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Bovine coronavirus uses *N*-acetyl-9-*O*-acetylneuraminic acid as a receptor determinant to initiate the infection of cultured cells

Beate Schultze and Georg Herrler*

Institut für Virologie, Philipps-Universität Marburg, Robert-Koch-Strasse 17, 3550 Marburg, Germany

The importance of *N*-acetyl-9-*O*-acetylneuraminic acid ($Neu5,9Ac_2$) as a receptor determinant for bovine coronavirus (BCV) on cultured cells was analysed. Pretreatment of MDCK I (Madin Darby canine kidney) cells with neuraminidase or acetylesterase rendered the cells resistant to infection by BCV. The receptors on a human (CaCo-2) and a porcine (LLC-PK1) epithelial cell line were also found to be sensitive to neuraminidase treatment. The susceptibility to infection by BCV was restored after resialylation of asialo-MDCK I cells with $Neu5,9Ac_2$. Transfer of sialic acid lacking a 9-*O*-acetyl group was ineffective in this respect. These results demonstrate that 9-*O*-acetylated sialic acid is used as a receptor determinant by BCV to infect cultured cells. The possibility is discussed that the initiation of a BCV infection involves the recognition of different types of receptors, a first receptor for primary attachment and a second receptor to mediate the fusion between the viral envelope and the cellular membrane.

Introduction

Bovine coronavirus (BCV) is an enteropathogenic coronavirus causing severe diarrhoea in newborn calves (Siddell *et al.*, 1983). The mechanisms by which BCV infects cells and causes disease are not well characterized, nor are the factors known which determine the host and tissue specificity. The cellular receptor which is a crucial determinant of the tropism of several viruses, is not known in the case of BCV.

The only receptor for coronaviruses which has been identified so far on susceptible cells is that for a murine coronavirus, mouse hepatitis virus (MHV). Strain MHV-A59 has been shown to bind to a 100K to 110K protein present in membranes from hepatocytes and enterocytes of susceptible mice, but absent in comparable preparations from resistant mice (Boyle *et al.*, 1987). A monoclonal anti-receptor antibody that prevents MHV from infecting susceptible mouse cells was used for affinity purification of the receptor (Williams *et al.*, 1990), which has been shown to be a member of the carcinoembryonic antigen family of glycoproteins (Williams *et al.*, 1991). It is not known whether a related receptor is required for other coronaviruses to initiate infection. BCV and MHV-A59 have quite different binding activities; they differ not only in the spectrum of cells susceptible to infection but also in their interactions with erythrocytes. MHV-A59 is unable to agglutinate erythrocytes (Yokomori *et al.*, 1989), whereas BCV is a potent haemagglutinating agent (Sato *et al.*, 1977).

The crucial step in the elucidation of the erythrocyte receptors for BCV was the finding that BCV and influenza C virus have a receptor-destroying enzyme in common. Influenza C virus inactivates its own receptors by a virus-encoded acetylesterase, which releases *O*-acetyl residues from position C9 of *N*-acetyl-9-*O*-acetylneuraminic acid ($Neu5,9Ac_2$) (Herrler *et al.*, 1985*b*). The same enzyme is also present on the surface of BCV (Vlasak *et al.*, 1988*b*; Schultze *et al.*, 1991*a*). Pretreatment of erythrocytes with virions or purified acetylesterase renders the cells resistant against agglutination by BCV (Vlasak *et al.*, 1988*a*; Schultze *et al.*, 1990). Restoration of coronavirus receptors was achieved by resialylation of erythrocytes with $Neu5,9Ac_2$ (Schultze *et al.*, 1990). These results indicated that $Neu5,9Ac_2$ is a receptor determinant for attachment of BCV to erythrocytes. However, the relevance of this interaction for infection of cells has remained unknown.

In the present report we provide evidence that $Neu5,9Ac_2$ serves as a receptor determinant for BCV on cultured cells. The receptor can be inactivated by pretreatment with acetylesterase or neuraminidase. Resialylation of cells with $Neu5,9Ac_2$ restores the susceptibility to virus infection. This finding is discussed in the context of the protein receptor described for MHV-A59.

