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Vaccinia Virus Preferentially Enters Polarized Epithelial Cells through the Basolateral Surface

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The uptake of vaccinia virus in polarized epithelial cells was studied to determine whether the site of entry was confined to either the apical or the basolateral membrane. Virus infection was monitored with a recombinant vaccinia virus carrying the luciferase reporter gene. Using cell lines MDCK and MDCK-D11, a clonal line with high transepithelial electrical resistance, we determined that vaccinia virus preferentially enters through the basolateral membrane. The possibility that there is a polarized cell surface distribution of vaccinia virus receptors which may be involved in systemic poxvirus infections is discussed.

Poxviruses can produce a systemic disease or a localized lesion in the skin at the site of inoculation (6). Little is known about the uptake of these viruses in vivo (21). In cultured cells, vaccinia virus (VV), a member of the *Orthopoxvirus* group, has a wide host range and is able to infect cells of many different origins. Studies with specific neutralizing antibodies suggest that viral binding and subsequent entry can be dissociated into two separate events, since it has been proposed that several polypeptides localized in the outer envelope of the viral particle play a role in either virus adsorption (13, 16, 24) or penetration (29, 30, 36). Because VV can be used as a live recombinant vaccine against many different diseases (22), understanding the mechanism of virus entry and tropism will be of major importance for the better usage of this virus as a vaccine.

The entry of several animal viruses has been shown to be restricted to only one of the two plasma membrane domains of polarized epithelial cells: the basolateral membrane, which faces the blood supply, or the apical membrane, which borders the luminal surface. It has been reported that vesicular stomatitis virus (7), Semliki Forest virus (8), and canine parvovirus (1) preferentially enter epithelial cells through the basolateral surface, while only simian virus 40 has been shown to enter solely through the apical surface of polarized epithelial cells (4). Polarized uptake in trypanosomes has also been shown (33).

The Madin-Darby canine kidney (MDCK) cell line appears to have been derived from the distal nephron (11, 28, 37), and it is one of the best characterized epithelial cell lines (32, 34). It exhibits a functional polarity characteristic of transporting epithelia (2, 32, 34) that is reflected by differences in the lipid and protein compositions of each membrane domain (10, 12, 15, 32, 38, 39). The MDCK cell line has been very useful in many studies on polarity of viral entry and viral maturation. However, it is known that the MDCK cell line is not homogeneous, and clonal lines that have transepithelial electrical resistances characteristic of leaky epithelia (150 $\Omega \cdot cm^2$) can be isolated, whereas other clones form tight epithelial monolayers that can develop very high electrical resistances (>1,000 $\Omega \cdot cm^2$) (27, 37). These cell types are morphologically distinct (19, 23, 25, 37) and show differences in both membrane receptors (19, 20) and lipid composition (9, 23).

Because polarized viral entry is a quantitative phenomenon (7), in this investigation we have studied the route of VV entry in cells of the D11 line, a subclone of the MDCK cell line that is highly polarized with a transepithelial electrical resistance of 1,000 to 2,000 $\Omega \cdot cm^2$ (10, 25). A recombinant of VV harboring the luciferase reporter gene (recombinant WR-LUC) was used to monitor virus entry through the apical and basolateral membrane domains of D11 cells. Since luciferase expression can be easily measured, we were able to utilize its measurement as a sensitive method to evaluate the extent of viral infection (31).

To study the ability of VV to enter polarized cells through either the apical or basolateral surface, confluent and subconfluent monolayers of MDCK cells and D11 cells were infected with WR-LUC. Cells were seeded in 12-well plates at densities of 5×10^5 (confluent) or 5×10^4 (subconfluent) cells per cm^2. After 3 days of growth, MDCK and D11 cells were infected with different multiplicities (1, 10, and 50 PFU per cell) of the WR-LUC recombinant virus. At 24 h postinfection (p.i.), cells were washed with phosphate-buffered saline (PBS), and cell extracts were tested for luciferase activity as previously described (31). In confluent MDCK monolayers, the amount of luciferase produced (measured in nanograms per microgram of cell protein) was only 25 to 60% of that produced by subconfluent monolayers, with the greater differences being observed at lower multiplicities of VV infection (Fig. 1A). However, when confluent D11 monolayers were infected, luciferase production represented <5% of that obtained with subconfluent monolayers at any of the three multiplicities of virus infection used (Fig. 1B).

