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Cell surface influenza haemagglutinin can mediate infection by other animal viruses

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We have used filter-grown Madin-Darby canine kidney (MDCK) cells to explore the mechanism by which influenza virus facilitates secondary virus infection. Vesicular stomatitis virus (VSV) and Semliki Forest virus (SFV) infect only through the basolateral surface of these polarized epithelial cells and not through the apical surface. Prior infection with influenza virus rendered the cell susceptible to infection by VSV or SFV through either surface. The presence of both a permissive and a restrictive surface for virus entry in the same cell allowed us to determine how the influenza infection enhanced the subsequent infection of a second virus. Biochemical and morphological evidence showed that influenza haemagglutinin on the apical surface serves as a receptor for the superinfecting virus by binding to its sialic acid-bearing envelope proteins. Influenza virus also facilitates secondary virus infection in non-epithelial cells; baby hamster kidney cells (BHK-21), which are normally resistant to infection by the coronavirus (mouse hepatitis virus MHV-A59), could be infected via the haemagglutinin-sialic acid interaction. Facilitation of secondary virus infection requires only the sialic acid-binding properties of the haemagglutinin since the uncleaved haemagglutinin could also mediate virus entry.

Key words: alphavirus/coronavirus/polarity/receptor-mediated endocytosis/rhabdovirus/virus receptors

Introduction

The interactions between two animal viruses which infect the same tissue comprise a complex array of phenomena. Often the establishment of one virus infection in a tissue interferes with its susceptibility to a second animal virus infection. Many mechanisms of interference have been characterized and range from effects at the level of entry, such as the inactivation of cell surface receptors, to those at the level of replication, such as competition for host cell components required for viral protein synthesis, to those at the level of propagation, such as those mediated by the interferons (Fenner *et al.*, 1974). A complementary set of interactions by which infection with one virus increases the susceptibility of a tissue to a second virus infection has also been described. Complementation occurs in many systems and is particularly well studied in retroviral systems (Dickson *et al.*, 1982). A special type of complementation occurs between enveloped viruses of different families. Here a mutant virus, often vesicular stomatitis virus (VSV), with a defective envelope protein can replicate only in cells bearing the envelope proteins of another virus. As a result, mixed or pseudotypic virions are produced bearing the envelope proteins of one virus and the internal proteins of the other (Zavada, 1982). Enhancement, as distinguished from complementation, denotes an interaction in which mixed infection by unrelated viruses results in increased virus production and/or cytopathic effects (Fenner *et al.*, 1974). Although such phenomena are clearly important for an understanding of viral pathogenesis, the mechanism of enhancement is known in few cases. The replication of interferon-sensitive viruses such as VSV can be enhanced in tissues pre-infected with paramyxoviruses, which suppress the production of interferon (Hermodsson, 1963; Cantell and Valle, 1965; Frothingham, 1965). Facilitation, or non-interferon-mediated enhancement, has also been described (Ginder and Friedewald, 1951; Padgett and Walker, 1970; Tsuchiya and Tagaya, 1970). However, the lack of convenient and well-defined model systems has made the elucidation of mechanisms for these processes difficult.

Simple (Rodriguez-Boulan, 1983) and multilayered (Klenk, 1980) epithelial cells provide model systems for the virus infections of the organized tissues found *in vivo*. The best characterized of the model epithelial systems is the Madin-Darby canine kidney (MDCK) cell line, which was originally developed to study transport processes in epithelia and subsequently adapted to the study of virus infection (Rodriguez-Boulan, 1983). Confluent MDCK cells are linked by circumferential tight junctions into an epithelium-like sheet. The tight junction defines two distinct domains of plasma membrane: the apical, corresponding to the luminal surface of the renal tubule and the basolateral, corresponding to the serosal surface. Rodriguez-Boulan and Sabatini (1978) showed that virus maturation observed the polarity of the MDCK cell. Influenza and parainfluenza viruses mature only from the apical surface while VSV buds exclusively from the basolateral surface. Polarity of virus maturation appears to be a consequence of the polar localization of viral envelope proteins since the G protein of VSV is located predominantly on the basolateral surface, while the haemagglutinin and neuraminidase of influenza are predominantly on the apical surface (Rodriguez-Boulan and Pendergast, 1980). We extended these observations to the polarity of virus infection by using MDCK monolayers grown on large pore (3.0 μm) nitrocellulose filters to allow access of virus to either surface of the monolayer (Fuller *et al.*, 1984). Enveloped animal viruses exhibit an infection polarity which parallels their maturation polarity. VSV, for example, infects polar MDCK cells only through their basolateral surface. Influenza virus can infect the cell through both surfaces but sialic acid-bearing serum proteins on the filter inhibited infection in the model system as they would on the serosal side *in vivo*. VSV infection polarity appears to reflect receptor polarity (Fuller *et al.*, 1984).

We first observed the phenomenon described here during double infection experiments with MDCK cell monolayers grown on plastic (Fuller *et al.*, 1985). This system differs from the filter system in that only the apical surface of the monolayer is exposed for infection. Under these conditions primary VSV infection was relatively inefficient and developed slowly. In contrast, MDCK monolayers which had been pre-infected with the influenza virus, fowl plague virus (FPV), became susceptible to VSV

infection from the apical surface. Although interferon-mediated enhancement of VSV infection has been described (Valle and Cantell, 1965; Cantell and Valle, 1965), the rapidity with which the effect appeared and the fact that the proteins of both viruses are expressed in the same cell (Fuller *et al.*, 1985) exclude such a mechanism. Complementation can also be ruled out because each virus replicates efficiently when introduced into the cell through the appropriate surface. Therefore this system presented a previously uncharacterized type of enhancement and the phenomenon seemed to be tied to the epithelial nature of the system and might therefore be relevant to dual virus infections of epithelia *in vivo*.

Here we employ highly polarized, filter-grown MDCK cells to demonstrate that the enhancement of VSV infection in influenza-infected MDCK cells results from the ability of influenza haemagglutinin on the cell surface to act as a receptor for the entry of VSV by binding to the sialic acid-binding VSV G protein. This is the first demonstration that such an interaction can mediate infection of a previously resistant cell surface. The mechanism is not limited to VSV or to epithelial cells; cell surface haemagglutinin can also mediate apical infection by Semliki Forest virus (SFV) of MDCK cells as well as infection of the non-polarized baby hamster kidney cell (BHK-21) by mouse hepatitis virus (MHV-A59), a coronavirus.

Results

Influenza infection mediates VSV entry through the apical surface of MDCK

The ability of influenza infection to mediate apical infection of MDCK by VSV is shown in Figure 1. VSV was applied to the apical (a) or basolateral (b) surface of MDCK monolayers grown on 3.0 μm pore size filters at various times after infection with the avian influenza, FPV, or after mock infection. Protein synthesis in the cells 5 h after VSV application was monitored by a pulse-chase experiment. In the absence of FPV infection, VSV only infected the monolayers efficiently when applied to the basolateral surface. Prior infection with FPV rendered the cell susceptible to VSV infection from either surface. The greater polarity of the filter-grown cells renders the effect much clearer, since apical VSV infection is absolutely dependent on FPV pre-infection. The rates of VSV protein synthesis after basolateral infection in the presence or absence of FPV pre-infection were similar (data not shown) suggesting that the major effect of FPV pre-infection was limited to facilitating entry through the previously resistant apical surface. The mobility shift in G protein in the presence of FPV infection reflects intracellular contact between the envelope proteins (FPV neuraminidase and VSV G protein) of the two viruses and confirms that both are expressed in the same cell (Fuller *et al.*, 1985).

