We have analysed the uptake of influenza C virus and bovine coronavirus (BCV) by a polarized epithelial cell line, Madin–Darby canine kidney (MDCK) cells. Both viruses use N-acetyl-9-O-acetyl-neuraminic acid as a receptor determinant for attachment to cells. Virus binding assays with immobilized proteins indicated that a glycoprotein of 40 kDa is the major surface protein containing the receptor determinant for the two viruses. MDCK cells grown on filters for permeable support were found to have differential sensitivity to infection by these viruses. Both viruses were able to initiate infection via the apical domain of the plasma membrane, but only influenza C virus also accomplished infection via the basolateral plasma membrane. The resistance of MDCK cells to BCV infection from the basal filter chamber was overcome when the cell polarity was abolished by maintaining the cells in calcium-free medium. This finding indicates that the resistance to basolateral infection by BCV is a property of the cell line and not due to a technical problem related to the use of filters. Our results indicate that two viruses which use the same receptor for attachment to cells may differ in their ability to enter polarized cells. The possible involvement of an accessory molecule in the entry of BCV is discussed.

Introduction

Epithelial cells line the body cavities of higher eukaryotes and, therefore, represent the primary barrier to infection of vertebrate hosts by micro-organisms. The polarized organization of these cells involves the division of the plasma membrane into an apical and a basolateral portion that are separated by tight junctions (Simons & Wandinger-Ness, 1990). As the two membrane domains differ in their protein and lipid composition, they may also differ in their receptors for infecting micro-organisms. The presence of suitable receptors is thought to determine whether epithelial cells are infectable from the lumenal or the serosal surface as has been shown for several viruses (Tucker & Compans, 1993). If virus receptors are lacking or if they are present exclusively on the basolateral side, the epithelium is resistant to infection from the environment. This resistance does not necessarily protect the organism from infection, because the virus may enter the host through an epithelial breach mediated by an animal bite, an injection needle or any other physical trauma. Examples of this mode of entry are provided by rabies virus, hepatitis B virus and vaccinia virus.

A number of viruses are able to infect epithelial cells from the lumenal surface, indicating the presence of receptors on the apical domain of the plasma membrane. The epithelium of the respiratory tract is the primary target for many viruses including orthomyxoviruses (influenza), paramyxoviruses (mumps, measles) and herpesviruses (Tucker & Compans, 1993). Although the initial site of infection is the same, the course of infection may be quite different. Measles virus and herpesviruses are able to cross the barrier of epithelial cells and spread to other cells and tissues. Infections by human influenza viruses, irrespective of the serotype (A, B or C), are usually restricted to the respiratory tract. Other viruses that infect epithelial cells via the apical domain have a preference for the intestinal, rather than the respiratory, epithelium. Bovine coronavirus (BCV), for example, may initially infect the respiratory tract, but the disease caused by this virus is due to infection of the intestinal epithelium resulting in severe diarrhoea in newborn calves (Siddell et al., 1983). The preference for a certain type of epithelium may depend on
different factors, e.g. the ability to survive in the gastrointestinal tract, which exposes the infecting micro-organism to such unfavourable conditions as acidic pH, proteases and bile salts. Other factors that are not well characterized may also contribute to the differential susceptibility of epithelial cells to virus infection.

We compared the initial stage in the infection of epithelial cells by influenza C virus and BCV. Both viruses possess a lipid envelope with protruding glycoproteins. The spike proteins are essential for the initial interaction of the viruses with the cell. The S protein of coronavirus and the HEF protein of influenza C virus mediate attachment to the cell surface by binding to specific receptors (Boyle et al., 1987; Herrler et al., 1988). Following the adsorption step, the glycoproteins induce fusion between the viral envelope and the cellular membrane and thus influence the introduction of the viral genome into the cytoplasm. In the case of influenza C virus, fusion is an acid-dependent event. The fusion reaction is triggered by the acidic environment encountered within endosomes after endocytotic uptake (reviewed by Herrler & Klenk, 1991). The fusion activity of BCV is not acid-dependent (Payne & Storz, 1988).

