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Polarized Entry of Canine Parvovirus in an Epithelial Cell Line

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The binding and uptake of canine parvovirus (CPV) in polarized epithelial cells were investigated by growing the cells on a permeable support and inoculating with the virus either from the apical or basolateral surface. Binding of radiolabeled CPV occurred preferentially on the basolateral surface. In contrast, when a similar experiment was carried out on nonpolarized A72 cells, virus binding occurred regardless of the direction of virus input. Binding appeared to be specific for CPV and could not be competitively inhibited by either bovine or porcine parvovirus. Analysis of the binding data revealed a high-affinity receptor (10^5 per cell) for CPV on the basolateral surfaces of MDCK cells (K_d, 29 pM). In indirect immunofluorescence studies, virus entered only from the basolateral surfaces of MDCK cells. These results provide evidence for a functional CPV-specific receptor that is expressed only on the basolateral surfaces of polarized epithelial cells, a result that has interesting consequences for viral pathogenesis.

The initial event in the life cycle of a virus is attachment to specific receptors on a host cell. Although such binding alone does not necessarily initiate a successful infection, this step may nevertheless be a major determinant of virus tropism. Autonomous parvoviruses are known to have a very restricted host range (17); infection is usually restricted to one host species, as indicated by the name of the virus. Moreover, they appear to require certain cellular factors expressed during the S phase of the cell cycle (6). Since parvoviruses cannot induce resting cells to enter the S phase, virus growth is restricted to the population of mitotically active cells (18). Parvovirus growth is also dependent on the differentiated state of the host cell, which indicates that the expression of developmentally regulated host factors also plays a role in productive replication (8, 9, 19). Therefore, the restricted host range for these viruses could be determined either at the level of the receptor or by specific interactions with certain other host factors (12, 19). Linser et al. (7) have presented evidence for a specific receptor for minute virus of mice in mouse A-9 and in Friend erythroleukemia cells. However, the biochemical nature of the receptor as well as the receptor specificity for this and other autonomous parvovirus types are not known.

Canine parvovirus (CPV) is known to cause enteric infections in dogs. The lesions in the intestine are characterized by destruction of the epithelial cells lining the crypts (2). The route of virus entry to these cells as well as the route of spread of infection to the adjacent cells are not known. Epithelial cells exhibit polarity because of the presence of tight junctions between the cells that define as well as separate the apical domain from the basolateral domain. Recent studies have shown that some viruses enter polarized epithelial cells preferentially from only one domain, apical or basolateral. Of the viruses that have been previously reported to enter cells in a polarized manner, vesicular stomatitis virus and Semliki forest virus were reported to enter preferentially from the basolateral surfaces of MDCK cells (4, 5). Similarly, reovirus serotype 1 was found to bind to the basolateral membrane of intestinal epithelial cells (13). In contrast, simian virus 40 was found to enter exclusively from the apical surface of polarized Vero C1008 or AGMK cells (1). The polarity of virus entry is presumed to be the result of polarized expression of the viral receptor. In this study, we have investigated whether CPV binds to specific receptors and whether such receptors are polarized in epithelial cells. The results of such studies should help our understanding of the virus infection process and the spread of infection.

To investigate the binding of CPV to MDCK cells, the cells were grown in 12-mm-diameter Millicell-HA filter chambers (Millipore Corp., Bedford, Mass.) in 24-well plates. After the cells reached confluency, the apical or basolateral surfaces were incubated with purified [^{35}S]methionine-labeled CPV at 4°C; at different times postinfection, the cell-associated radioactivity was determined. Virus binding to the basolateral surfaces of MDCK cells was about sixfold higher than to the apical surface at 2 h postinfection (Fig. 1A). In addition, indirect intracellular immunofluorescence studies as described below indicated that CPV can bind to and enter these cells only when the basolateral surface is accessible. Scatchard analysis of the binding to apical surfaces suggested low-affinity binding, which probably resulted from nonspecific interactions of the virions with the cell surface (data not shown). In contrast, in nonpolarized A72 cells, binding was not found to be dependent on the direction of virus input (Fig. 1B). The increased binding to the upper surfaces of A72 cells (Fig. 1B) may have been due to the fact that the available surface area was greater than the area of the smoother lower surface. Moreover, access of virions to the basal surface may have been lower because of interference by the filter. Thus, the results in Fig. 1 suggest that there is a specific receptor for CPV and that this receptor is located primarily on the basolateral surfaces of polarized epithelial cells.