Infection with FPV increases VSV binding to the apical surface of MDCK

Incubation of uninfected MDCK monolayers with high concentrations of VSV (100 μg/ml for 1 h at 4°C; i.e., $>10^6$ particles/cell) resulted in no detectable binding of virions to the apical surface. Electron microscopic examination of cells in several such experiments has never revealed bound VS virions (data not shown). In contrast, parallel experiments with MDCK monolayers after 4 h of FPV infection showed extensive binding of VSV to the apical surface (not shown). Hence, the increase in susceptibility to apical infection seen in Figure 1 is accompanied by the appearance of VSV binding sites on this surface.

These apical VSV binding sites could represent receptors capable of mediating VSV entry at this normally resistant surface. These receptors which appear after FPV infection, would then be the basis of the enhancement of VSV infection. We therefore compared the characteristics of FPV-induced binding with FPV-induced infection. The issues of binding and infection must be considered separately, particularly for the case of VSV whose normal cell surface receptors remain uncharacterized (see however, Schlegel *et al.*, 1983) and whose binding to the cell surface is not saturable (Miller and Lenard, 1980; Matlin *et al.*, 1982).

One plausible basis for the binding of VSV would be the well characterized affinity of surface expressed influenza haemagglutinin for sialic acid-bearing glycans (for reviews see Compans and Choppin, 1975; Schulze, 1975) such as those of the G protein of VSV. This interaction is sufficiently stable to enable the inhibition of influenza haemagglutination by VSV to be used as an assay for this virus (Compans, 1974). In the MDCK system influenza haemagglutinin would then act as the VSV receptor and the characteristics of FPV-mediated infection would mirror those of this interaction. Alternatively the polarity of the cell surface or the integrity of the tight junctions might be destroyed through the cytopathic effects of FPV infection. Apically applied VSV would then have access to receptors which are basolateral in the uninfected and polar cell. The characteristics of the FPV-induced receptors would then match those of basolateral VSV receptors in the uninfected cell. A second possibility would be that the infected cell contains receptors on its apical surface for VSV which are masked in the uninfected cell. In this case, influenza infection would modify the surface to uncover existing receptors. The influenza neuraminidase, for example, could remove sialic acid from the gangliosides of the apical surface allowing closer approach and binding of the negatively charged VS virions (Bücher and Palese, 1975). The experiments below were designed to distinguish between these alternatives.

VSV binding to influenza-infected MDCK cells is sialic acid-dependent

The expression of influenza haemagglutinin on the apical surface as a function of time was measured by an indirect radiometric assay using an anti-FPV haemagglutinin antibody (Figure 2). Figure 2b shows the amount of [^{35}S]methionine-labelled VSV bound by the apical surface of the MDCK cell monolayer at increasing times after FPV infection. The qualitative correlation between influenza haemagglutinin expression and the binding of VSV to the apical surface shown by our electron microscopic results is confirmed by the quantitative results shown in Figure 2. The expression of influenza haemagglutinin preceded the increase in VSV binding by ~30 min and thereafter the two rose together.

The sialic acid-dependence of VSV binding to FPV-infected monolayers was assayed using neuraminidase-treated VSV. Pretreatment of VSV with *Clostridium perfringens* neuraminidase under the conditions described in Materials and methods abolished the binding between VS virions and FP virions as assayed by equilibrium centrifugation (Figure 3) as well as the binding to FPV-infected monolayers (Figure 2b). The FP virion-VS virion complex was stable once it had formed. The FPV neuraminidase was not able to disrupt the interaction even after overnight incubations at 37°C. This must reflect a lack of accessibility of the neuraminidase to haemagglutinin-bound G protein in the virus since solubilized FPV proteins readily cleared the sialic acid from solubilized VSV G protein (Fuller *et al.*, 1985).

VSV binding to its normal receptors in MDCK and in other

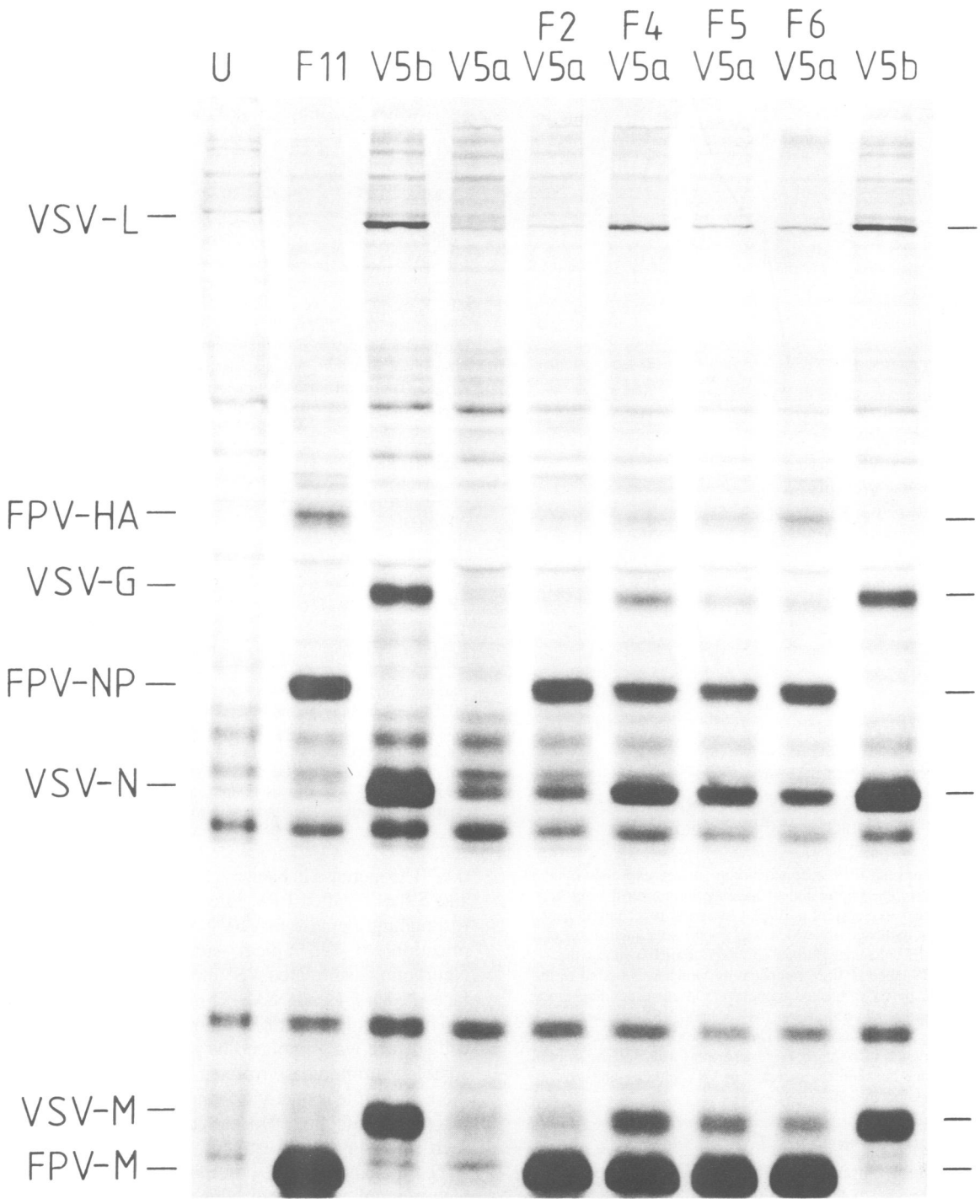

Fig. 1. Effect of FPV pre-infection on VSV protein synthesis. MDCK cell monolayers were removed from holders and infected by apical application of 20 p.f.u./cell of FPV for 1 h and then incubated at 37°C for a further 1 h (F2), 3 h (F4), 4 h (F5) or 5 h (F6). 20 p.f.u./cell of VSV was then applied to the apical surface of the monolayers and the infection allowed to proceed for a further 5 h (V5a). The cells were then pulsed with ^{35}S-labelled methionine for 5 min, chased for 40 min and analysed by SDS-polyacrylamide gel electrophoresis. Approximately equal numbers of cells (10^5) were loaded on each lane. Samples which were infected with FPV alone for 11 h (F11), with VSV alone from the apical (V5a) or basal (V5b) surface for 5 h as well as mock-infected (U) samples were included for comparison. Equal counts were loaded onto each lane to compensate for differences in methionine incorporation. The individual viral proteins are labelled at the left. Comparison of the FPV-pre-infected samples with the non-FPV-infected ones clearly shows an increased efficiency of VSV infection from the apical surface as reflected by viral protein synthesis. The maximum effect is seen between 4 h (F4 V5a) and 5 h (F5 V5a) after FPV infection.