Although influenza C virus and BCV have a different cell tropism, both viruses use N-acetyl-9-O-acetyleneuraminic acid (Neu5,9Ac₂) as a receptor determinant for attachment to cells (Rogers et al., 1986; Herrler & Klenk, 1987; Vlasak et al., 1988; Schultze & Herrler, 1992). Here we show that two viruses which recognize the same receptor determinant may nevertheless differ in their ability to enter epithelial cells. While influenza C virus is able to infect MDCK I cells from both the apical and basolateral plasma membrane, entry of BCV is restricted to the apical domain. A further difference was found when infection of another subline, MDCK II cells, was analysed. This cell line is resistant to infection by influenza C virus because of a lack of receptors on the cell surface. The resistance can be overcome by coating the cells with bovine brain gangliosides (BBG), which contain Neu5,9Ac₂ (Haverkamp et al., 1977), the receptor determinant for this virus. In contrast to influenza C virus, BCV was unable to infect MDCK II cells coated with gangliosides. These findings indicate that the two viruses differ in the way they enter epithelial cells. The implications for the cell tropism are discussed.

Methods

**Cells.** MDCK I and II cells, sublines of Madin–Darby canine kidney cells, were obtained from K. Simons (Heidelberg, Germany) and grown in minimum essential medium (MEM) containing 10% fetal calf serum. Polycarbonate membrane filters (tissue culture treated; pore size 0.4 μm; diameter 24.5 mm) placed in a 6-well cluster plate were purchased from Costar. Cells were grown for 3–4 days; the apical and basal media were replaced daily. Electrical resistance was measured using a Millicell ERS apparatus. Only cell monolayers with a resistance higher than 1000 Ω cm² were used for experiments.

**Viruses.** Strain Johannesburg/1/66 of influenza C virus was grown in 8-day-old embryonated eggs by allantoic inoculation. The allantoic fluid was harvested after incubation of the eggs for 3 days at 33 °C (Herrler & Klenk, 1987). This virus was stored at −80 °C and used to infect MDCK cells. Strain L-9 of BCV was grown in MDCK I cells as described previously (Schultze et al., 1990). The kinetics of virus growth in MDCK I cells was similar for both viruses.

- **Virus infection.** Cells grown on a filter membrane with an electrical resistance higher than 1000 Ω cm² were incubated with virus at an m.o.i. of about 10 TCID₅₀ per cell. After an adsorption time of 60 min, cells were washed with PBS and incubated with MEM in a CO₂-incubator to allow the virus infection to proceed. In experiments involving the inactivation of virus receptors, cells were incubated with virus for 30 min at room temperature. Following three washes with PBS, cells were incubated for 10 min at room temperature with rabbit antiserum (diluted 1:100 with PBS) directed against the infecting virus. After three washes with PBS, cells were incubated with MEM to allow virus infection to proceed. The efficiency of infection was judged by the yield of virus released into the medium as indicated by the haemagglutinating activity of the cell supernatant.

- **Inactivation of cellular receptors.** Cells grown on a filter membrane were washed three times with PBS and incubated for 60 min at 37 °C with sialidase (neuraminidase) from *Clostridium perfringens*. After having been washed with PBS, the cells were infected with virus as described above.

- **Haemagglutination assays.** Haemagglutination titration was performed in microtitre plates. Serial twofold dilutions of virus suspensions were prepared in PBS. Each dilution (50 μl) was mixed with an equal volume of a 0.5% suspension of chicken erythrocytes. After incubation for 60 min at 4 °C, the erythrocytes were analysed for agglutination. The haemagglutination titre (HA units/ml) indicates the reciprocal value of the maximum dilution that caused complete agglutination.

- **Virus binding assay.** Sialylated glycoproteins were isolated by affinity chromatography with wheat germ agglutinin bound to agarose. Surface proteins were isolated after surface biotinylation followed by precipitation with streptavidin–agarose. Both methods have been described in detail (Zimmer et al., 1995). The isolated cellular proteins were subjected to SDS–PAGE and then transferred to nitrocellulose by Western blotting. The immobilized proteins were incubated with either BCV or influenza C virus. Bound virus was detected by a colour assay based on the viral acetyl esterase as described elsewhere (Schultzze et al., 1993).