To investigate the concentration dependence of the binding of CPV to cells, increasing amounts of [^{35}S]methionine-labeled CPV were added to the basolateral surfaces of the MDCK monolayers at 4°C for 2 h. The available binding sites were saturable at higher input multiplicity of virus (Fig. 2A). Replotting of these data by the method of Scatchard (15) (Fig. 2B) indicated that there were two distinct interactions between CPV and MDCK cells, with differing binding affinities. Approximately 10^5 high-affinity binding sites were present per cell, with an apparent K_d of 29 pM. The low-affinity sites probably represent nonspecific binding, as reported for other viruses such as vesicular stomatitis virus,

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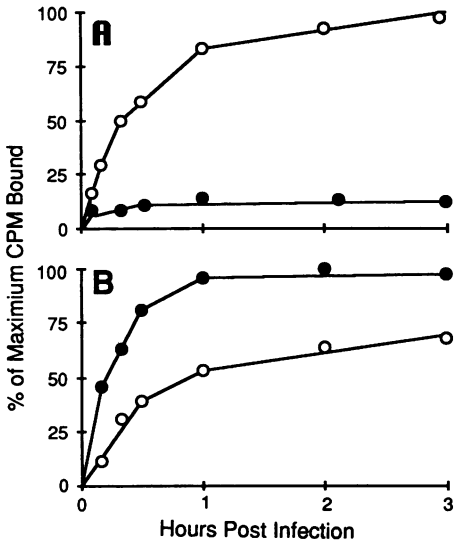

FIG. 1. (A) CPV binding to plasma membranes of MDCK cells. [^{35}S]methionine-labeled CPV was purified by a modification of the procedure of Paradiso (10). Briefly, A72 cells were infected with CPV in the presence of [^{35}S]methionine. At 48 h postinfection, cells were disrupted by freezing and thawing, and the supernatant was clarified. Virions were pelleted from the supernatant at 100,000 × *g* and further purified by banding in a CsCl gradient containing 0.2% sarcosine. Cells were grown on Millicell-HA filter chambers and inoculated with purified virions from either the apical (●) or basal (○) chamber at 4°C. At intervals postinfection, the inoculum was removed and the cell-associated radioactivity was determined by dissolving the cells with 0.1 N NaOH counting in a liquid scintillation counter. (B) CPV binding to plasma membranes of A72 cells. Methods were as described above. Binding of CPV to upper surfaces of A72 cells (●) is higher than to lower surfaces (○).

adenovirus, and reovirus, which have approximately 3.5 × 10^5 sites per cell (3, 11, 16).

The specificity of binding of CPV to MDCK cells was also examined by using a competitive inhibition assay (Fig. 3). In

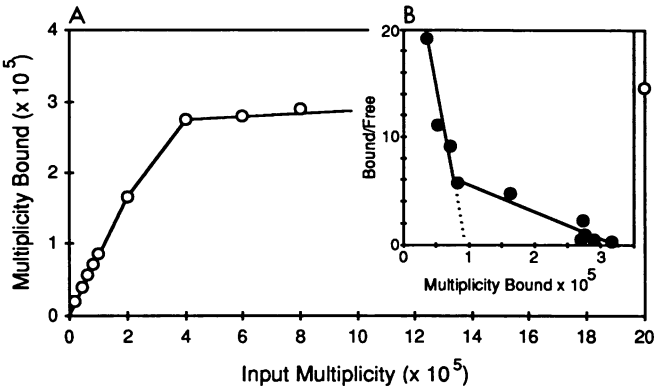

FIG. 2. Saturation of CPV-binding sites. (A) Confluent monolayers of MDCK cells were inoculated with increasing amounts of ^{35}S-labeled CPV at 4°C. At 2 h postinfection, the inoculum was removed and the cell-associated radioactivity was determined. Saturation of virus binding indicates that binding to cell surfaces is due to the presence of specific receptors. (B) Scatchard plot of the same data. The *x* intercept indicates the number of receptor sites per cell. The equilibrium dissociation constant (K_d, 29 pM) was calculated from the slope of the line and is indicative of high-affinity binding.

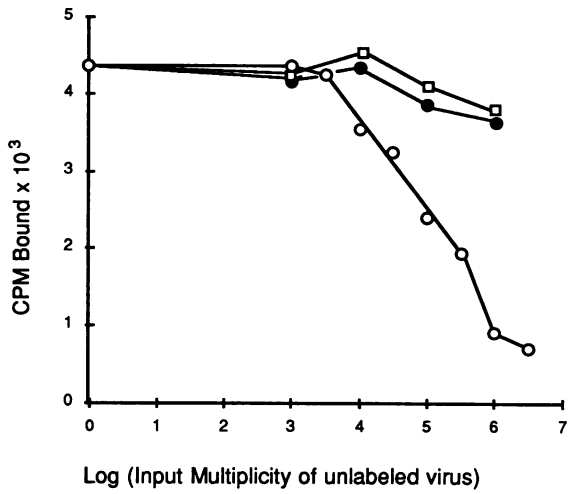

FIG. 3. Competition for CPV-binding sites. A fixed concentration of [^{35}S]methionine-labeled CPV was added to the confluent monolayers of MDCK cells in the presence of various concentrations of unlabeled CPV (○), PPV (●), or BPV (□). PPV and BPV were grown in ST and Buffalo lung cells, respectively, and purified by the procedure described in the legend to Fig. 1. After incubation at 4°C for 2 h, the inoculum was removed and the cell-associated radioactivity was determined. Multiplicities of unlabeled CPV greater than 10^5 could effectively compete for more than 50% of the available binding site. In contrast, no significant inhibition was observed with either PPV or BPV even at multiplicities of 10^6.