Methods

Cells. MDCK I cells, a subline of Madin Darby canine kidney cells, were maintained as described previously (Schultze *et al.*, 1991*a*). CaCo-2 cells were purchased from ATCC and grown in RPMI medium containing 10% foetal calf serum (FCS). LLC-PK1 cells were kindly provided by Dr D. Jans (Max Planck Institut für Biophysik, Frankfurt, Germany) and grown in Dulbecco's medium containing 10% FCS.

Virus. Strain L-9 of BCV and strain Johannesburg/1/66 (JHB/1/66) of influenza C virus were grown on MDCK I cells as described previously (Schultze *et al.*, 1990). Strain WSN of influenza A virus was grown in 11-day-old embryonated chicken eggs. Virus was harvested from the allantoic fluid 2 days after inoculation.

Virus infections. For the inactivation and regeneration of virus receptors, cells were grown in 35 mm plastic Petri dishes and infected with virus (about 10 $TCID_{50}$/cell). After an adsorption time of 20 min at room temperature, cells were washed with PBS and incubated for 10 min at room temperature with rabbit antiserum (diluted 1:100 with PBS) directed against the virus used for infection. Following two washes with PBS, the cells were incubated for 24 h at 37 °C with MEM containing trypsin (1 μg/ml). The efficiency of infection was judged by the yield of virus released into the medium as indicated by the haemagglutination activity of the cell supernatant.

Haemagglutination assays. These were performed as described previously (Herrler *et al.*, 1985*a*). The haemagglutination titre (HA units) indicates the reciprocal value of the maximum dilution that caused complete agglutination.

Purification of the acetylesterase of BCV. The purification of the acetylesterase of BCV has been described previously (Schultze *et al.*, 1991*a*). The amount of acetylesterase required to release 1 μmol of acetate from paranitrophenyl acetate in 1 min at 25 °C was defined as 1 unit of enzyme.

Inactivation of cellular receptors. Cells grown on culture dishes (35 mm) were washed twice with PBS and incubated for 60 min at 37 °C with 200 μl of PBS in the presence of either neuraminidase from *Clostridium perfringens* [150 milli units (mU)], acetylesterase from BCV (1·3 U), or trypsin (50 μg). After being washed with PBS, the cells were infected with virus. In some experiments, neuraminidase-treated cells were resialylated prior to infection.

Resialylation of cultured cells. After pretreatment with neuraminidase to remove cellular receptors for BCV, cells were washed twice with PBS. Following addition of 5 mU of Galβ1,4GlcNAc α2,6-sialyltransferase and the amount of CMP-sialic acid indicated, the cells were incubated at 37 °C for various times. Prior to use, the sialyltransferase was freed of detergent as described previously (Schultze *et al.*, 1990). Cells were again washed twice and infected with virus.

Materials. Rat liver Galβ1,4GlcNAc α2,6-sialyltransferase were purchased from Boehringer Mannheim; CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac) and *C. perfringens* neuraminidase from Sigma; CMP-$Neu5,9Ac_2$ was kindly provided by Drs R. Brossmer and H. J. Gross, Heidelberg, Germany.

Results

Inactivation of cell surface receptors by neuraminidase

As BCV uses $Neu5,9Ac_2$ as a receptor determinant for attachment to erythrocytes (Vlasak *et al.*, 1988*a*; Schultze *et al.*, 1990), the importance of this type of sialic acid for the infection of cells was analysed. MDCK I cells were pretreated with neuraminidase from *C. perfringens* to remove sialic acid from the surface glycoproteins and glycolipids prior to infection with BCV. The effect of the enzyme on virus replication was determined 24 h post-infection (p.i.) by measuring the haemagglutinating activity of the virus released into the medium. As shown in Fig. 1, the yield of virus obtained from MDCK I cells was reduced after pretreatment with neuraminidase. When the enzyme was used in an amount as high as 40 mU, the cells became resistant against infection by BCV as shown by the lack of haemagglutination activity in the supernatant. This result indicates that sialic acid present on the cell surface is essential for BCV to initiate an infection. We were interested to find out whether sialic acid also plays an important role in the infection of cell types other than MDCK I cells. Human colon carcinoma cells, CaCo-2, and porcine kidney cells, LLC-PK1, are both susceptible to BCV, although the virus yield is not as high as that from MDCK I cells. These different cell types were pretreated with neuraminidase to remove sialic acid from the cellular surface prior to infection with BCV. The enzyme treatment had the same effect on LLC-PK1 and CaCo-2 cells as described above for MDCK I cells (Table 1). The virus yield from neuraminidase-treated cells was reduced fourfold in the case of