The tight junctions that separate the apical and basolateral membrane domains of epithelial cells can be disrupted after treatment with EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] (2). Thus, to expose the basolateral surface to VV, confluent D11 cells were treated with 30 mM EGTA for 30 min at 37°C and infected with

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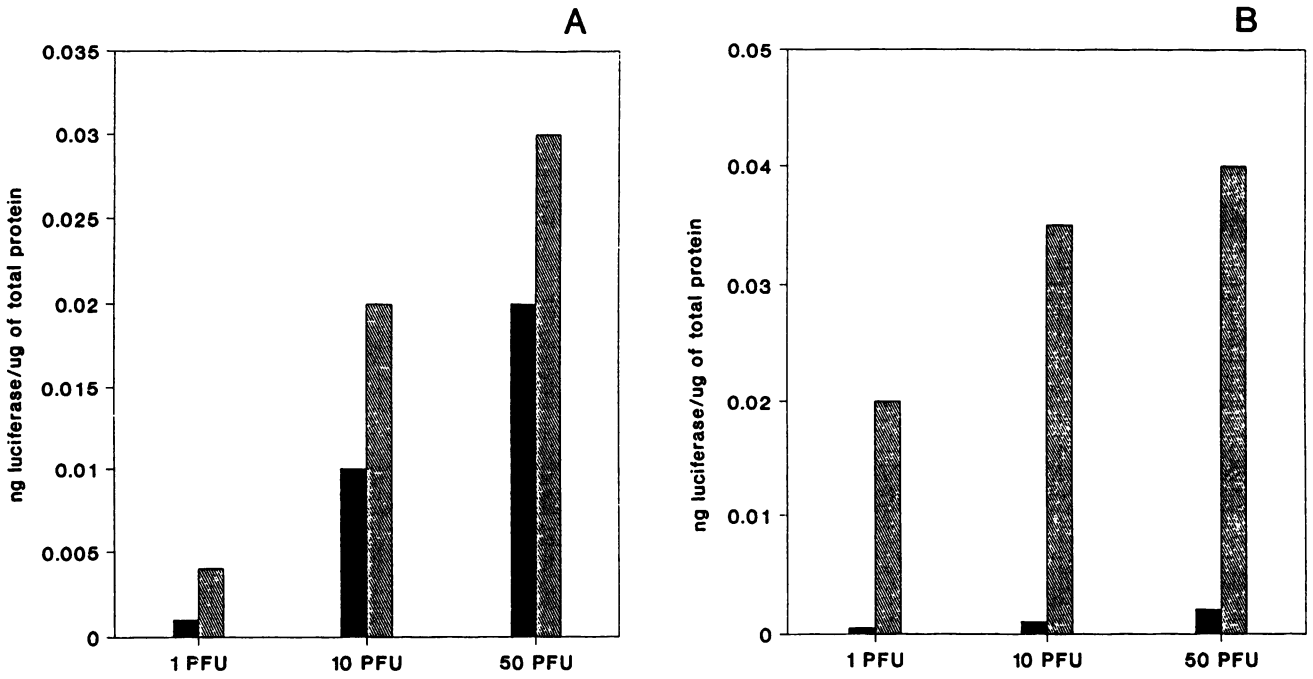

