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VIRUS 00580

# Hemagglutinating encephalomyelitis virus attaches to *N*-acetyl-9-*O*-acetylneuraminic acid-containing receptors on erythrocytes: comparison with bovine coronavirus and influenza C virus

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(Accepted 20 February 1990)

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## Summary

The receptors for the hemagglutinating encephalomyelitis virus (HEV, a porcine coronavirus) on chicken erythrocytes were analyzed and compared to the receptors for bovine coronavirus (BCV) and influenza C virus. Evidence was obtained that HEV requires the presence of *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac<sub>2</sub>) on the cell surface for agglutination of erythrocytes as has been previously shown for BCV and influenza C virus: (i) Incubation of red blood cells with sialate 9-*O*-acetyl-esterase, the receptor-destroying enzyme of influenza C virus, rendered the erythrocytes resistant against agglutination by each of the three viruses; (ii) Human erythrocytes which are resistant to agglutination by HEV acquire receptors for HEV after resialylation with Neu5,9Ac<sub>2</sub>. Sialylation of red blood cells with limiting amounts of sialic acid indicated that strain JHB/1/66 of influenza C virus requires less Neu5,9Ac<sub>2</sub> for agglutination of erythrocytes than the two coronaviruses, both of which were found to be similar in their reactivity with Neu5,9Ac<sub>2</sub>-containing receptors.

Bovine coronavirus; Hemagglutinating encephalomyelitis virus; receptor; Sialic acid; *N*-acetyl-9-*O*-acetylneuraminic acid

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## Introduction

There are great variations among members of the family Coronaviridae in their ability to agglutinate red blood cells. Some strains such as A59 or JHM of mouse hepatitis virus are completely devoid of hemagglutinating activity. Other coronaviruses have been reported to be poor hemagglutinins, for instance transmissible gastroenteritis virus (Noda et al., 1987) or infectious bronchitis virus (Bingham et al., 1975). Several members of this virus family, however, are quite potent hemagglutinating agents. These include bovine coronavirus (BCV), human coronavirus OC43 (HCV-OC43), hemagglutinating encephalomyelitis virus (HEV), and some murine coronaviruses (Kaye and Dowdle, 1969; Pensaert and Callebaut, 1974; Sato et al., 1977; Sugiyama and Amano, 1980). Information about the nature of the erythrocyte receptors recognized by these viruses became available only recently, when BCV was reported to contain a sialate 9-*O*-acetyl esterase (Vlasak et al., 1988a). The same enzyme, which releases 9-*O*-acetyl residues from sialic acid, has previously been shown to be present on influenza C virions and to inactivate cellular receptors for influenza C virus (Herrler et al., 1985c; Herrler and Klenk, 1987). These findings indicated that *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac<sub>2</sub>) is a receptor determinant for attachment of influenza C virus to cells, which was confirmed by resialylation studies (Rogers et al., 1986). The esterases of both BCV and influenza C virus have been shown to inactivate the receptors on erythrocytes for BCV as well as for HCV-OC43 (Vlasak et al., 1988a). This result indicates that Neu5,9Ac<sub>2</sub> is a receptor determinant for attachment of BCV and HCV-OC43 to erythrocytes.

Here we report that the hemagglutinating activity of HEV is also dependent on the presence of Neu5,9Ac<sub>2</sub> on the erythrocyte surface. Evidence is presented that strain JHB/1/66 of influenza C virus is more efficient in recognizing Neu5,9Ac<sub>2</sub>-containing receptors than HEV and BCV, which both require similar amounts of 9-*O*-acetylated sialic acid on the cell surface in order to cause hemagglutination.