cells is strongly pH dependent and is > 10-fold greater at pH 6.3 than at pH 7.4 (Miller and Lenard, 1980; Matlin *et al.*, 1982). In contrast, influenza haemagglutinin binding to sialic acid varies only slightly over this pH range. Figure 3b shows a weak dependence of VSV binding on pH and hence is consistent with the behaviour of binding to influenza haemagglutinin.

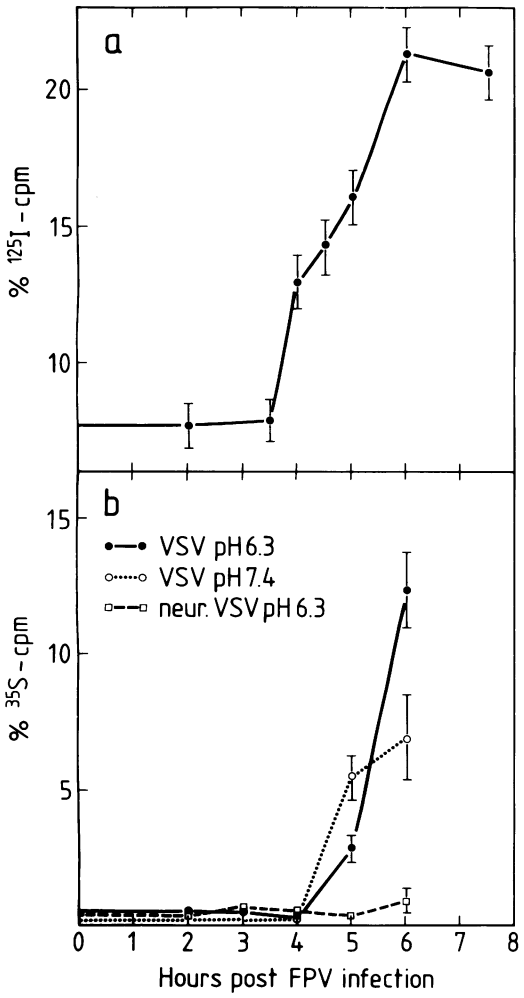

Fig. 2. Correlation between FPV haemagglutinin surface expression and VSV binding. **Panel a**. The expression of haemagglutinin on the apical surface of FPV-infected monolayers is displayed as the percentage of total ^{125}I-labelled protein A radioactivity which was bound after incubation of the monolayer with anti-FPV haemagglutinin antibody. **Panel b** shows the percentage of total ^{35}S-labelled VSV radioactivity which was bound to the apical surface at 4°C by FPV-infected monolayers at corresponding times. Binding was performed at pH 6.3 and at pH 7.4 with VSV and with neuraminidase-treated VSV (neur.VSV). The standard error of the mean for each point is shown when its value is larger than the width of the symbol. A clear correspondence between the increase in VSV binding and haemagglutinin expression is seen.

Table I. FPV infection does not increase monolayer permeability

Absorption	Resistance (ohm cm^2)	
	Before FPV infection	After VSV
Mock-infected	2492 ± 219	3220 ± 268
2 h FPV	2026 ± 207	2733 ± 293
3 h FPV	1970 ± 78	2498 ± 63
4 h FPV	2051 ± 134	2463 ± 136
5 h FPV	2095 ± 272	2387 ± 399
6 h FPV	2270 ± 441	2348 ± 165

MDCK monolayers were infected with FPV for various times or mock-infected and then VSV was applied to the apical surface for 1 h at 37°C. The electrical resistance of the monolayers was measured before the initiation of FPV infection and after the absorption of VSV to the apical surface. The mean for each time point (3–5 samples) and the standard error of the mean are shown.

Table II. SFV infection is more efficient from the basal surface than from the apical

P.f.u./cell	Fields counted	SFV-infected cells/field
200 p.f.u./cell basal	20	95.4 ± 22
40 p.f.u./cell basal	20	127 ± 40
8 p.f.u./cell basal	20	5.2 ± 1.5
200 p.f.u./cell apical	100	0.02

Various amounts of SFV were applied to the apical or basal surface of MDCK cell monolayers and the cells held at 37°C for 5 h to allow the infection to develop. The cells were fixed and processed for immunofluorescence by application of reagents to the basal surface of the cell. Infected cells were scored at a magnification of 100× so that each field contained ~1000 cells. Duplicate or triplicate filters were used for each determination. The standard errors of the means are shown.

Surface haemagglutinin mediates VSV infection

The characteristics of influenza-mediated VSV infection were compared with those of VSV binding to the apical surface. Immunofluorescence experiments (Figure 4) showed that VSV infection through the apical surface was dependent on the expression of the influenza haemagglutinin and on sialation of VSV. Either VSV (Va) or neuraminidase-treated VSV (N-Va) was applied to the apical surface of MDCK cell monolayers after either 4 or 5 h of influenza infection. The monolayers were incubated for a further 5 h to allow the VSV infection to develop and then fixed and processed for immunofluorescence. Monolayers which had not been infected with FPV (top row) did not show VSV protein expression when VSV (Va) or neuraminidase-treated VSV (N-Va) was applied to the apical surface. Neuraminidase-treated VSV (N-Vb) and VSV (Figure 1 and Fuller *et al.*, 1984) both infected efficiently through the basolateral surface. FPV infection for either 4 h (F4) or 5 h (F5) mediated apical VSV infection. The increase in haemagglutinin expression between 4 h (F4) and 5 h (F5) after FPV infection was accompanied by a corresponding increase in VSV protein expression 5 h after its application to the apical surface (Va-F4 versus Va-F5). Neuraminidase-treated VSV never infected from the apical surface (N-Va-F4 and N-Va-F5).

The possible effect of influenza neuraminidase action on the cell surface was simulated by treatment with 10 units/ml of the soluble neuraminidase from *C. perfringens*. This is >100 times the neuraminidase activity contained in the FPV produced during 6 h of infection. No increase in apical VSV infection was seen following this treatment.

The intactness of the cell monolayer throughout the FPV infection and the VSV absorption period was monitored by measuring the transepithelial resistance. The resistance of the monolayers remained >2000 ohm cm^2 (Table I). All of these characteristics of apical VSV infection mirror those of the VSV binding seen in Figures 2 and 3. They support a mechanism in which binding of VSV through its sialic acid to the influenza haemagglutinin on the cell surface mediates the entry of the virus. Neither the effects of influenza neuraminidase action nor of depolarization of existing receptors can account for the influenza-mediated infection. The time dependence of the process excludes the possibility that binding to whole FP virions mediates VSV infection. At early times more input FPV would be present on the cell surface than is produced during the first 4 h of infection yet apical VSV infection is not seen until newly synthesized haemagglutinin appears.