FIG. 1. Luciferase expression in confluent and subconfluent monolayers of MDCK and D11 cells infected with the recombinant VV WR-LUC. Cells seeded on 12-well plates and grown in Dulbecco's modified Eagle medium were infected with different multiplicities of WR-LUC either as confluent (■) or subconfluent (▧) monolayers. At 24 h p.i., cells were harvested, washed with PBS, and suspended in 100 μl of luciferase buffer (100 mM potassium phosphate, pH 7.8, 1 mM dithiothreitol). Luciferase activity in cell extracts was determined as previously described (31), with a luminometer apparatus (Monolight 2010; Analytical Luminescence Laboratory, San Diego, Calif.). The mean of duplicate samples (standard error, <5%), represented as nanograms of luciferase per microgram of total cell protein, was determined by comparison with a standard curve made with purified luciferase enzyme. (A) MDCK cells; (B) D11 cells.

different multiplicities (1, 10, and 50 PFU per cell) of the WR-LUC recombinant virus, and luciferase levels were measured in cell extracts prepared at 8 h p.i. Low levels of luciferase activity were observed when intact monolayers were infected with WR-LUC, while disruption of the tight junctions by EGTA treatment resulted in a significant increase (5- to 10-fold) of luciferase expression (Fig. 2). These results suggest that VV may preferentially enter polarized epithelial cells through the basolateral membrane.

Epithelial cells grown on permeable supports have been used to study the polarity of virus entry (1, 7, 8). We have used permeable supports to compare the efficiency of VV infection from the apical and basolateral surfaces of D11 cells. Cells grown on Nuclepore filters (3-μm pores) were infected from either the apical or the basolateral side of monolayers with different multiplicities (1, 10, and 50 PFU per cell) of WR-LUC. At 8 h p.i., cells were washed and luciferase activity in cell extracts was measured. When cells were infected from the apical side, little luciferase expression was detected and almost no differences in luciferase activity were observed between cells infected with multiplicities of either 10 or 50 PFU per cell (Fig. 3). However, high levels of luciferase were obtained when cells were infected from the basolateral side (through the filter), and the levels of luciferase expression were directly dependent on the multiplicity of VV infection (Fig. 3).

Because VV induces morphological damage in infected cells (6, 21), we examined luciferase expression during the time course of virus infection in D11 cells infected through either the apical or the basolateral surface. This was possible because the luciferase gene is under the control of the VV early-late promoter p7.5 (3). When confluent D11 cells grown on filters were infected with WR-LUC (50 PFU per cell) through the basolateral surface, luciferase expression was detected as early as 2 h p.i. and increased with time (Fig. 4). When cells were infected from the apical surface, measurable levels of luciferase were detected between 4 and 6 h p.i., but these levels were 5- to 10-fold lower than those in cells infected through the basolateral surface (Fig. 4). Although the experiments described above were carried out with intracellular virions, similar results were observed with extracellular enveloped virions (data not shown).

Previous reports have shown the usefulness of VV recombinants as an expression system for studying the directional transport of envelope glycoproteins from heterologous viruses in MDCK cells (14, 26, 35). On the basis of these studies and of the ability of VV recombinants to infect confluent monolayers of MDCK cells, it appears that receptors for VV are present on the apical surface of MDCK cells. This is not surprising in view of previous observations that some basolateral membrane proteins can be found on the apical surface during development of MDCK cell surface polarity (12, 39). The low levels of luciferase expression in apically infected cells might also be due to the existence of a subpopulation of nonpolarized or incompletely polarized cells, as has been previously reported with other virus-cell systems (4, 7). Furthermore, it is also possible that nonspecific uptake of some virus particles from the apical surface could lead to low levels of luciferase expression. Our results clearly demonstrate that entry of VV into D11 cells occurs preferentially at the basolateral surfaces in a polarized