this experiment, a fixed amount of radiolabeled virus was added to the cells in the presence of various amounts of unlabeled virus. Unlabeled CPV virions were able to effectively compete with labeled particles, which indicated that a finite number of binding sites were present per cell. In contrast, binding of CPV to the basolateral surfaces of MDCK cells could not be competitively inhibited by other autonomous parvoviruses such as porcine parvovirus (PPV) or bovine parvovirus (BPV).

To investigate the entry of CPV into MDCK cells, the cells were grown on glass cover slips. After the cells reached confluency, they were treated with ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) to disrupt the tight junctions, thereby making the basolateral surface accessible to infection. After 2 h of adsorption at 4°C, cells were shifted to 37°C; at intervals postadsorption, cells were fixed and stained to determine the intracellular location of the input virions or viral antigens. No viral antigens were detected in cells infected from the apical surface (Fig. 4A). In contrast, viral antigens were detected in cells in which the basolateral surfaces were made accessible to the virus before infection (Fig. 4B), which indicated that the functional receptors for CPV were exclusively localized on the basolateral surfaces of these cells. There was some variation apparent in the intensity of fluorescence among different cells in the monolayer, which suggested that there may have been variation in the number of receptors per cell. At different times postincubation at 37°C, viral antigens were found to accumulate in the cytoplasm (Fig. 4C and D). However, even at 6 h postinfection, we were unable to detect any nuclear fluorescence in MDCK cells. In contrast, in A72 cells, which support virus replication, the input viral antigens were detected in the nucleus by immunofluorescence at 2 h postinfection (data not shown).

In an attempt to stimulate CPV replication in infected MDCK cells, cells infected from either the apical or baso-