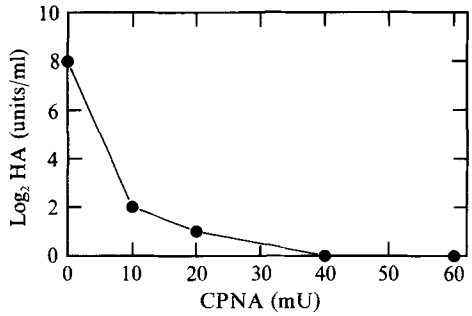

Fig. 1. Inactivation of cell surface receptors for BCV by neuraminidase treatment. After incubation with different amounts of neuraminidase from *C. perfringens* (CPNA) for 40 min, MDCK I cells were infected with BCV. The virus yield was analysed by determining the haemagglutinating activity of the medium.

Table 1. *Effect of neuraminidase treatment of different cells on the susceptibility to infection by BCV*

	Haemagglutinating activity (HA units/ml)	
Cell type	Control	Neuraminidase-treated
MDCK I	256	<2
LLC-PK1	16	<2
CaCo-2	48	12

CaCo-2 cells and more than eightfold with LLC-PK1 cells. These results indicate that sialic acid is necessary for BCV to infect cultured cells.

Inactivation of cell surface receptors by acetylesterase

The receptor-destroying enzyme of BCV inactivates the Neu5,9Ac_2-containing receptors on erythrocytes (Vlasak *et al.*, 1988*a*; Schultze *et al.*, 1991*a*). We analysed whether this enzyme is able to inactivate receptors for BCV on MDCK I cells. In parallel experiments, we compared the effect of acetylesterase and neuraminidase on the cellular receptors for BCV as well as for influenza C virus (JHB/1/66), which requires Neu5,9Ac_2 to initiate an infection (Herrler & Klenk, 1987), and for influenza A virus (WSN), which recognizes Neu5Ac as a receptor determinant. After pretreatment with neuraminidase, no virus was detectable by haemagglutination titration of the supernatant of MDCK I cells infected with either of the three viruses (Table 2). Incubation of MDCK I cells with acetylesterase resulted in resistance to infection by BCV and influenza C virus. On the other hand, strain WSN was still able to infect the pretreated cells. As influenza A virus does not require a 9-*O*-acetyl group for recognition of sialic acid, the receptors for WSN are not sensitive to acetylesterase treatment. The result from Table 2 indicates that BCV resembles influenza C virus, i.e. it uses acetylesterase-sensitive receptors for infection of MDCK I cells. We conclude therefore that BCV requires Neu5,9Ac_2 on the surface of cells to initiate an infection.

In contrast to the enzymes mentioned above, treatment of cells with trypsin had no effect on the ability of the three viruses to infect MDCK I cells.

Restoration of cellular receptors by resialylation

To provide further evidence that Neu5,9Ac_2 is the crucial receptor determinant for BCV on cultured cells, resialylation experiments were carried out. This method has already been used to characterize the receptors for influenza C virus and BCV on erythrocytes (Rogers *et al.*, 1986; Schultze *et al.*, 1990). Cells were pretreated with neuraminidase to remove sialic acid from the cellular surface. The asialo cells were resialylated using purified rat liver Galβ1,4GlcNAc α2,6-sialyltransferase and activated sialic acid (CMP-Neu5Ac or CMP-Neu5,9Ac_2, respectively). Neither BCV nor influenza C virus was able to infect MDCK I cells resialylated to contain Neu5Ac on the surface (Table 3). As expected for influenza A virus, strain WSN could infect cells resialylated with Neu5Ac, but not cells containing Neu5,9Ac_2. In contrast, replication of BCV and influenza C virus was observed following attachment of Neu5,9Ac_2 to cell surface glycoproteins of asialo cells, whereas cells containing Neu5Ac were found to be resistant to infection.