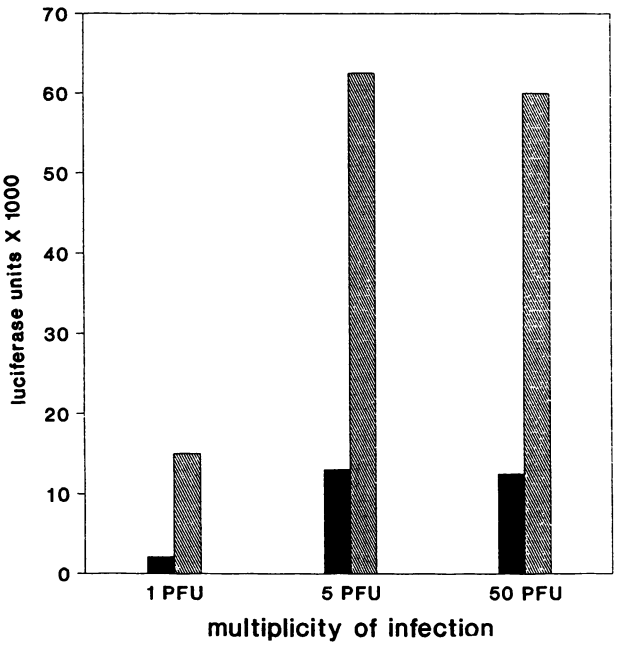

FIG. 2. Luciferase expression in D11 cells infected with WR-LUC after disruption of the tight junctions. Cells grown to confluency in 24-well plates were washed with Ca^{2+}- and Mg^{2+}-free PBS and treated with 30 mM EGTA in Dulbecco's modified Eagle medium, for 30 min at 37°C and then infected with various multiplicities of WR-LUC. At 8 h p.i., luciferase activity in cell extracts was measured. Results from duplicate samples are expressed in luciferase units per 5×10^4 cells with mean values varying by <5%. Symbols: ■, untreated cells; ▨, EGTA-treated cells.

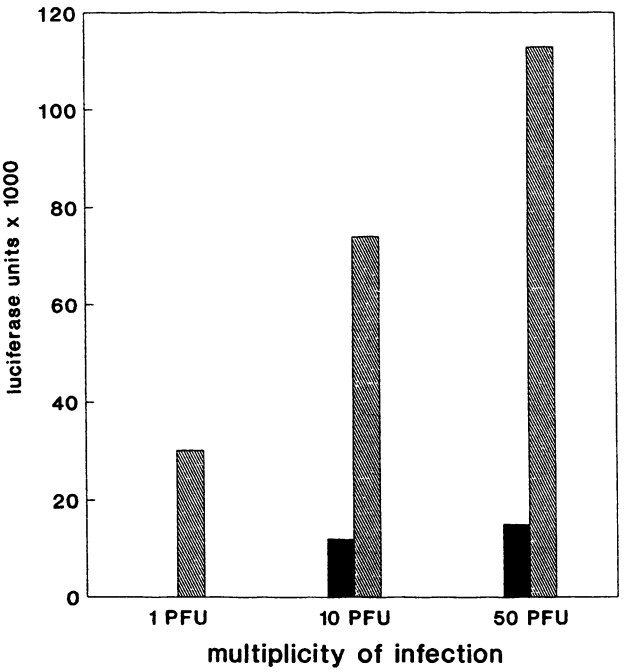

FIG. 3. Expression of luciferase in polarized cells infected with WR-LUC through either the apical (■) or basal (▨) surfaces. D11 cells grown to confluency on gelatin-coated Nuclepore filters (12-mm diameter, 3-μm pores) attached to plastic cylinders in 24-well plates were infected with different multiplicities of WR-LUC. After 1 h of virus adsorption at 37°C, the inoculum was removed, cells were washed with Dulbecco's modified Eagle medium, and fresh medium containing 2% newborn calf serum was added. At 8 h p.i., cells were washed with PBS and cell extracts were prepared in 150 μl of luciferase buffer (31). Results from duplicate samples are expressed in luciferase units per 5×10^4 cells, with mean values differing by <5%.

fashion. However, our results do not distinguish which step in virus entry (binding, membrane fusion, or postfusion events) is blocked at the apical surface.