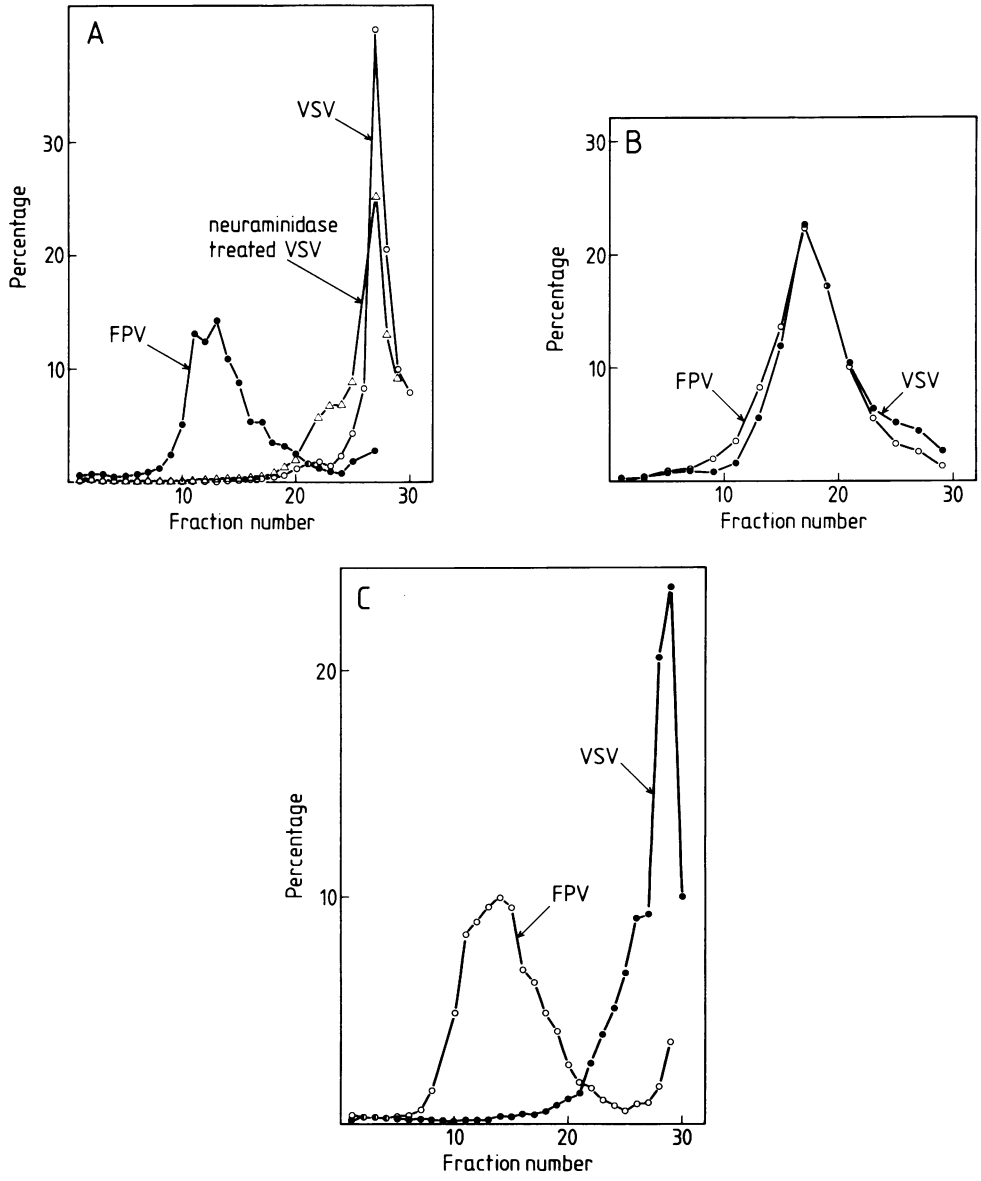

Fig. 3. Sialic acid-mediated binding of VSV by FPV. The distribution of [^{3}H]leucine-labelled FPV (●——●) and [^{35}S]methionine-labelled VSV (○——○) or neuraminidase-treated [^{35}S]methionine-labelled VSV (△——△) after separate equilibrium centrifugation on 20% (fraction 30) to 28% (fraction 1) potassium tartrate gradients are shown in **panel A**. The results for mixtures of FPV and VSV (**panel B**) and of FPV and neuraminidase-treated VSV (**panel C**) are shown. The distributions are expressed as a percentage of the total counts for each virus. The interaction between FPV and VSV, seen by their co-migration in **panel B**, is abolished by pre-treatment of the VSV with neuraminidase.

Apical infection of MDCK cells by SFV can be mediated by influenza virus pre-infection

The infection of MDCK cell monolayers by the alphavirus, SFV, displays a polarity which parallels that of VSV (Figure 5). SFV-infected MDCK monolayers when applied to the basolateral surface (Sb) but not after application to the apical surface (Sa). The SFV infection results in viral protein synthesis but not in the production of virus particles (data not shown). Immunofluorescent staining of the cell surface reveals that the envelope proteins are predominantly expressed on the basolateral surface although a small fraction can be seen on the apical surface of some cells. The relative efficiencies of apical and basolateral infection were compared by counting the number of cells infected after application of varying amounts of SFV to either surface (Table II). This quantitation showed that basolateral SFV infection was at least 1000 times more efficient than apical.

The low background level of SFV apical infection made the effect of influenza pre-infection very striking. Five hours of influenza infection dramatically increased (> 100 times) the number of cells which could be infected by SFV from the apical surface (Sa-F5). Neuraminidase treatment of the SFV abolished the influenza-mediated apical infectivity (N Sa-F5), while leaving its basolateral infectivity unaltered (N Sb). Electron microscopy showed no binding of SFV to the apical surface of uninfected MDCK cell monolayers (data not shown). Five hours of FPV infection, however, resulted in extensive apical SFV binding. High transmonolayer electrical resistance >2000 ohm cm^2 was also maintained throughout these experiments (data not shown).

Mouse hepatitis virus infects BHK cells infected with influenza virus

The influenza-mediated infection mechanism we have characterized with MDCK cells should not be restricted to polarized,

