JOURNAL OF VIROLOGY, Aug. 1980, p. 547-550
0022-538X/80/08-0547/04$02.00/0

Vol. 35, No. 2

Antibody-Resistant Spread of Vesicular Stomatitis Virus Infection in Cell Lines of Epithelial Origin

MICHAEL G. ROTH AND RICHARD W. COMPANS*

Department of Microbiology, University of Alabama in Birmingham, Birmingham, Alabama 35294

In MDCK cells, vesicular stomatitis virus (VSV) buds exclusively from the basolateral plasma membranes beneath tight junctions, whereas influenza virus forms only at the free apical surface. Anti-VSV antiserum did not prevent the formation of plaques on MDCK cell monolayers infected with VSV, whereas plaque formation in BHK-21 cells was completely inhibited by such antiserum. Under similar conditions, homologous antiserum completely prevented plaque formation by influenza virus on MDCK cells. In several other epithelioid cell lines, VSV also formed plaques in the presence of specific antiserum. These results suggest that VSV receptors are present on basolateral membranes in the cells studied and that junctional complexes present between cells may exclude antibody from intercellular spaces and thus permit the lateral spread of virus infection in the presence of neutralizing antibody.

Cells of the Madin-Darby canine kidney (MDCK) line retain many of the features of normal secretory epithelia (2, 5). MDCK cells in culture form tight junctions between adjacent cells, produce a spontaneous electrical potential between the upper and lower cell surfaces, exhibit a vectorial transport of fluid from the apical to basal surface, and maintain contact inhibition (1–4). When injected into baby nude mice, these cells form epithelial sheets lining fluid-filled glands with tight junctions between cells and with apical cell surfaces oriented toward the central lumen (5).

Recently it has been reported that the assembly of vesicular stomatitis virus (VSV) occurs exclusively at the basolateral membranes of MDCK cells in confluent monolayers, whereas influenza and several parainfluenza viruses bud only from the free apical surface (6). We have previously investigated the possible role of differences in glycosylation of viral glycoproteins in determining the maturation sites of enveloped viruses in MDCK cells (7). Using tunicamycin, an inhibitor of formation of asparagine-linked oligosaccharides (8), we demonstrated that the maturation site of VSV or influenza virus is not altered by complete inhibition of glycosylation. In the course of these studies we observed that MDCK monolayers infected with VSV contain large numbers of virus particles in the intracellular spaces beneath intact tight junctions. Since intact tight junctions prevent the passage of large molecules, this observation suggested that VSV might be able to spread laterally and form plaques on MDCK monolayers in the presence of anti-VSV antibody, provided that VSV receptors are present on basolateral cell surfaces.

Plaque-purified VSV of the prototype Indiana serotype and the A/WSN (H0N1) strain of influenza virus were grown and assayed as previously described (7). For all experiments, multiplicities of infection were calculated by assays performed on the same cell line as that being tested, since VSV titers on MDCK cells were consistently 1 to 2 logs lower than on BHK-21 cells (data not shown). Madin-Darby bovine kidney (MDBK), MDCK, Vero, and BHK-21 cells were obtained from Flow Laboratories (Rockville, Md.), LLC-MK_2 cells were obtained from J. Clerx, and PS-EK porcine kidney cells were obtained from P. Walker. All cell lines were maintained in Dulbecco-modified Eagle minimal medium with 0.1 μg of streptomycin, 500 U of penicillin, and 0.5 μg of gentamicin per ml. MDCK and PS-EK cells were grown in medium containing 2% newborn calf serum, and other cell lines were grown in 10% newborn calf serum. Virus-specific antiserum was prepared by intravenous inoculation of rabbits with purified influenza A/WSN or VSV-Indiana virions (~0.25 mg of protein) followed by intraperitoneal injection with an equal amount of virus after 2 weeks. Serum was obtained 2 weeks after the booster injection. A 1:16,000 dilution of the VSV antiserum produced a 50% reduction in the number of VSV plaques that formed in BHK-21 cell monolayers, and the anti-influenza antiserum had a hemagglutination inhibition titer of 4,800. Both antisera were heat inactivated at 56°C for 30 min before being used in neutralization assays.