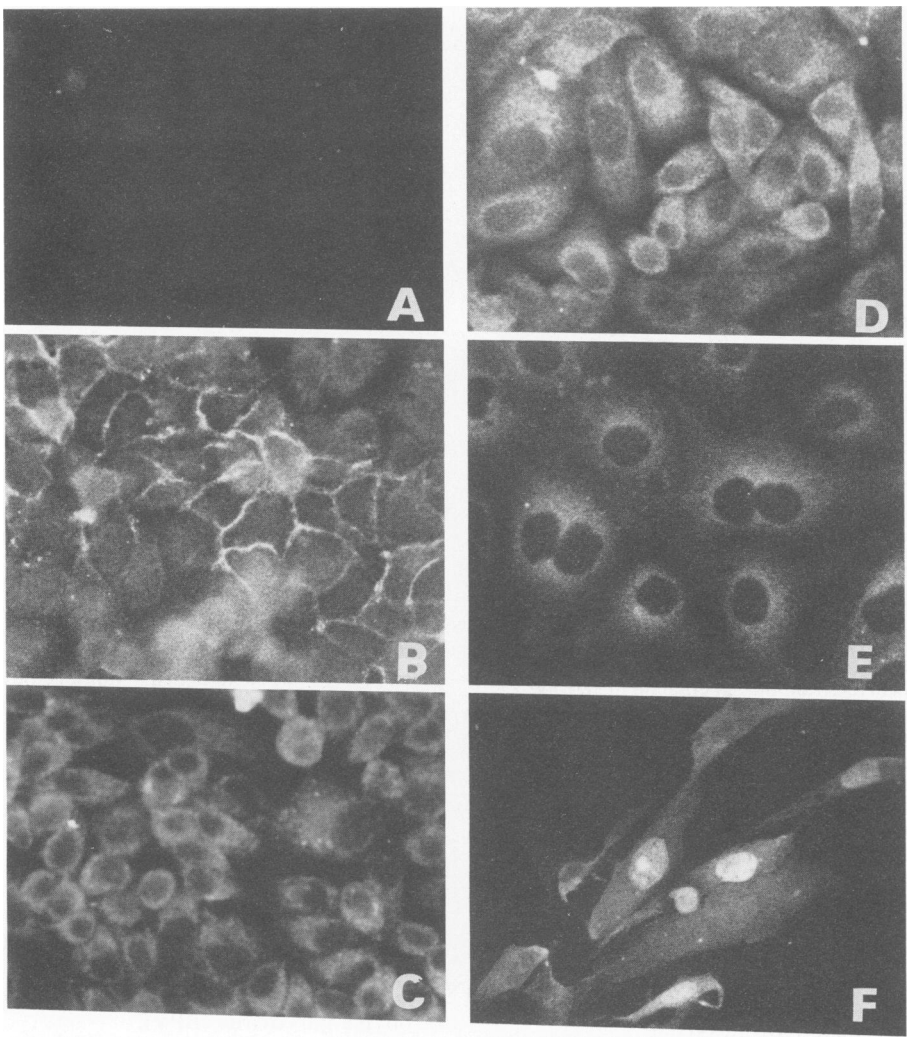

FIG. 4. Indirect immunofluorescence studies of the entry of CPV in a permissive (A72) and a nonpermissive (MDCK) cell line. Confluent monolayers of MDCK cells were infected with CPV at 4°C from either the apical surface or both surfaces (EGTA treated). At 2 h postinfection, cells were carefully washed to remove unadsorbed virions. Cells were then warmed to 37°C and, at intervals postadsorption, fixed with methanol-acetic acid (95:5) at −20°C for 30 min. Fixed cells were stained for CPV antigens with polyclonal rabbit anti-CPV serum (antiserum raised against CsCl gradient-purified CPV), followed by fluorescein-conjugated goat anti-rabbit immunoglobulin G, and examined for immunofluorescence. (A) Untreated cells; (B) EGTA-treated cells infected with CPV at 0 min postadsorption; (C and D) EGTA treated infected cells at 2 (C) and 6 (D) h postinfection; (E) MDCK cells (infected and passaged) at 16 h postinfection showing only cytoplasmic fluorescence; (F) A72 cells at 8 h postinfection showing nuclear fluorescence.

lateral surface were trypsinized at 2 h postinfection and reseeded at a lower density to stimulate cell division, a requirement for parvovirus replication. Again, no viral antigens were detected in cells infected from the apical surface (data not shown), whereas viral antigens were present in the cytoplasm 6 to 8 h postinfection when cells were infected from the basolateral surface (Fig. 4E). However, no viral antigen was detected in the nuclei of MDCK cells by immunofluorescence even 24 h postinfection. In addition, we were unable to detect viral DNA in the nucleus by in situ hybridization studies using a ^{32}P-labeled CPV DNA fragment as a probe (results not shown), which indicated the absence of viral replication. Moreover, using antiserum to nonstructural protein (obtained as a gift from P. Tattersall), we were unable to detect any newly synthesized nonstructural protein in the infected cells (results not shown). Under similar conditions, we have detected viral DNA in the nuclei of infected A72 cells at 8 h postinfection (data not shown) as well as newly synthesized viral antigens in nuclei at 24 h postinfection (Fig. 4F). Therefore, these results suggest that although virus entry into MDCK cells depends on the direction of virus input, entry into a dividing cell is not sufficient to support CPV replication.

The pathogenesis of a virus may be correlated with the route of entry as well as release. Some viruses, such as vesicular stomatitis virus, enter and are released from the basolateral surfaces of epithelial cells such that the spread of infection to underlying tissues and blood supply can be easily achieved. With other viruses, such as influenza and parainfluenza, which are released only from the apical surfaces of infected tissues, the spread of infection may be limited to the epithelial lining. Little is known about the pathogenesis of CPV in its natural host. Since CPV infects the epithelial cell lining of the small intestine, it was of interest to determine whether virus entry is polarized. Our results demonstrate that CPV binding occurs primarily on

the basolateral surfaces of MDCK cells, suggesting that a receptor for CPV is exclusively expressed on the basolateral surfaces of these epithelial cells. This study has also demonstrated that the receptor for CPV on the basolateral surfaces of MDCK cells is not shared by other parvoviruses such as PPV or BPV. Therefore, the restricted host range of these viruses may be determined at least in part at the level of the receptor. In contrast, Ridpath and Mengeling (12) have demonstrated that PPV can enter efficiently both in permissive and a nonpermissive cell line. The polarized uptake of CPV may also be relevant to virus infection in vivo. CPV grows predominantly in crypt cells of the epithelial lining of the small intestine in a manner similar to that of reovirus type 1. Rubin et al. (14) have demonstrated that reovirus type 1 first infects M cells in lymphoid patches of intestinal epithelium. Later, infection of adjacent crypt cells occurs through binding to basolateral surfaces. Whether CPV follows the same route in vivo remains to be determined. It will be of interest to determine whether other parvoviruses that infect epithelial cells in vivo also preferentially enter the cells through one of the cell surfaces, i.e., whether the receptors are localized in one domain. Such studies will contribute to a better understanding of the initial events of parvovirus pathogenesis as well as spread of the infection.

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