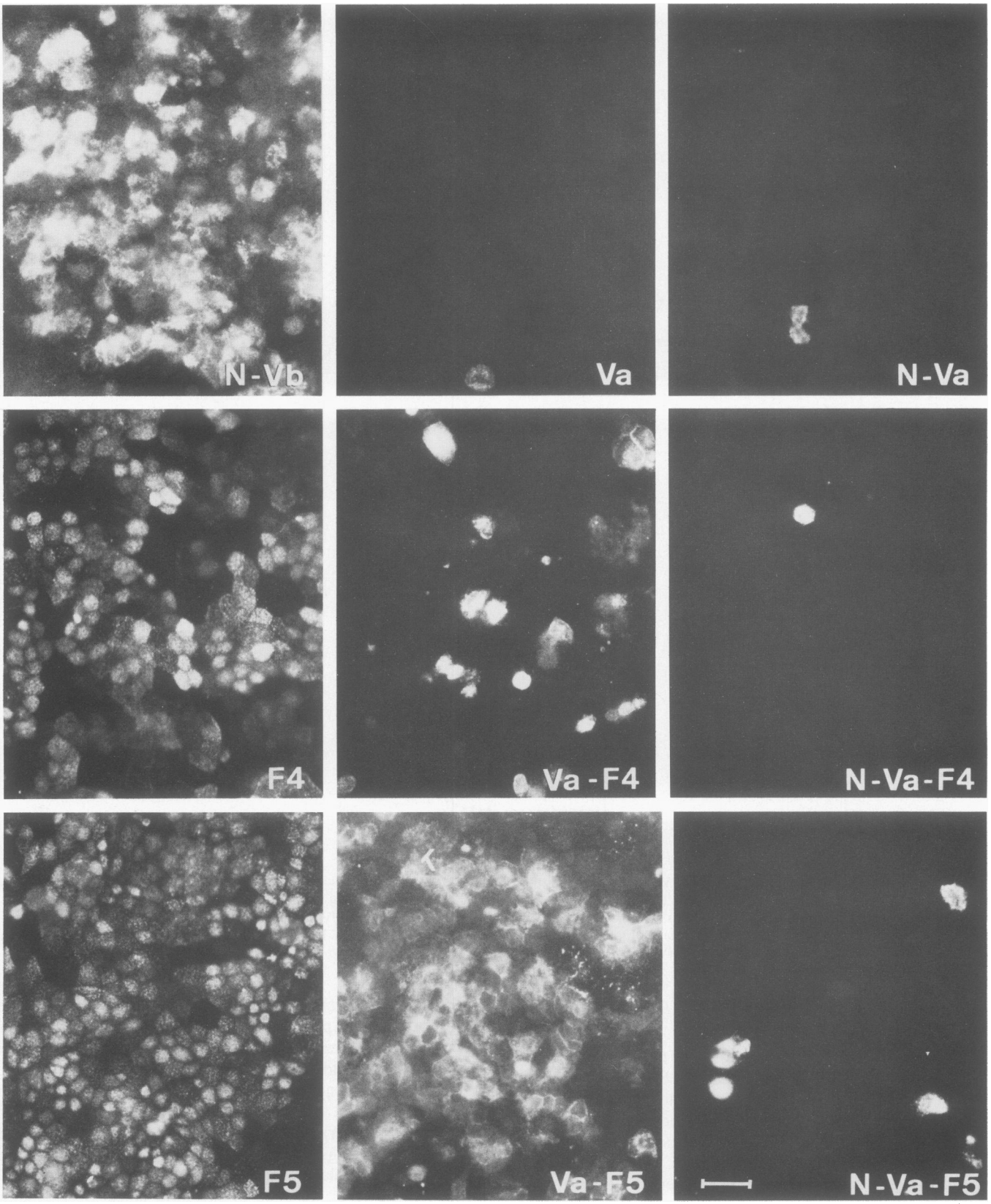

Fig. 4. VSV infection in FPV-pre-infected cells. MDCK cell monolayers were infected with VSV and/or FPV for various periods, fixed, permeabilized with Triton X-100 and processed for indirect immunofluoresence using antibodies directed against the FPV haemagglutinin (F4, F5) or against the VSV-G protein (all others). **Top row**: monolayers challenged with neuraminidase-treated VSV from the basal surface (N-Vb), VSV from the apical surface (Va) or neuraminidase-treated VSV from the apical surface (N-Va) and incubated for 5 h at 37°C to allow the infection to develop before fixation. **Middle row**: monolayers infected with FPV for 4 h and fixed (F4) or challenged with VSV (Va-F4) or neuraminidase-treated VSV (N-Va-F4) from the apical side and incubated for 5 h to allow the VSV infection to develop. **Bottom row**: monolayers infected with FPV for 5 h and fixed (F5), or challenged with VSV (Va-F5) or neuraminidase-treated VSV (N-Va-F5) from the apical side and incubated for a further 5 h to allow the VSV infection to develop. All images are presented at the same magnification. Each field contains >500 cells. The bar represents 25 μm.

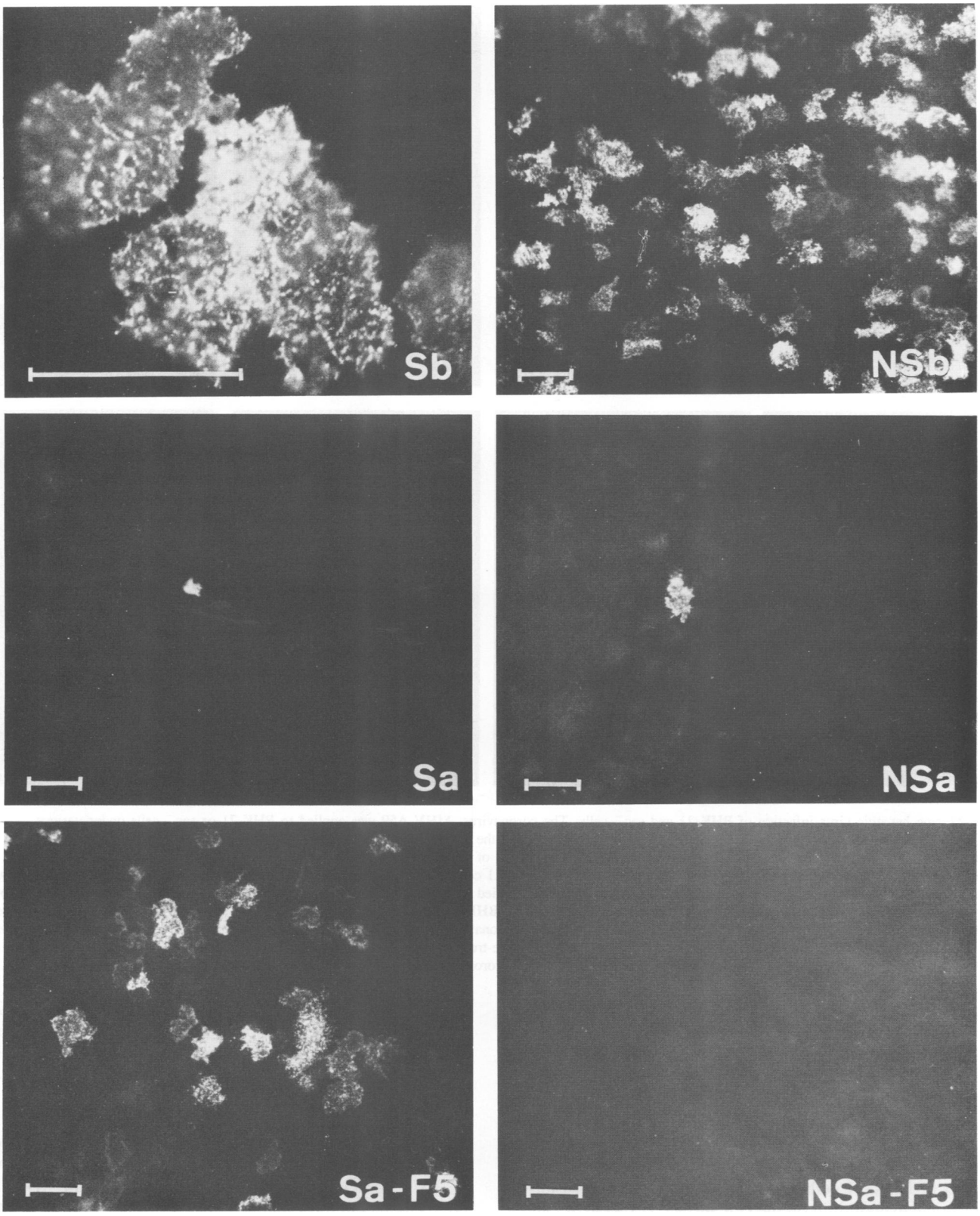

Fig. 5. SFV infection of MDCK cells. SFV was applied to MDCK monolayers and the cells held for 5 h at 37°C to allow development of infection. The monolayers were fixed and processed for surface immunofluorescence by application of reagents to the basolateral surface. An antibody directed against the envelope proteins of SFV was used for all pictures. **Sb**: high magnification image of the basal surface of a monolayer infected with SFV from the basal side. Although both VSV and SFV proteins are expressed basolaterally, the SFV proteins appear more basally localized due to less swelling of the paracellular spaces during infection (cf. Fuller *et al.*, 1984, Figure 4). **Sa**: occasional field which contained an infected cell after application of SFV to the apical surface of the monolayer. **Sa-F5**: result of the application of SFV to the apical surface of a monolayer which had been infected with FPV 5 h previously. **NSb**: infection caused by application of neuraminidase-treated SFV to the basolateral surface of the monolayer. **NSa**: result of application of neuraminidase-treated SFV to the apical surface of the monolayer. **NSa-F5**: result of application of neuraminidase-treated SFV to the apical surface of a monolayer which had been infected with FPV 5 h previously. All fields, except Sb, are at the same magnification as in Figure 4 and contain >500 cells. Bars represent 25 μm.