- **Gangliosides.** MDCK I and MDCK II cells were pretreated with sialidase from *C. perfringens* (1 U/ml) for 1 h at 37 °C. Following incubation with BBG (type III, Sigma; 2 mg/ml) for 40 min at 37 °C, cells were infected for 20 min at room temperature with either BCV or influenza C virus. After three washes with PBS, cells were incubated with MEM to allow the virus infection to proceed.

Results

**Inactivation of cell surface receptors by sialidase**

Studies with MDCK I cells cultured on plastic Petri dishes have indicated that both influenza C virus (Herrler & Klenk, 1987) and BCV (Schultze & Herrler, 1992) use 9-O-acetylated sialic acid as a receptor determinant for infection of cells. To confirm this result for filter-grown cells, MDCK I cells were
cultured on filters and treated with sialidase from *C. perfringens* added to either the apical or basolateral medium. Following desialylation, cells were incubated with BCV or influenza C virus from the apical side. The supernatant was removed 24 h post-infection (p.i.) to determine the amount of virus released into the medium by measuring the haemagglutinating activity. As shown in Fig. 1, removal of sialic acid from the apical surface inhibited viral infection, whereas sialidase treatment of the basolateral domain did not affect virus replication. This result indicates that Neu5,9Ac2 is involved as a receptor determinant for BCV and influenza C virus in the infection of filter-grown MDCK I cells. The inability of the sialidase in the basal filter chamber to inactivate the apical receptors for either virus confirmed that the filter-grown cells were polarized and can be used to analyse the polarity of virus entry.

**Identification of a surface receptor for BCV and influenza C virus**

BCV and influenza C virus attach to the same receptor determinant, but they differ somewhat in their preference for a certain linkage type. Sialic acid connected by an α2,3-linkage to galactose is recognized more efficiently by BCV, whereas influenza C virus recognizes the α2,6-linkage more efficiently (Schultze & Herrler, 1994). Therefore, it was of interest to know whether the two viruses attach to the same or different surface glycoproteins. Recently, a binding assay with proteins immobilized on nitrocellulose has been described that allows the sensitive detection of cellular proteins recognized by influenza C virus (Schultze et al., 1993). Using this assay, influenza C virus has been shown to recognize several glycoproteins from MDCK I cells. Only one of these proteins was expressed at the cell surface. Other surface sialo-glycoproteins were not recognized by influenza C virus (Zimmer et al., 1995). Applying this approach to BCV we found that BCV is also able to attach to several glycoproteins (Fig. 2, lane C). The spectrum of proteins recognized by the two viruses is not identical (compare lanes A and C), which may reflect the difference in the preference for certain linkage types. However, when the recognized surface proteins were analysed, no difference was observed. As in the case of influenza C virus, binding of BCV was restricted to a single glycoprotein (compare lanes B and D). The protein, which is characterized in more detail in the discussion, has an estimated molecular mass of 40 kDa and has been designated ‘gp40’. From this result we conclude that BCV and influenza C virus use the same receptor for attachment to MDCK I cells.

**Polarity of virus entry**

In order to determine how the two viruses enter polarized epithelial cells, MDCK I cells were grown on a filter membrane for 3 days until the monolayers acquired a resistance of more than 1000 Ω cm². After incubation of either the apical, or the basolateral, domain with virus for 60 min, the inoculum was removed and cells were incubated with medium to allow the virus infection to proceed. Virus production was determined 24 h p.i. by measuring the haemagglutinating activity of the virus released into the medium of the apical chamber. No virus was detectable in the basolateral chamber. This finding...
Fig. 3. Attempts to infect MDCK I cells from the apical or basolateral surface with either BCV (cross-hatched bars) or influenza C virus (hatched bars). The cells were grown on a permeable support for 3 days and incubated for 1 h with one of the two viruses. After an incubation time of 24 h the amount of virus released into the apical chamber was determined by measuring the haemagglutinating activity of the supernatant.

Fig. 4. Infection of MDCK I cells after opening the tight junctions. MDCK I cells were grown on filters until they developed an electrical resistance $> 1000 \ \Omega \ \text{cm}^2$. Tight junctions were opened by incubation in medium deficient in calcium ions for 24 h (hatched bars). Control cells were incubated in medium containing calcium ions (cross-hatched bars). After incubation with BCV from either the apical or basolateral domain, cells were maintained in Ca$^{2+}$-containing medium. The virus yield in the apical medium was determined 24 h p.i. by measuring the haemagglutinating activity.