Table 2. *Effect of neuraminidase, acetylesterase and trypsin on the susceptibility of MDCK I cells to infection by BCV, influenza C virus (JHB/1/66) and influenza A virus (WSN)*

Treatment	Haemagglutinating activity (HA units/ml)		
	BCV	JHB/1/66	WSN
None	256	64	512
Neuraminidase	<2	<2	<2
Acetylesterase	<2	<2	512
Trypsin	348	64	512

Table 3. *Generation of receptors for BCV, influenza C (JHB/1/66) and influenza A virus (WSN) by resialylation of MDCK I cells*

Type of sialic acid attached to cells	Haemagglutinating activity (HA units/ml)		
	BCV	JHB/1/66	WSN
None	<2	<2	2
Neu5Ac	<2	<2	32
Neu5,9Ac_2	32	64	<2

In a previous study we have shown that influenza C virus requires less Neu5,9Ac_2 on the cell surface for agglutination of erythrocytes than BCV (Schultze *et al.*, 1990). We were interested to find out whether such a difference is also observed with cultured cells. Resialylation experiments were carried out with different amounts of CMP-Neu5,9Ac_2 to obtain cells with different amounts of 9-*O*-acetylated sialic acid on the surface. As shown in Fig. 2, infection by BCV was detected when 0·5 nmol CMP-Neu5,9Ac_2 had been used for resialylation of MDCK I cells. The minimum concentration of CMP-Neu5,9Ac_2 required to render asialo cells susceptible to influenza C virus infection was more than 1 nmol. This result suggests that BCV is more efficient in utilizing low amounts of Neu5,9Ac_2 for the initiation of an infection. However, when 10 nmol of CMP-Neu5,9Ac_2 had been used for the resialylation of cells, the yield of influenza C virus was 50% compared to untreated control cells, whereas in the case of BCV a yield of only 12·5% was obtained. Thus, an optimum infection by influenza C virus requires less sialic acid on the cell surface than does a BCV infection. The same observation was made when the time course of the resialylation reaction was analysed. For this purpose, asialo cells were incubated with sialyltransferase and 20 nmol of CMP-Neu5,9Ac_2,

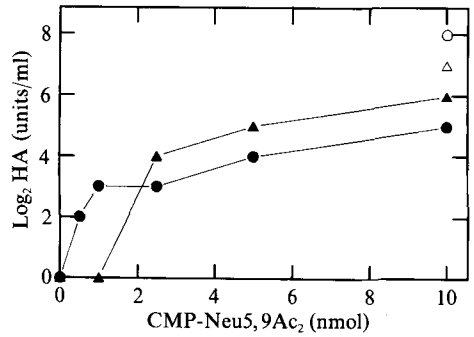

Fig. 2. Restoration of cell surface receptors by resialylation with Neu5,9Ac$_2$. After incubation of asialo cells with different amounts of CMP-Neu5,9Ac$_2$, cells were infected with either BCV (filled circles) or influenza C virus (filled triangles). The virus yield was determined by the haemagglutination assay. Open symbols indicate the virus yield obtained from untreated cells after infection with the corresponding virus.