Our findings suggest the possibility that a receptor(s) for VV is present on the basolateral membrane of both MDCK and D11 cells. The identity of the receptor for VV is still unknown. It has been proposed that the epidermal growth factor receptor can mediate VV infection (5, 18). Although the general importance of the epidermal growth factor receptor in VV infection remains uncertain, it is relevant to point out that the epidermal growth factor receptor is expressed primarily on the basolateral surface of both MDCK cells (17) and other epithelial cells (34). Regardless of the nature of the cell receptor(s) used by VV, it is clear from our findings that cellular determinants important for virus infection are localized on the basolateral membrane of epithelial cells.

It has been reported that there is a differential distribution of at least one cell surface glycoprotein (gp23) between high-resistance cell lines and leaky MDCK cell lines (10, 25). gp23 is restricted to the basolateral domain of D11 cells, while on leaky MDCK cell lines it shows a nonpolarized distribution. On epithelial cells of the renal distal and collecting tubule, the proposed origin of MDCK cells (11, 28), gp23 also shows a basolateral distribution (25), suggesting that this glycoprotein is correctly targeted only on the high-electrical-resistance cell lines (10, 25). Because of the high polarity exhibited by D11 cells, it seems possible that the suggested basolateral expression of VV receptor(s) on this cell line closely resembles what the virus encounters in vivo.

It should be noted that measuring viral infections by plaque assay is an insensitive method for the VV-MDCK cell system, because the differences between cell-associated virus and virus yields at 24 h p.i. are very small, under any condition of virus infection used. For example, D11 cells infected in 12-mm-diameter Nuclepore filters with 10 PFU per cell had titers for input virus of 8×10^3 PFU per ml (apical) and 7.4×10^3 PFU per ml (basolateral), while titers of the infectious virus produced at 24 h p.i. were 4.2×10^4 PFU per ml (apical) and 7.6×10^4 PFU per ml (basolateral).

The pathogenesis of poxvirus infections has been elegantly defined with ectromelia (mousepox) (6). In this model, invasion of the virus through the skin is followed by local multiplication in situ, which serves to spread the virus to sites, such as the reticuloendothelial system, where additional replication causes the virus to enter the blood and invade the liver and spleen, which are targets for virus multiplication. Following extensive replication in these organs, a secondary viremia occurs with resultant peripheral spread of virus to produce focal lesions (pox) of the skin and mucous membranes (rash). Since virus particles are likely to be transported in the blood by macrophages (6), preferential infection at the basolateral surface of epithelial cells might facilitate the outcome of the systemic disease caused by poxviruses.

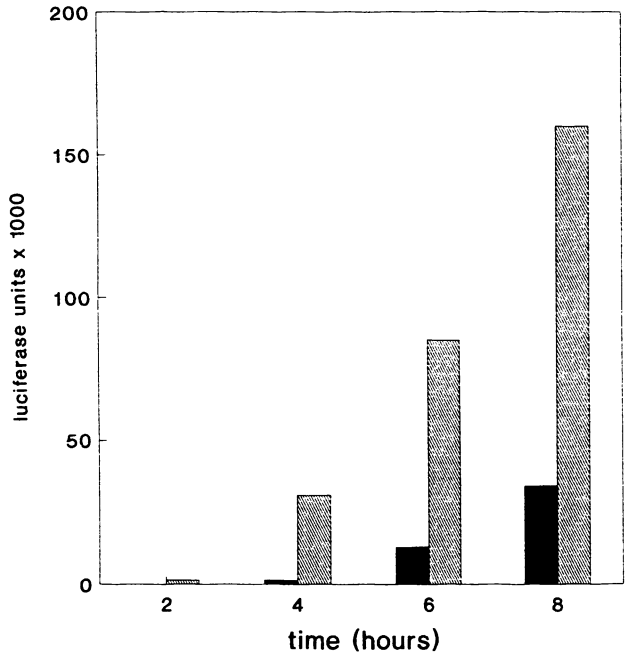

FIG. 4. Time course of luciferase expression in D11 polarized cells infected with WR-LUC through either the apical (■) or basal (▨) surfaces. Cells seeded to confluency on filters were infected with 50 PFU per cell (Fig. 3). At different times p.i., cell extracts were prepared and luciferase activity was measured in duplicate samples (standard error, <5%).

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