In initial experiments designed to assess the effect of antibody on plaque formation, confluent 60-mm monolayers of MDCK cells were infected with VSV or influenza virus, and BHK-21 cell

monolayers were infected with VSV. After an adsorption period of 1 h for VSV or 2 h for influenza virus, the inoculum was removed, and each plate received the appropriate overlay (7). A 1:100 dilution of the antiserum specific for the infecting virus was included in the overlay of half of the plates. After 30 h, VSV had produced large plaques on BHK-21 monolayers lacking antiserum, but no plaques were detected on plates to which antiserum had been added (Fig. 1a). After 2 days, VSV had produced numerous small plaques on MDCK monolayers in the presence or absence of antiserum (Fig. 1b). In the presence of antiserum, the VSV plaques were smaller in size, although the average plaque number was not reduced significantly in most experiments. Increasing the antiserum concentration fivefold gave similar results. In contrast to VSV, influenza virus, which does not bud beneath the tight junctions of MDCK cells, did not form any plaques in these cells in the presence of anti-influenza antiserum (Fig. 1c). We also observed that VSV-infected MDCK monolayers overlaid with liquid maintenance medium lacking antibody formed plaque-like lesions during the first 24 h postinfection. Subsequently, secondary sites of infection were observed that spread rapidly, and the monolayer was destroyed. However, when anti-VSV antiserum was included in the liquid overlay, VSV produced plaques similar in number and size to those formed with agar in the overlay. These results indicate that VSV receptors are present on the basolateral surfaces of MDCK cells, and that VSV infection can spread laterally through MDCK monolayers in the presence of neutralizing antibody.

The experiment described below was designed to test our assumption that tight junctions are responsible for the antiserum-resistant plaque formation by VSV on MDCK cells. VSV-infected monolayers were treated with 3 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid in phosphate-buffered saline deficient in Mg^{2+} and Ca^{2+} for 1 h after removal of the virus inoculum. Such treatment has been reported to disrupt tight junctions in these cells for a period of 6 h or more (I. Meza, M. Sabanero, A. Martinez-Palomo, and M. Cereijido, J. Cell Biol. **79:** 243a, 1978). After 1 h at room temperature under these conditions, cells were observed to have rounded slightly and pulled apart. At