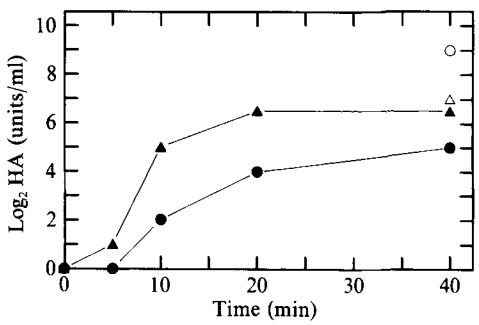

Fig. 3. Resialylation of MDCK I cells and restoration of cell surface receptors. Neuraminidase-treated MDCK I cells were resialylated for various times with CMP-Neu5,9Ac$_2$ and infected with either BCV (filled circles) or influenza C virus (filled triangles). The virus yield was determined by measuring the haemagglutination titre of the supernatant. Open symbols indicate the virus yield obtained from untreated cells after infection with the corresponding virus.

for various times. As shown in Fig. 3, the time required to restore enough Neu5,9Ac$_2$-containing receptors on the cell surface for a detectable infection was roughly the same for both BCV and influenza C virus (5 to 10 min). An incubation period of 20 min was sufficient to transfer enough Neu5,9Ac$_2$ to the cell surface to obtain a 50% yield of influenza C virus compared to control cells. For BCV, even after an incubation time of 40 min, the amount of virus released from resialylated cells was only 10% of the virus yield obtained from untreated cells. Because of difficulties in synthesizing CMP-Neu5,9Ac$_2$, only a limited amount of this compound was available for our experiments. Therefore, we were not able to determine the amount of CMP-Neu5,9Ac$_2$ required for a complete restoration of the receptors for BCV.

Discussion

The available information about the cellular receptor for BCV is based on work with erythrocytes (Vlasak *et al.*, 1988*a*; Schultze *et al.*, 1990). These studies have shown that Neu5,9Ac$_2$ is a receptor determinant for BCV, but the relevance of these findings for BCV infection was unknown.

The results presented above provide evidence that Neu5,9Ac$_2$ is the primary receptor determinant on cells which are infected by BCV: (i) pretreatment of cells with acetylesterase or neuraminidase rendered cells resistant against infection and (ii) resialylation of asialo cells with Neu5,9Ac$_2$ restored the susceptibility to virus infection. Thus, binding to Neu5,9Ac$_2$-containing receptors enables BCV to initiate an infection.

The enzyme used for generation of receptors on MDCK cells is specific for resialylation of proteins. Therefore, glycoproteins can serve as cellular receptors for BCV. However it cannot be dismissed that BCV is able to interact also with glycolipids, as shown for Sendai virus (Markwell *et al.*, 1981). This possibility has to be considered also for BCV, because bovine brain gangliosides have been shown to act as haemagglutination inhibitors (Vlasak *et al.*, 1988*a*). For convenience we have used only α2,6-sialyltransferase to restore receptors for BCV on asialo cells. Our results should not be interpreted as indicating a linkage specificity in the recognition of receptors. It is possible that BCV recognizes 9-*O*-acetylated sialic acid in other linkage types such as an α2,3 linkage as well as in an α2,6 linkage.

Influenza A and B viruses, some paramyxoviruses as well as several other viruses use Neu5Ac for binding to cells. So far BCV and influenza C virus are the only viruses reported to use 9-*O*-acetylated sialic acid for the initiation of infection. In addition to the receptor determinant, both viruses have an acetylesterase in common, which acts as a receptor-destroying enzyme. This similarity is surprising considering the fact that BCV is a positive-stranded RNA virus and influenza C virus a negative-stranded RNA virus. There are, however, differences in the distribution of the biological activities among the surface proteins. The HEF protein of influenza C virus is a haemagglutinin, an esterase and a fusion factor (reviewed by Herrler & Klenk, 1991). In the case of BCV, these activities are distributed among two glycoproteins. The S protein has fusion activity (Payne & Storz, 1988) and is the major haemagglutinin (Schultze *et al.*, 1991*b*). The HE protein is also a haemagglutinin (King *et al.*, 1985). It has the same binding specificity, but it is much less efficient in this respect than the S protein requiring a higher amount of Neu5,9Ac$_2$ for agglutination of cells (Schultze *et al.*,

1991*b*). Therefore, HE may function primarily as a receptor-destroying enzyme.

The target tissue for influenza C virus is the epithelium of the upper respiratory tract. BCV is able to infect cells of the respiratory tract as well as intestinal cells. These viruses appear to grow preferentially in epithelial cells. Our results imply that these cells contain 9-*O*-acetylated sialic acid. Whether the presence of $Neu5,9Ac_2$ is a determinant of the tissue tropism of BCV remains to be shown.