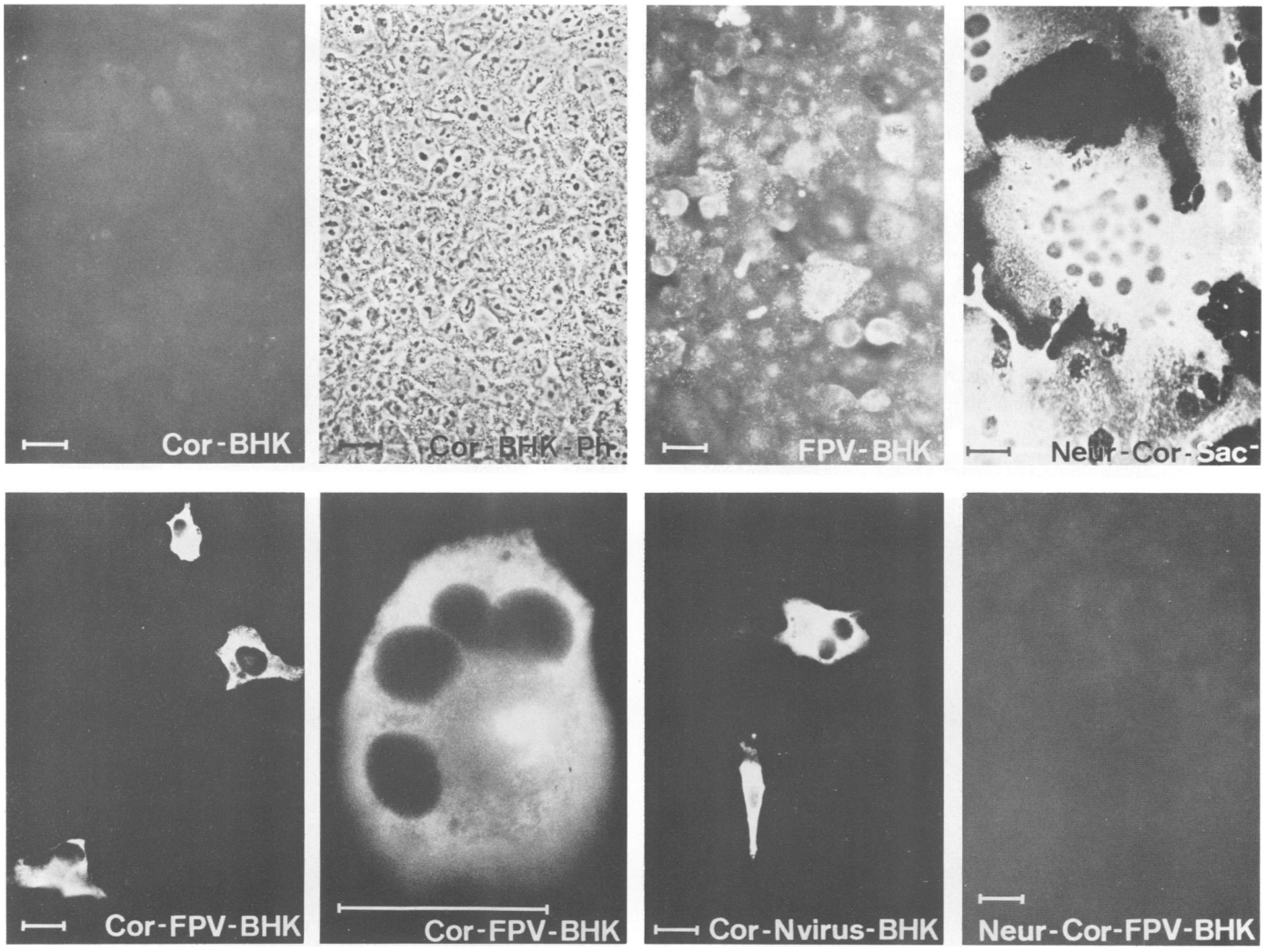

Fig. 6. Murine hepatitis virus infection of BHK-21 and sac$^-$ cells. The coronavirus, MHV-A59 was applied to BHK-21 or sac$^-$ cells under various conditions. The cells were then held at 37°C for 8 h to allow development of the infection, fixed, permeabilized and processed for indirect immunofluorescence using antibodies directed against either the haemagglutinin of FPV (FPV-BHK) or against all the coronavirus proteins (all others). **Cor-BHK**: the result of application of 500 p.f.u./cell of coronavirus to BHK-21 cells. **Cor-BHK-Ph**: the phase micrograph of the field in Cor-BHK showing that it contains >100 cells. **Cor-FPV-BHK**: 25 p.f.u./cell of coronavirus applied to BHK-21 cells which had been infected with FPV 6 h previously. Note the polykaryon formation shown in the panel with higher magnification. **FPV-BHK**: another field from the same filter as in Cor-FPV-BHK which has been stained wth anti-FPV haemagglutinin. **Cor-N virus-BHK**: 25 p.f.u./cell of coronavirus applied to BHK-21 cells which had been infected with the influenza virus N 6 h previously. **Neur-Cor-FPV-BHK**: 500 p.f.u./cell of neuraminidase-treated coronavirus applied to BHK-21 cells which had been infected with FPV 6 h previously. **Neur-Cor-Sac$^-$**: 25 p.f.u./cell of neuraminidase-treated coronavirus applied to sac$^-$ cells. Scale bar in each panel represents 10 μm.

epithelial cells. Any cell which is resistant to a virus because it lacks appropriate surface receptors may be rendered susceptible to that virus following influenza virus infection so long as the super-infecting virus has sialic acid residues on its envelope.

Coronaviruses typically have very limited host ranges (Sturmann and Holmes, 1983). BHK-21 cells were completely resistant to infection by the coronavirus MHV-A59 even when 500 p.f.u./cell were applied (Figure 6, Cor-BHK, Cor-BHK-Ph). Infection of BHK-21 cells with FPV resulted in expression of influenza haemagglutinin on the cell surface (FPV-BHK) and a limited production of virus particles (van Meer *et al.*, 1985). Application of 25 p.f.u./cell of the murine coronavirus after 8 h of FPV infection yielded coronavirus infection of 5 – 10% of the cells, as shown by staining with an antibody directed against the whole virus (Cor-FPV-BHK). The formation of syncytia by the infected cells demonstrated that the coronavirus E2 protein, which is responsible for fusion, is expressed and transported to the cell surface. These features matched those of coronavirus single infection in permissive cells, such as sac$^-$ (Tooze *et al.*, 1984). Influenza-dependent infection of BHK-21 cells was abolished by the treatment of the virus with neuraminidase (Neur-Cor-FPV-BHK) although infection of the permissive sac$^-$ cells was unaffected (Neur-Cor-Sac$^-$).