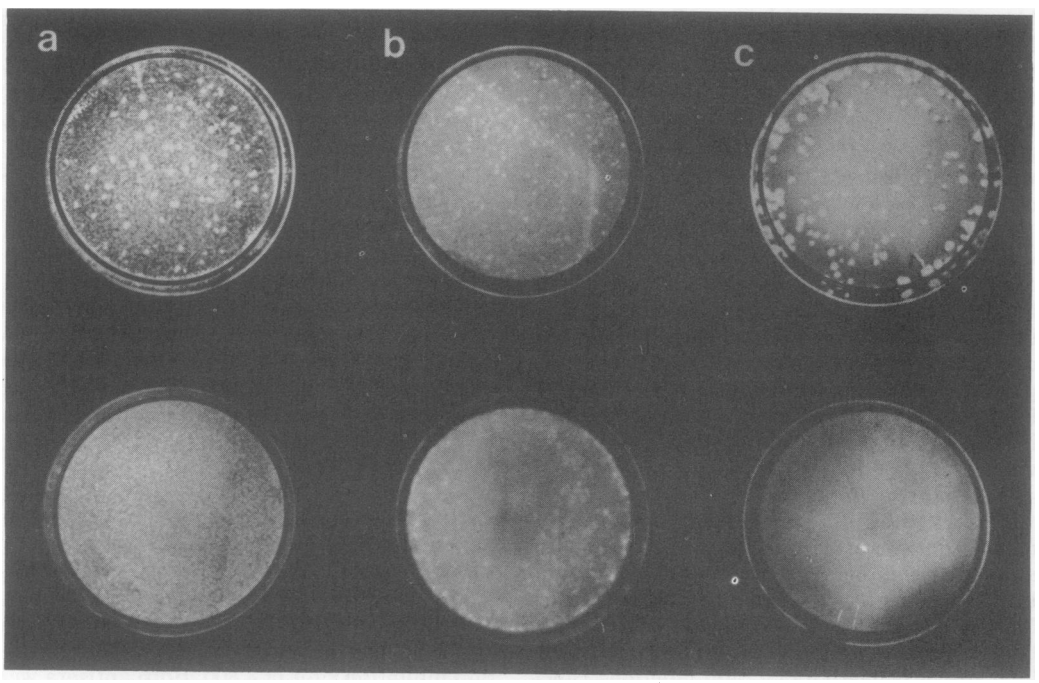

FIG. 1. *Effect of antibody on plaque formation by VSV and influenza virus. Confluent monolayers were infected with VSV and incubated with agar overlays in the absence (upper row) or presence (lower row) of antiviral antiserum. In the case of influenza virus, 0.0005% trypsin was included in the overlay. Plates were fixed with 7% formaldehyde and stained with Giemsa stain at 2 days postinfection for BHK-21 and 4 days postinfection for MDCK cells. (a) VSV plaques in BHK-21 cells. (b) VSV plaques in MDCK cells. (c) Influenza virus plaques in MDCK cells.*

this time the monolayers were washed with phosphate-buffered saline and overlaid with agar containing or lacking anti-VSV antiserum. The presence of agar between rounded cells prevented them from resuming a normal, flattened morphology. Pretreatment with ethylene glycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid followed in this way with an agar overlay containing anti-VSV antiserum resulted in a reduction of plaque number to less than 10% of the number present on control monolayers treated with ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid and overlaid without antiserum. Where plaques did form in the presence of antiserum, microscopic observation revealed that the surrounding monolayer had a normal morphology with tightly packed, flattened cells at the edge of the plaque, suggesting that plaque formation occurred in areas where the tight junctions had not been disrupted.

It seemed possible that other cell lines that have retained tight junctions might also exhibit polarity of maturation of enveloped viruses as a general feature. Monolayers of four cell lines known or suspected to form tight junctions were infected with VSV, fixed in situ for electron microscopy (7), removed from their plastic substrate with propylene oxide, and embedded in an epoxy resin. By electron-microscopic observation of thin sections, we found no polarity of VSV maturation in Vero or LLC-MK_2 cells. In PS-EK and MDBK cells, VSV showed a strong preference for maturation at basolateral membranes, although occasional particles were seen budding from apical surfaces. We determined whether VSV could form antiserum-resistant plaques on these four cell lines, none of which is known to retain specialized functions characteristic of its tissue of origin. All of these cell lines will overgrow; therefore, the experiments were performed in duplicate using both heavily overgrown cultures and newly confluent monolayers. In addition, both freshly confluent and heavily overgrown cultures of BHK-21 cells were used as controls. VSV was able to form plaques in the presence of anti-VSV antiserum on all of the epithelioid cell lines tested, but the presence of antiserum prevented plaques in BHK-21 cells even in heavily overgrown layers. Results of a representative experiment are shown in Table 1 and in Fig. 2. In each case, the plaques formed in the presence of antiserum enlarged more slowly than those in its absence, as shown in Fig. 2. This observation is consistent with the idea that progeny virus budding from the apical membranes of these cell types will be neutralized by antibody. Thus it is likely that virus will spread from an infected cell only to adjacent cells where antibody is excluded from the intercellular spaces. In contrast, Fig. 1b shows that plaque size in the presence and absence of antiserum does not differ as much in MDCK cells, indicating that the spread of virus along apical surfaces might contribute less to plaque formation in this cell type than in LLC-MK_2 or PS-EK cells. We have noticed some variability in the number of antibody-resistant plaques formed by VSV in repeated experiments in the same cell line, which may reflect the age or condition of the cell monolayers used. Thus the reduction in plaque number by antibody in Vero cells in Table 1 was not consistently observed in other experiments.

TABLE 1. *Plaque formation by VSV in the presence and in the absence of anti-VSV antiserum*[a]

Cell type	No. of plaques	
	VSV antibody	No antibody
LLC-MK_2	30	33
PS-EK	33	37
Vero	77	133
BHK-21/c13	0	65

[a] Each plaque number represents the average from three plates counted on day 2 (Vero, BHK-21) or day 4 (LLC-MK_2, PS-EK) after infection. A 1:100 dilution of anti-VSV rabbit serum was used. Plates were stained with neutral red before plaques were counted.