Table 1. Ability of BBG to serve as receptors for influenza C virus and BCV on MDCK I and MDCK II cells

Asialo cells were obtained by treatment of monolayers of the two cell types with sialidase. Ganglioside-treated cells were obtained by incubation of asialo cells with BBG as described in Methods. Native, asialo and BBG-treated cells were infected with influenza C virus or BCV. The yield of virus released from the cells was determined 20 h p.i. by measuring the haemagglutinating activity of the supernatant.

<table>
<thead>
<tr>
<th>Virus yield (HA units/ml)</th>
<th>MDCK I</th>
<th>MDCK II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>Asialo</td>
<td>BBG-treated</td>
</tr>
<tr>
<td>BCV</td>
<td>256</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Influenza C virus</td>
<td>128</td>
<td>&lt; 2</td>
</tr>
</tbody>
</table>

indicates that both viruses are released only from the apical membrane of MDCK cells, which has previously been demonstrated for influenza C virus by electron microscopy (Herrler et al., 1981). As shown in Fig. 3, infection of MDCK I cells with BCV from the apical surface resulted in a high virus yield, whereas no virus production was detectable after incubation with virus from the basolateral side. On the other hand, influenza C virus was able to infect filter-grown cells from both sides. Virus was also detected in the supernatant when the inoculum was applied to the basolateral domain of the plasma membrane. Thus, although BCV and influenza C virus use the same receptor, they differ in their ability to initiate an infection from the basolateral surface.

Infection of cells after disruption of tight junctions

The inefficient basolateral infection by BCV may be due to a surface component present on the apical, but absent from the basolateral, domain of the plasma membrane. Alternatively, it might reflect the difficulties of the virus in passing through the pores of the filter membrane. To prove that the filter membrane itself is no hindrance to virus infection from the basolateral surface, cells were incubated in calcium-deficient medium for...
24 h. The lack of calcium ions resulted in a loss of electrical resistance, indicating that tight junctions were leaky. After incubation of either the apical or the basolateral surface of the cells with BCV, the inoculum was removed and replaced with medium containing calcium ions. After incubation at 37 °C for 24 h, the amount of virus released into the medium was determined by measuring the haemagglutinating activity of the virus in the apical chamber. With cells kept in calcium-free medium, inoculation from the basolateral surface resulted in an efficient virus infection as indicated by an HA titre of 128 HA units (Fig. 4). Cells with intact tight junctions (calcium ions present) were resistant to infection from the basolateral surface. This result indicates that the filter membrane itself does not prevent the virus from reaching the basolateral plasma membrane of filter-grown cells. Therefore, we conclude that MDCK I cells are susceptible to infection by BCV from the apical surface, but resistant to infection from the basolateral domain of the plasma membrane.

**Infection of MDCK I and MDCK II cells coated with BBG**

Different sublines of MDCK cells have been described that differ in their functional and morphological characteristics (Richardson et al., 1981; Simmons, 1981; Valentich, 1981). MDCK I cells are derived from an early passage and can develop a high electrical resistance, whereas MDCK II cells are derived from later passages and display a much lower resistance. MDCK I and II cells differ in their susceptibility to infection by influenza C virus. MDCK I cells are readily infected and release a large amount of virus into the supernatant. MDCK II cells are resistant to infection because of a lack of virus receptors on the cell surface (Szepeński et al., 1992). Receptors for influenza C virus can be generated by enzymatic transfer of Neu5,9Ac~9 to surface glycoproteins (Szepeński et al., 1992) or by coating the cells with BBG, which are known to contain Neu5,9Ac~9 (Herrler & Klenk, 1987). MDCK II cells treated in either way are susceptible to infection as are MDCK I cells. Both cell types were incubated with either BCV or influenza C virus. The amount of virus released into the supernatant was determined 24 h p.i. by haemagglutination titration. No virus was detectable in early MDCK II cells incubated with either BCV or influenza C virus (Table 1) indicating that these cells are resistant to infection, not only by influenza C virus, but also by BCV. To find out whether the lack of infectibility was due to a lack of receptors on the cell surface, MDCK II cells were coated with BBG. This treatment was sufficient to render the cells susceptible to infection by influenza C virus. BCV, on the other hand, was unable to infect these cells. However, in contrast to MDCK II cells, BBG were able to serve as receptors for BCV on MDCK I cells. After inactivation of endogenous receptors by sialidase treatment, MDCK I cells are resistant to infection by BCV (Schultze & Herrler, 1992) and this resistance was overcome by coating the cells with BBG (Table 1). Thus, the resistance of ganglioside-treated MDCK II cells is not due to the inability of BCV to recognize BBG as receptors. There must be another factor present in MDCK I, but absent from MDCK II cells, that is crucial for the infection by BCV.

