release of poliovirus by utilization of the currently accepted mechanism for the vectorial delivery of endogenous and exogenous membrane-bound and secretory proteins. The process by which poliovirions might become enclosed in, or associated with, vesicles bearing targeting signals remains to be elucidated. However, there is considerable evidence that poliovirus replication complexes are associated with host membranes, which are most likely derived from the RER (1, 5, 6, 16, 49–51, 54). Poliovirus-infected cells also characteristically exhibit a highly vesiculated cytoplasm (42), and continuous phospholipid biosynthesis to form membranes of defined composition appears essential for viral RNA synthesis (23, 24). Since polarized epithelial cells also transport lipids in a vectorial fashion (47), poliovirus transport to the apical plasma membrane may be a consequence of interaction with these or similar membranes. Alternatively, the vectorial transport of poliovirus may be mediated by an as yet uncharacterized process involving the recognition of targeting signals contained within the virion structure. We are also unable to exclude the possibility that vectorial lysis and/or extrusion of infected cells prior to lysis contribute to vectorial release.

Our observations on the effect of BFA on poliovirus replication are consistent with a requirement for an unperturbed RER or Golgi complex membrane for virus replication. BFA is known to significantly affect the secretory pathway in mammalian cells (for reviews, see references 29 and 39), causing disruption of the Golgi complex and the redistribution of Golgi enzymes to the RER in many cell types, and has been shown to have an inhibitory effect on the release of a variety of enveloped viruses (11, 12, 38, 52, 58, 60). BFA inhibition of poliovirus replication in Caco-2 cells occurred at low concentrations of the compound and appeared to be due to the specific inhibition of viral transcription or translation. We obtained no evidence that BFA has a significant effect on the release of progeny poliovirus from infected polarized epithelial cells. While this report was in preparation, Maynell et al. (34) reported the effects of BFA on poliovirus replication in HeLa cells. These authors also observed the inhibition of poliovirus replication following BFA treatment and presented evidence that the compound acts by inhibition of poliovirus RNA synthesis. More recently, Irurzun et al. (28a) have reported similar findings on the effect on BFA on poliovirus-infected HeLa cells which are in agreement with those of Maynell et al. The inhibition of capsid protein synthesis (Fig. 6) can thus be explained by the inhibition of virus-specific mRNA synthesis. Some minor differences between their results and ours are apparent. They reported that BFA inhibition of poliovirus replication was readily reversible and involved a step which occurred

prior to approximately 3 hpi, whereas we observed significant inhibition at 4 hpi and detected no effective increase in the yield of intracellular virus up to 8 h after the removal of the inhibitor. These differences are most likely a reflection of differences in experimental protocols but may be due to differences in the cell types, MOI, and concentration of BFA used. However, a comparison of our results with those reported by Maynell et al. and Irurzun et al. reveals that the potency of BFA as an inhibitor of poliovirus replication is approximately equivalent in HeLa, Vero C1008, and Caco-2 cells. The cellular targets for BFA action differ depending on the cell types examined and the concentration of the inhibitor used. In some cell types, for example, the Golgi complex is reported to be resistant to BFA (29, 39). It may therefore be significant that the effects of BFA on poliovirus replication are strikingly similar in three quite different cell types. The mechanism of BFA inhibition of poliovirus replication is currently unclear. We observed the induction of a polypeptide of approximately 80 kDa in uninfected and infected Caco-2 cells treated with BFA. Maynell et al. (34) reported a very similar finding and noted that the BFA-induced protein comigrated on two-dimensional gels with a protein induced during glucose deprivation, an observation suggesting that it may be glucose-regulated protein 78 (grp78), or immunoglobulin heavy-chain binding protein (BiP). Indeed, Liu et al. (30a) have provided evidence that this hypothesis is correct. Interestingly, the increase of detectable grp78 (BiP) upon BFA treatment appears to be regulated at transcriptional and posttranscriptional steps (30a). The role of the 80-kDa protein in poliovirus replication or in BFA-mediated perturbation of cell functions remains to be established.

The polarized release of poliovirus from the apical surface of a human intestinal epithelial cell line provides interesting implications for viral pathogenesis. We have recently demonstrated that the poliovirus receptor is present on both surfaces of this cell type, allowing infection to be mediated by adsorption to either surface (57). A simple model for poliovirus infection and dissemination from the human gut may therefore be proposed. From the observations of Sabin (43) and Bodian (3), lymphoid structures (such as Peyer's patches) located in the gastrointestinal epithelium comprise the initial replication site and are probably the portal of entry. Traversal of the epithelium at these sites may be cell mediated (46) or occur following necrosis of the epithelial barrier. The results presented in this report suggest that in the absence of extensive necrosis, the cell-mediated route may be preferred since only a small proportion of virus may be released at the basolateral surface of the infected epithelial cell. Dissemination of progeny virus into the gut may be mediated by infection of intestinal wall epithelial cells via the basolateral surface, followed by the vectorial release of virus from these cells into the apical or luminal environment. It is anticipated that further studies will provide greater insights into the mechanisms responsible for the polarized release of poliovirus and other nonenveloped viruses and into the role of viral capsid proteins in this process and its significance for viral pathogenesis.

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