The murine coronavirus MHV replicates in the intestinal and respiratory epithelium, the liver, and some strains replicate also in the brain and spinal cord of susceptible mice. A 110K receptor protein has been isolated from the brush border membrane of hepatocytes and enterocytes (Williams *et al.*, 1990) and was shown to belong to the carcinoembryonic antigen family of glycoproteins (Williams *et al.*, 1991). In the case of MHV it is not known whether $Neu5,9Ac_2$ is involved in virus binding or infection of cells. On the other hand, it is also not known whether a protein similar to the 110K protein plays a role in the infection of cells by BCV.

In comparing BCV and MHV, the question arises whether two serologically related viruses are using two different receptors. This appears to be unlikely. A possible explanation might be that there are two receptors which are involved in the initiation of an infection. A first receptor may mediate the primary attachment of virus. $Neu5,9Ac_2$ is a good candidate for this kind of binding, because it is a more frequent component of the cells surface than a specific protein. A second receptor might be required for a closer contact between virus and cell to facilitate or even induce the fusion of the membranes. In the case of influenza virus, a conformational change of the HA protein takes place at acidic pH, which is believed to induce the fusion (Skehel *et al.*, 1982). Fusion caused by BCV is not dependent on a low pH (Payne & Storz, 1988). Therefore, the interaction with a second receptor might be required for the exposure of a fusogenic epitope.

This model does not imply that the two proposed receptors have to be of equal importance for all strains. For example, increasing the efficiency in the recognition of the second receptor may render the first receptor dispensable for infection. This may be true for some murine coronaviruses. Several strains have both acetylesterase and haemagglutinating activity, which suggests that they recognize $Neu5,9Ac_2$ (Sugiyama & Amano, 1980). Other MHV strains such as A59 or JHM have no haemagglutinating activity and they either lack an esterase or have only low amounts of it (Yokomori *et al.*, 1989). Maybe for the latter viruses recognition of the 110K receptor is sufficient for the initiation of an infection. However, it should be kept in mind that these viruses might also have an affinity for $Neu5,9Ac_2$-containing receptors despite the lack of haemagglutinating activity. Interestingly, following passage of strain JHM though neural cell cultures, viral isolates were obtained which differed from the original virus by the expression of high levels of HE protein and by an increased size of the S protein (Taguchi *et al.*, 1986). There is no information about the receptor-binding activity of these isolates. These findings suggest, however, that the importance of $Neu5,9Ac_2$ for an MHV infection may vary between cell types.

A second receptor, in addition to $Neu5,9Ac_2$-containing surface components, may also play a role in the case of BCV. Support for this concept may come from the observation that inactivation and restoration of sialic acid-containing receptors was detectable only by following the stringent conditions of the infection protocol: (i) a short period of virus adsorption at room temperature, (ii) a short resialylation time at 37 °C and (iii) inactivation of residual virus by neutralizing antibodies. Similar experimental difficulties have been reported for influenza A viruses, which do not appear to require a second receptor for virus infection (Carroll & Paulson, 1985). However, endogenous restoration of virus receptors was found to be less critical in the case of influenza C virus compared to BCV (B. Schultze, unpublished observation). Furthermore, despite the lower efficiency in recognizing $Neu5,9Ac_2$ on erythrocytes, BCV was somewhat more efficient than influenza C virus in utilizing low amounts of 9-*O*-acetylated sialic acid for a low level of infection (Fig. 2). Influenza C virus, however, required less $Neu5,9Ac_2$ to reach optimum values of infection. This finding is compatible with the existence of a second receptor, which may interact with BCV following primary virus attachment to sialic acid-containing receptors. If such a receptor exists, it differs from the receptor for MHV-A59 in species specificity. Whereas the latter receptor is restricted to murine cells, BCV is able to infect cells from different species, e.g. canine (MDCK I), porcine (LLC-PK1) and human cells (HRT-18, CaCo-2). Future work will be aimed at the identification of a potential second receptor for BCV.

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