We used the BHK-21-coronavirus system to show that only the binding function of the haemagglutinin is required to mediate entry of other viruses. The haemagglutinin of the influenza virus N (A/Chick/Germany/49) is not cleaved into HA_1- and HA_2-subunits in most mammalian cell lines and, as a result, influenza N haemagglutinin retains its ability to bind sialic acid but not its ability to catalyse low pH-mediated fusion (Klenk *et al.*, 1975; White *et al.*, 1983). Infection of BHK-21 cells with N virus led to production of surface haemagglutinin as it did for FPV, but this haemagglutinin was incapable of catalysing fusion of lipid vesicles containing influenza virus receptors with the cell surface unless exogenous protease was added to cleave the haemagglutinin (G. van Meer, personal communication). Never-

theless, the N virus-infected BHK cells were also susceptible to coronavirus infection (Cor-N virus-BHK).

Discussion

We have shown that surface influenza haemagglutinin can mediate the entry and infection of sialic acid-bearing viruses through normally resistant cell surfaces. Infection by VSV and SFV is a multistep process which involves receptor-mediated endocytosis (Lenard and Miller, 1983; White *et al.*, 1983). In fact, fusion of VSV directly with the cell surface does not mediate infection (Matlin *et al.*, 1982; Lenard and Miller, 1983). The nature of the receptors involved in virus infection remains unclear. Since viruses have evolved to overcome cellular defences, it has been assumed that they infect via a broad range of potential receptors (Lenard and Miller, 1983; Kielian and Helenius, 1985). Indeed, binding experiments with VSV and SFV reveal thousands of binding sites on permissive cells (Miller and Lenard, 1980; Matlin *et al.*, 1982; Marsh and Helenius, 1980). A lipid, phosphatidylserine, has even been proposed as the receptor for VSV (Schlegel *et al.*, 1983). Whether all of these binding sites can mediate infection is unknown. However, their multiplicity has led to a picture of viral endocytosis which differs from the specific receptor-mediated endocytosis of ligands such as insulin or transferrin (Lenard and Miller, 1983). The difference was emphasized in a recent review (Bretscher and Pearse, 1984) where multiple weak interactions between the virus and its receptors are invoked to allow virus entry without receptor clustering. The authors suggest that a virus rolls around on the cell surface, continually making and breaking weak bonds with individual receptors until it encounters a cluster of receptors in a coated pit and endocytosis ensues. Such a mechanism would be difficult to examine in previously available systems containing many potential receptors. The system described here allows the study of viral entry through a receptor whose binding and localization is characterized. The G protein-haemagglutinin interaction is quite stable even at 37°C (Figure 4). Immunoelectron microscopy has shown that the majority of the cell surface influenza haemagglutinin is not clustered in coated pits (Rindler *et al.*, 1984), but is spread over the plasma membrane surface. These two facts taken together suggest that the virus must gather a cluster of receptors before entering a coated pit (Helenius *et al.*, 1980). Hence, the endocytosis of a virus with its receptors would be no different from that of ligands such as epidermal growth factor or insulin whose receptors also cluster before entering coated pits (Maxfield *et al.*, 1978; Schlessinger *et al.*, 1978; Hopkins *et al.*, 1981).

The present results also confirm our previous supposition that virus infection polarity is a consequence of virus receptor polarity (Fuller *et al.*, 1984). The elaboration of apical VSV binding sites was sufficient to mediate apical infection. Further, the coronavirus experiments with uncleaved haemagglutinin showed that only the binding activity of the protein was necessary. Together, these results validate infection polarity as a measure of cell surface polarity.

Our previous work with VSV infections on 3.0 μm pore size filters showed that basolateral infection was at least 100-fold more efficient than apical infection. The smaller size of the SF virion enabled it to infect efficiently through 0.45 μm pore size filters, which appear to give the MDCK cell monolayer greater mechanical stability. This minimized the problem of monolayer leakiness and allowed us to demonstrate that MDCK cells displayed at least a 1000-fold greater efficiency for basal SFV infection than for apical. The interpretation of this ratio in terms of receptor concentration must take into account the measured ratio of apical to basolateral surface area in these cells (< 1:7, von Bonsdorff *et al.*, (1985) as well as the possibility that more than one receptor may be required for the initial binding event. However, the results demonstrate that an epithelial cell is capable of exquisite control over the composition of its surface domains. tion of its surface domains.

The potential importance of a phenomenon such as haemagglutinin-mediated virus entry in initiating infection in an epithelium is illustrated by the magnitude of this infection polarity. An epithelium *in vivo* should be at least as polar as our *in vitro* model. Hence influenza infection could increase the susceptibility of an epithelium to luminal infection. After initiation, the infection could then spread by its usual route, e.g., along the serosal surface.

The examples we chose to demonstrate that viral proteins at the cell surface can mediate the entry of other viruses were intended to be illustrative rather than exhaustive. Parainfluenza viruses should, for example, be able to mediate infection by this mechanism since the envelope protein also has a sialic acid-binding activity (Choppin and Compans, 1975). The phenomenon should apply to many combinations of viruses and must be considered along with immunosuppressive and interferon-suppressing activities as a mechanism for increasing susceptibility to secondary infection.

Materials and methods

Cells and viruses

BHK-21, Madin Darby canine kidney cells-strain I (MDCK), vesicular stomatitis virus Indiana strain (VSV) and FPV (A/FPV/Rostock, H7, N1) were as in Fuller *et al.* (1984). Growth of cells on filters was as described in Fuller *et al.* (1984) for 4 days except that monolayers were grown on 25 mm diameter, 0.45 μm pore size Millipore filters for some experiments. The cells reach a higher density (2.5 × 10^6 cells/filter) after 4 days growth than they do on 3.0 μm pore size filters (Balcarova-Ständer *et al.*, 1984). Sac^- cells, a line of murine fibroblast non-productively transformed with murine sarcoma virus, and MHV-A59 were grown from stocks provided by S.Tooze (EMBL). Growth of MHV-A59 was as described in Tooze *et al.* (1984) except that virus was produced in Eagle's minimum essential medium supplemented with 0.2% w/v bovine serum albumin (BSA), 10 mM Hepes, 100 U/ml penicillin and 100 μg/ml streptomycin (EMEM). SFV was prepared as described in White *et al.* (1980) and N virus [A/Chick/Germany/49 (H10, N7)], was isolated as in van Meer and Simons (1983). [^{35}S]-Methionine-labelled VSV was grown in BHK-21 cells as described in Pesonen and Simons (1983). [^{3}H]Leucine-labelled FPV was grown in and harvested from strain II MDCK cells using a procedure which parallels that described for the [^{35}S]methionine-labelled FPV in Matlin *et al.* (1981).

Virus infections

Infections of filter-grown monolayers of strain I MDCK cells were performed in mini-Marbrook chambers to allow monitoring of the transepithelial resistance (Richardson and Simmons, 1979; Fuller *et al.*, 1984). The filter and chamber were rinsed by dipping, edgewise, into beakers containing EMEM. Excess medium was aspirated from the basal and apical sides of the filter without touching the filter. Virus, in EMEM, was applied to one surface in a 50 μl aliquot and 50 μl of EMEM was applied to the other surface. The filter and holder were placed feet down in a deep 6 × 35 mm diameter well dish (NUNC) containing 100 μl of EMEM. A 25 mm 3MM filter (Whatman) was soaked in EMEM and placed atop the collar of the chamber. The 6-well dish was then covered and placed in a 37°C, 5% CO_2 incubator for 1 h to allow virus absorption and then rinsed three times with growth medium. The filters in holders were incubated in 10 ml of growth medium in a 37°C, 5% CO_2 incubator between measurements. To avoid the development of hydrostatic pressure which could damage the monolayer during manipulation, the chamber is always inserted edgewise into solutions and kept filled and floating when maintained for long times in solution. The monolayers were always incubated in the presence of BSA or of serum because loss of resistance was observed during incubation in protein-free solutions. When these precautions were observed, filter-grown monolayers which displayed initial resistances of > 1800 ohm cm^2 could routinely be manipulated and maintained their resistances for at least 10 h. Monolayers with lower initial original resistances appeared to be more fragile.