## Materials and Methods

### *Viruses*

Strain NT-9 of HEV was obtained from Dr R.G. Hess (Koblenz, F.R.G.). Strain L9 of BCV was provided by Dr R. Rott (Giessen, F.R.G.). Both coronaviruses were grown in MDCK I cells, a subline of Madin-Darby canine kidney cells, which were maintained as described previously (Herrler et al., 1988). After an adsorption period of 60 min at 37°C, the inoculum was removed, and the infected cells were incubated at 37°C with serum-free Eagle's minimal essential medium (MEM) in the presence of trypsin (1 µg/ml). Virus was harvested 48 h p.i. The supernatant, which usually had an HA-titer of 256–512 HA-units/ml, was clarified by low speed centrifugation and, following addition of fetal calf serum (10%), stored at –80°C.

Strain Johannesburg/1/66 (JHB/1/66) of influenza C virus and fowl plague virus (influenza A/FPV/Rostock/34 (H7N1)) were grown in embryonated eggs as described previously (Herrler and Klenk, 1987; Klenk et al., 1972).

#### *Hemagglutination and hemagglutination-inhibition assays*

The hemagglutination assay was performed as described previously (Herrler et al., 1985a). The hemagglutination titer (HAU/ml) indicates the reciprocal value of the maximum dilution that caused complete agglutination.

The hemagglutination-inhibition assay was performed as described previously (Herrler et al., 1985b).

#### *Purification of the acetylerase of influenza C virus*

The purification of the acetylerase of influenza C virus has been described previously (Herrler et al., 1988; Schauer et al., 1988). The enzyme activity was defined as the amount of acetylerase required to release 1  $\mu$ mol of acetate from bovine submaxillary gland mucin (BSM) in 1 min at 37°C.

#### *Inactivation of erythrocyte receptors by acetylerase*

Samples containing 100  $\mu$ l of a 10% suspension of chicken erythrocytes in PBS were incubated at 37°C in the presence of different amounts of purified acetylerase from influenza C virus. After 60 min the red blood cells were washed twice with PBS and resuspended in 2 ml of the same buffer. These erythrocytes were used to determine the HA-titer of BCV, HEV and influenza C virus.

#### *Synthesis of Neu5,9Ac<sub>2</sub>*

The diphenylmethylester of *N*-acetyl-2- $\alpha$ -benzyl-neuraminic acid was acetylated using acetic anhydride/pyridine (U. Rose and R. Brossmer, in preparation). Hydrogenation over palladiumoxide then afforded *N*-acetyl-9-*O*-acetylneuraminic acid. A detailed description will be published elsewhere.

#### *Synthesis and purification of CMP-Neu5,9Ac<sub>2</sub>*

CMP-Neu5,9Ac<sub>2</sub> was prepared enzymatically on a preparative scale using a partially purified CMP-sialic acid synthase from bovine brain (Gross et al., 1987). The reaction mixture was composed essentially as described previously (Gross et al., 1987), except that the pH was shifted from 9 to 7.5, as the 9-*O*-acetyl group is labile at pH-values above 7.5; the preparative assay contained 7 MnCl<sub>2</sub> instead of 40 mM MgCl<sub>2</sub>, and only 6 mM CTP in accordance to the analytical CMP-sialic acid synthase assay developed for pH 7.5 (Gross et al., 1988).

Purification of CMP-Neu5,9Ac<sub>2</sub> was achieved by semipreparative HPLC and gel filtration (Gross et al., 1987). The final CMP-glycoside preparation was char-

acterized by analytical HPLC systems as outlined earlier (Gross et al., 1987). After acidic hydrolysis, the molar ratio of CMP to Neu5,9Ac<sub>2</sub> was 1/1.02; contaminations of free CMP or Neu5,9Ac<sub>2</sub> were below 5%, of CTP even below 0.3%, and the preparation was free of further impurities as judged by TLC (Gross et al., 1987).