**Discussion**

Attachment sites for viruses are crucial factors for the susceptibility of a cell to infection. They may even determine the cell tropism of a virus as in the case of human immunodeficiency virus, which has a tropism for CD4~9 cells (Dalgleish et al., 1984; Klatzmann et al., 1984). With polarized epithelial cells, it is generally assumed that the presence of suitable receptors determines whether a virus can enter the cell via the apical and/or the basolateral domain of the plasma membrane. Studies with SV40, for example, have shown that this virus infects polarized cells only from the apical side (Clayson & Compans, 1988). This restriction is reflected in the ability of the virus to bind to the apical, but not to the basolateral domain of the plasma membrane. Though the receptor for SV40 has not been identified, it appears to be present only on the apical surface (Basak et al., 1992).

For viruses that recognize the same receptor, one might expect that they enter the cell in the same way. Influenza C virus and BCV have both been shown to use 9-O-acetylated sialic acid as a receptor determinant for the infection of cells. Nevertheless, we found that they differ in the mode of virus entry. Whereas influenza C virus was able to infect MDCK cells from both surfaces, infectious entry of BCV was restricted to the apical surface. The resistance of the cells to basolateral infection by BCV cannot be explained by the virus being obstructed by the filters. Influenza C virus, which is similar in size, passed through the filter pores and infected MDCK I cells very efficiently from the basolateral side. When cells were maintained in a nonpolarized state, BCV was also able to initiate infection from the basal filter chamber. Therefore, the restriction of virus entry observed under normal conditions is an intrinsic property of the cell. Obviously a factor that is required at the early stage of infection is present on the apical surface, but absent from the basolateral plasma membrane.

The resistance of MDCK cells to basolateral infection by BCV is not due to a lack of 9-O-acetylated sialic acid on the respective domain of the cell surface. Influenza C virus uses the same receptor determinant for binding to cells and entry is not restricted. The two viruses differ from each other somewhat in the linkage specificity. Small amounts of Neu5,9Ac~9 attached by an a2,6-linkage to surface glycoproteins are recognized more efficiently by influenza C virus, whereas BCV is more efficient in the recognition of the a2,3-linkage type (Schultze & Herrler, 1994). Whether these minor differences in the receptor specificity are due to the different origin of the samples — egg-adapted influenza C virus and MDCK-grown BCV — is not known. In the case of influenza A viruses, which recognize N-
acetyleneuraminic acid (sialic acid without a 9-O-acetyl residue) as the receptor determinant, linkage specificity has been suggested to be correlated with the host tropism (Rogers et al., 1983). Avian strains of the H3 subtype of influenza A viruses preferentially recognize receptors containing α2,3-linked sialic acid. The human strains preferentially bind α2,6-linked sialic acid. This binding specificity correlates with the predominant linkage type of sialic acid present on the target cells for human influenza viruses, i.e. the ciliated cells of the respiratory epithelium (Couceiro et al., 1993). As BCV has a preference for the α2,3-linkage type, the inability of this virus to infect MDCK cells via the basolateral domain of the plasma membrane might be explained by a lack of α2,3-linked Neu5,9Ac₂ on the basolateral surface. However, to our knowledge there are no reports of polarized distributions of glycosidic linkages connecting sialic acid with surface components on MDCK cells. Moreover, the binding assays indicate that both viruses attach to the same receptor, a glycoprotein designated ‘gp40’. This protein has been detected on both domains of the plasma membrane, but the majority is present on the apical side (Zimmer et al., 1995). The lower receptor density in the basolateral membrane is sufficient for influenza C virus infection. It may not be sufficient for infection by BCV. However, it is also possible that an additional factor determines the polarized entry of BCV. This interpretation is also suggested by the studies with MDCK II cells. This subline of canine kidney cells was found to be resistant to infection by both BCV and influenza C virus. In the case of the latter virus, the resistance was due to a lack of surface receptors. MDCK II cells lack gp40 (G. Zimmer, H.-D. Klenk and G. Herrler, unpublished results). After coating the cells with BBG, these glycolipids served as the virus receptors and resistance to virus infection was overcome. The gangliosides can also function as receptors for BCV as shown with MDCK I cells, the endogenous receptors of which were inactivated by sialidase treatment. MDCK II cells, however, were resistant to infection by BCV regardless of whether or not gangliosides were added. This finding indicates that virus entry is not just a matter of binding to sialic acid. MDCK II cells obviously differ from MDCK I cells not only by a lower amount of 9-O-acetylated sialic acid on the cell surface, but also by the lack of an additional factor that is required for a BCV infection. Though we cannot exclude that resistance of MDCK II cells is determined at a later stage, it is an interesting possibility that the missing factor in MDCK II cells acts at the virus entry stage. If this factor is a defined protein that is present predominantly on the apical surface of MDCK cells, it would explain not only the resistance of MDCK II cells to BCV infection but also the resistance of MDCK I cells to basolateral infection.