Infection of subconfluent sac^- cells with the coronavirus MHV-A59 was per-

formed as described by Tooze *et al.* (1984) except that virus was applied in EMEM.

Infections of BHK-21 cells with FPV, influenza N virus or with MHV-A59 were performed with barely confluent monolayers 1.5×10^5 cells/cm^2 on glass coverslips in 25 mm dishes. The monolayers were rinsed twice with EMEM and 50 p.f.u./cell of influenza or 20 – 500 p.f.u./cell of MHV-A59, diluted in EMEM, was applied in a volume of 0.2 ml. The dishes were covered and placed in a 37°C, 5% CO_2 incubator for 1 h to allow absorption. The monolayers were then rinsed with and further incubated in growth medium.

Neuraminidase digestion of VSV and SFV was performed by incubating virus for 8 h at 37°C in Hank's basal salt solution containing 0.2% BSA (Sigma GmbH, FRG) and 10 mm Hepes, pH 7.4 (BSS/BSA) at a concentration of at least 1×10^9 p.f.u./ml with 10 mg/ml of *C. perfringens* neuraminidase (Type IV-Sigma GmbH, FRG). Similar treatment of the coronavirus MHV-A59 (or indeed, incubation in BSS/BSA for 8 h at 37°C in the absence of neuraminidase) caused essentially complete loss of infectivity. For this reason, coronavirus digestions were performed on ice for 7 h at the same concentrations of virus and neuraminidase. In all cases the virus was diluted so that 0.1 mg/ml of the neuraminidase was applied to the cell surface during absorption. Control viruses were always used after parallel incubations in EMEM without neuraminidase. Parallel infection with serial dilutions of control and neuraminidase-treated viruses were assayed by immunofluorescence and showed no loss of infectivity due to neuraminidase treatment under the digestion conditions described.

Analysis of FPV-VSV binding on tartrate gradients

20 – 28% (w/w) continuous potassium tartrate gradients in 0.15 M NaCl, 25 mM Tris-Cl (pH 7.4) were prepared in 14 ml Beckman SW40 tubes at 4°C. Virus samples were mixed in BSS/BSA and either loaded atop the gradients immediately or incubated at 37°C overnight and then loaded.

Gradients were centrifuged for 17 h at 40 000 r.p.m. in a Beckman SW40 rotor (284 000 *g*) at 4°C. A centrifugation time of >5 h was necessary to observe any separation of FPV and VSV peaks. 0.25 ml fractions were taken across each gradient and applied to 25 mm diameter 3MM Whatman filters with 100 μl of BSS/BSA in three applications. Between applications the filter was allowed to dry. The filters were then processed for scintillation counting as described by Mans and Novelli (1961) and counted in a Mark III-6800 liquid scintillation counter (Searle) operated in a 2-channel mode. The counts are displayed as a percent of the total counts recovered from the gradient after correction for spillover between channels. Typically 2×10^6 c.p.m. per virus were used for each gradient and >90% of this was recovered in the fractions.

Virus binding assay

A plexiglass filter holder, constructed in the EMBL workshop, was used for virus binding assays. The device consists of an upper block containing 12.3-cm-deep 18-mm-diameter holes which clamps against the lower block with corresponding 0.5-mm, 25-mm-diameter depressions. A rubber O-ring of 20 mm diameter is fitted around the bottom of each hole forming a seal with the cell monolayer and isolating each well from its neighbors.

The electrical resistance of each filter was measured in its individual mini-Marbrook chamber by the current clamp device described in Fuller *et al.* (1984) and moved to a 4°C room where all further operations were performed. The filters were then removed from chambers, rinsed in BSS/BSA, laid atop BSS/BSA-soaked 25 mm diameter 3MM filters (Whatman) with the apical surfaces up, and clamped into the pre-cooled plexiglass filter holder. The apical surface was rinsed with 1 ml of BSS/BSA, excess fluid aspirated from the wells and the radioactive virus added in 200 μl total volume. The filter holder was covered with Parafilm (Amer. Can. Co., Greenwich, CT) and set on ice atop a shaker to allow binding to occur. After incubation for 1 h, the virus was removed and the apical surface was washed twice with BSS/BSA. The filters were then removed from the plexiglass holder and washed in separate 10 ml 35 mm dishes (NUNC, Algade, Denmark) three times with 7 ml of BSS/BSA by shaking for 15 min on ice. The basal surface of each filter was blotted with Whatman # 1 paper and the entire filter with cells counted in Rotiscint 22 (Carl Roth, Karlsruhe, FRG) in a Mark III-6800 liquid scintillation counter.

Binding experiments for electron microscopy

MDCK monolayers were grown for 4 days on 3.0 μm pore size filters and infected with 20 p.f.u./cell of FPV from the apical surface or mock-infected. The filters were removed from their chambers after 4 h infection, rinsed in ice-cold PBS containing Ca^{2+} and Mg^{2+} (PBS^+) and transferred to a 4°C room where all further operations were performed. 2 mm square pieces were cut from each filter, blotted edgewise against filter paper and immersed in 50 μl of PBS^+ containing either 35 μg VSV or 25 μg SFV in 35 mm plastic dishes. The dishes were covered and held on ice for 1 h. After incubation with virus, the pieces of filter were rinsed in PBS^+ and fixed with 0.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) for 20 min. Processing of the fixed filters for electron microscopy was completed as described in Fuller *et al.* (1984).

Antibodies and immunofluorescence

Immunofluorescent staining of filter-grown MDCK monolayers was performed using affinity-purified antibodies as described by Fuller *et al.* (1984) except that 10% (w/v) newborn calf serum was used in place of gelatin. BHK-21 cells and sac^- cells were grown on coverslips and processed for immunofluorescence after permeabilization with Triton X-100 as described in Louvard (1980) using either affinity-purified rabbit anti FPV-haemagglutinin or a rabbit antibody directed against all the proteins of MHV-A59 which was described in Tooze *et al.* (1984). All antibodies were visualized by staining with goat anti-rabbit IgG conjugated to rhodamine (Louvard, 1980). No staining of either uninfected cells or of cells infected with other viruses was observed with any of these antibodies.

[^{125}I]protein A assay of FPV apical surface expression

Filter-grown monolayers were fixed with paraformaldehyde, quenched with ammonium chloride and rinsed with PBS^+ and PBS^+ containing 0.2% (w/v) gelatin (PBS-gelatin) as described in Fuller *et al.* (1984). Whole filters were laid apical surface down on 35 μl drops of anti-FPV-haemagglutinin antibody (1.3 μg/ml) in PBS-gelatin in a hydrated chamber and left at 37°C for 20 min. The filters were removed from the antibody and washed twice in PBS^+ gelatin for 10 min each with shaking. The filters were then laid atop 35 μl drops of PBS-gelatin containing 30 ng protein A and 300 000 c.p.m. of ^{125}I-labelled protein A (Amersham) in a hydrated chamber. After incubation with the protein A for 20 min at 37°C, the filters were then washed twice with PBS^+ gelatin and twice with PBS^- for 10 min each with shaking. The outer rim of each filter was removed with a cork bore and the central 18 mm of the filter counted in a gamma counter (Nuclear Science, Chicago).

Other methods

Pulse-chase experiments, cell counts, and gel electrophoresis were performed as described in Fuller *et al.* (1984).

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