#### *Preparation of the sialyltransferase*

Rat liver Gal $\beta$ 1,4GlcNAc  $\alpha$ 2,6-sialyltransferase was stored at a concentration of 2 mU/ $\mu$ l in the presence of Triton CF-54. The detergent was removed prior to the sialylation of the cells according to published methods (Holloway, 1973; Carrol et al., 1981). The enzyme was mixed with Biobeads SM-2 (1 mg wet beads per  $\mu$ l of enzyme) in PBS containing 1% bovine serum albumin (10  $\mu$ l of PBS per mg wet beads). After incubation for 30 min at 4°C, the supernatant was removed and a 1/4 volume of fresh PBS/BSA was added to the beads. After 10 min at 4°C, both supernatants were combined and used for sialylation of cells.

#### *Resialylation of erythrocytes*

Cells were sialylated essentially as described by Paulson and Rogers (1987) using rat liver Gal $\beta$ 1,4GlcNAc  $\alpha$ 2,6-sialyltransferase. A 10% suspension of human erythrocytes was prepared in a buffer containing 0.1 M NaCl, 20 mM CaCl<sub>2</sub>, 50 mM MES, pH 6.5. Samples containing 0.1 ml of the red blood cell suspension were incubated with 50 mU of neuraminidase from *Vibrio cholerae*. After 30 min at 37°C, the cells were washed twice with PBS and resuspended with 50  $\mu$ l PBS/BSA. Following addition of 2mU of Gal $\beta$ 1,4GlcNAc  $\alpha$ 2,6-sialyltransferase and the amount of CMP-sialic acid indicated, the erythrocytes were incubated at 37°C for 3 h. The cells were washed twice, resuspended in 5 ml of PBS, and used to determine the HA-titer of BCV, HEV and influenza C virus.

#### *Materials*

Rat liver Gal $\beta$ 1,4GlcNAc  $\alpha$ 2,6-sialyltransferase and bovine submaxillary gland mucin were purchased from Boehringer (Mannheim, F.R.G.); *Vibrio cholerae* neuraminidase from Behringwerke (Marburg, F.R.G.); Biobeads SM-2 from Biorad (München, F.R.G.); bovine serum albumin and CMP-Neu5Ac from Sigma (Deisenhofen, F.R.G.).

## **Results**

#### *Agglutination of different erythrocytes by coronaviruses and influenza viruses*

In order to characterize the hemagglutinating activity of HEV hemagglutination assays were performed with erythrocytes known to differ in their content of Neu5,9Ac<sub>2</sub>. For this purpose chicken red blood cells were chosen, because 9-O-

TABLE 1

Agglutination of red blood cells by hemagglutinating encephalomyelitis virus (HEV), bovine coronavirus (BCV), influenza A (FPV/Rostock), and C virus (JHB/1/66)

Erythrocytes	Hemagglutination titer (HA-units/ml)			
	HEV	BCV	JHB/1/66	FPV/Rostock
Chicken, adult	32	64	512	512
Chicken, 1-day-old	< 2	< 2	< 2	256
Human	< 2	< 2	< 2	1024

acetylated sialic acid has been shown to be a developmental marker on the surface of these cells being present only on erythrocytes from adult chicken, but not on cells from one-day-old chicken (Herrler et al., 1987). In addition to avian cells human erythrocytes were used, for which chemical analysis has failed to detect any *O*-acetylated sialic acid (Shukla and Schauer, 1982) though red blood cells of some individuals may have low amounts of Neu5,9Ac<sub>2</sub> as indicated by hemagglutination tests with influenza C virus (Nishimura et al., 1988). As shown in Table 1, among the viruses tested only influenza A virus is able to agglutinate cells from all three sources. HEV was found to have the same agglutination behavior as BCV and influenza C virus. HA-titers of these viruses could only be determined with erythrocytes from adult chicken, while cells from one-day-old chicken and human erythrocytes were resistant to agglutination. Considering the type of sialic acid present on the red blood cells (see above), the result from Table 1 is compatible with Neu5,9Ac<sub>2</sub> being a receptor determinant for attachment of HEV to erythrocytes.