Interaction with distinct surface proteins at the initial stage of infection is not unusual for coronaviruses. Several members of this virus family do not recognize sialic acid as a receptor determinant, but instead bind to defined surface proteins. A member of the carcinoembryonic antigen family of proteins has been identified as a receptor for the murine coronavirus mouse hepatitis virus (Williams et al., 1991). Aminopeptidase N has been shown to serve as a receptor for the porcine coronavirus transmissible gastroenteritis virus (Delmas et al., 1992) and a human coronavirus, designated 229E (Yeager et al., 1992). Both proteins have been localized to the apical surface of polarized epithelial cells (Hauri et al., 1985). Indeed, entry of transmissible gastroenteritis virus has recently been shown to be restricted to the apical surface of a polarized porcine cell line, LLC-PK1 (Rosen et al., 1994). Thus, it would fit quite well if BCV also reacted with a protein that is expressed on the apical domain of the plasma membrane. Interaction with a cellular protein has been suggested for BCV as a possible way to induce the viral fusion activity (Schultzze & Herrler, 1994). The fusion of the viral membrane with the plasma membrane of a cell is required for enveloped viruses in order to get their genome across the membrane barrier of the cell. Fusion is achieved by a conformational change of a viral surface protein, exposing a fusogenic domain that interacts with the membrane of the target cell. In the case of influenza viruses and several other viruses that enter the cell via the endocytotic pathway, the conformational change is triggered by the acidic pH encountered within endosomes (Skehel et al., 1982). The fusion activity of BCV is not acid-dependent (Payne & Storz, 1988) and it is not known what factor induces this activity. The interaction with a specific cellular protein could explain not only how the fusion activity of BCV is triggered, but also why influenza C virus, which has an acid-dependent fusion activity, does not require such an interaction. If this putative protein had a polarized distribution on the cell surface, it would determine the polarity of virus entry. The factor determining the polarized entry of BCV might be any surface protein that is located preferentially at the apical plasma membrane. It might even be gp40, provided that the penetration step of the BCV infection requires a higher receptor density than is achieved in the basolateral domain.

With increasing information about the interaction between viruses and cells, it has become obvious that more than one component on the cell surface may be required for a virus to infect a cell. This concept is based on results obtained with different viruses, e.g. HIV (Bhat et al., 1991; Callebaut et al., 1993), poliovirus (Shepley & Racaniello, 1994) and measles virus (Naniche et al., 1993; Döring et al., 1993; Dunster et al., 1994). The interplay between the different cellular components in the early stage of infection may vary from virus to virus. Surface proteins or lipids may be involved in virus attachment to the cell surface or in the subsequent fusion reaction described above. If any of these receptors—for attachment or for fusion—are distributed on epithelial cells in a polarized way, it would result in a polarized infection. Thus, whether a polarized cell is infected by viruses from the apical or basolateral surface is not necessarily determined at the level of virus attachment, it may also be determined at the level of penetration.
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