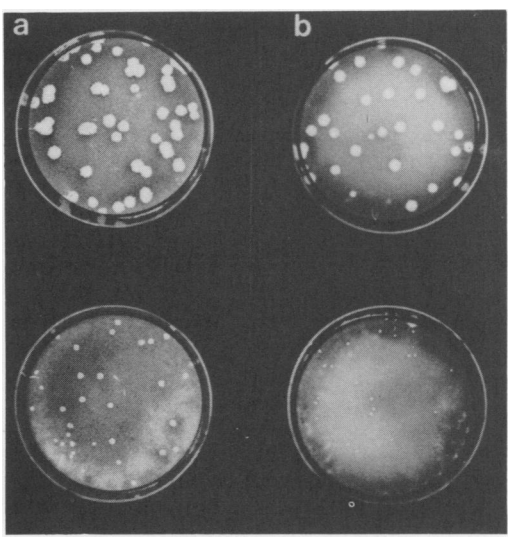

FIG. 2. *Plaque formation by VSV in LLC-MK_2 and PS-EK cells in the presence of antibody. The agar overlay in the upper row contained no antibody, and the overlay in the lower row contained anti-VSV rabbit serum at a dilution of 1:100. (a) LLC-MK_2 cells. (b) PS-EK cells. Plates were fixed and stained as in Fig. 1 and photographed on day 4 postinfection.*

Tight junctions establish two separate domains of the plasma membranes of MDCK cells in culture, which are similar to the functionally polar surfaces of epithelial tissues. The observations reported here indicate that in this cell type influenza virus, and presumably other viruses which do not form beneath tight junctions, will not be protected from neutralizing antiserum, whereas VSV and presumably other viruses capable of maturation and adsorption at basolateral membranes will not be neutralized. In other cell lines where VSV matures at both apical and basolateral membranes, the presence of junctional complexes will also permit the lateral spread of virus infection in the presence of heat-inactivated antiserum, where inactivation of complement prevents possible immune cytolysis. The latter phemonenon may not have a parallel in vivo, where immune cytolysis might play a role in preventing the lateral spread of infection.

This research was supported by research grants PCM79-13057 from the National Science Foundation, AI-12680 from the National Institute of Allergy and Infectious Diseases, and IN66 from the American Cancer Society. M.G.R. is supported by Public Health Service predoctoral training grant T32-AI-07150 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Cereijido, M., E. S. Robbins, W. J. Dolan, C. A. Rotunno, and D. D. Sabatini.** 1978. Polarized monolayers formed by epithelial cells on a permeable and translucent support. J. Cell. Biol. **77**:853-880.
2. **Leighton, J., L. W. Estes, S. Mansukhan, and Z. Brada.** 1970. A cell line derived from normal dog kidney (MDCK) exhibiting qualities of papillary adenocarcinoma and of renal tubular epithelium. Cancer **26**:1022-1028.
3. **Lever, J. E.** 1979. Inducers of mammalian cell differentiation stimulate dome formation in a differentiated kidney epithelial cell line (MDCK). Proc. Natl. Acad. Sci. U.S.A. **76**:1323-1327.
4. **Misfeld, D. S., S. T. Hamamoto, and D. R. Pitelka.** 1976. Transepithelial transport in cell culture. Proc. Natl. Acad. Sci. U.S.A. **73**:1212-1216.
5. **Rindler, M. J., L. M. Chuman, L. Shaffer, and M. H. Saier.** 1979. Retention of differentiated properties in an established dog kidney epithelial cell line (MDCK). J. Cell Biol. **81**:635-648.
6. **Rodriguez Boulan, E., and D. D. Sabatini.** 1978. Asymmetric budding of viruses in epithelial monolayers: a model system for study of epithelial polarity. Proc. Natl. Acad. Sci. U.S.A. **75**:5071-5075.
7. **Roth, M. G., J. P. Fitzpatrick, and R. W. Compans.** 1979. Polarity of virus maturation in MDCK cells: lack of a requirement for glycosylation of viral glycoproteins. Proc. Natl. Acad. Sci. U.S.A. **76**:6430-6434.
8. **Tkacz, J. S., and J. O. Lampen.** 1975. Tunicamycin inhibition of polyisoprenol *N*-acetylglucosaminyl pyrophosphate formation in calf liver microsomes. Biochem. Biophys. Res. Commun. **65**:248-257.