*Effect of sialate O-acetyltransferase on the erythrocyte receptors*

The importance of Neu5,9Ac<sub>2</sub> for the agglutination of red blood cells by HEV was further analyzed by pretreating chicken erythrocytes with sialate *O*-acetyltransferase. The enzyme was isolated from purified influenza C virions (Herrler et al., 1988). Following isolation, the HEF-protein of influenza C virus lacks hemagglutinating activity, but it retains the esterase activity. As shown in Table 2, incubation with acetyltransferase renders the red blood cells from adult chicken

TABLE 2

Effect of the acetyltransferase from influenza C virus on chicken erythrocyte receptors for HEV, BCV and influenza C virus (JHB/1/66)

Amount of acetyltransferase <sup>a</sup>	Hemagglutination titer (HA-units/ml)		
	HEV	BCV	JHB/1/66
—	256	256	128
2 mU	64	64	128
10 mU	< 2	< 2	32
25 mU	nt	nt	< 2

<sup>a</sup> Chicken erythrocytes were treated with acetyltransferase as described under Materials and Methods. nt, not tested.

TABLE 3

Generation of receptors for HEV, BCV and influenza C virus (JHB/1/66) by resialylation of human erythrocytes

Type of sialic acid attached to cells	Hemagglutination titer (HA-units/ml)		
	HEV	BCV	JHB/1/66
None	< 2	< 2	< 2
Neu5Ac	< 2	< 2	< 2
Neu5,9A <sub>2</sub>	256	256	512

resistant against agglutination by HEV, BCV, and influenza C virus. However, receptors for HEV and BCV are more sensitive to the enzyme treatment than the receptors for influenza C virus. Incubation of erythrocytes in the presence of 2 mU of acetylcholinesterase caused a 4-fold reduction of the hemagglutination titer of both HEV and BCV, whereas the titer of influenza C virus was unaffected. Increasing the amount of enzyme to 10 mU resulted in a partial reduction of the HA-titer of influenza C virus and in a complete resistance against agglutination by both coronaviruses. This result confirms the role of Neu5,9Ac<sub>2</sub> as a receptor determinant for attachment of HEV to cells. It indicates, however, that influenza C virus differs from both coronaviruses in the ability to recognize Neu5,9Ac<sub>2</sub>-containing receptors.

#### *Restoration of coronavirus receptors by resialylation of erythrocytes*

With influenza C virus direct evidence for the importance of Neu5,9Ac<sub>2</sub> as an essential part of the receptors on red blood cells was obtained by resialylation of human erythrocytes (Rogers et al., 1986). The same approach was applied to BCV and HEV. Human red blood cells were incubated with neuraminidase from *Vibrio cholerae* to release most of the native sialic acids. The asialo-cells were resialylated using purified rat liver Gal $\beta$ 1,4GlcNAc  $\alpha$ 2,6-sialyltransferase (specifically attaching sialic acid to galactose in an  $\alpha$ -2,6-linkage) and activated sialic acids (CMP-Neu5Ac and CMP-Neu5,9Ac<sub>2</sub>). As shown in Table 3, neither influenza C virus nor the coronaviruses BCV and HEV are able to agglutinate cells resialylated to contain Neu5Ac on the surface. High hemagglutination titers were obtained, however, following attachment of 9-*O*-acetylated sialic acids to cell surface glycoproteins of asialo-cells. This result provides further proof that attachment of these viruses to erythrocytes involves the same type of receptors.

The resialylation method was used to compare the efficiency of the two coronaviruses and influenza C virus in recognizing Neu5,9Ac<sub>2</sub>-containing receptors. The reaction conditions were changed as to reduce the amount of 9-*O*-acetylated sialic acid attached to the cell surface. For this purpose limiting concentrations of CMP-Neu5,9Ac<sub>2</sub> were used for the incubation of red blood cells with sialyltransferase. As shown in Table 4, optimal hemagglutination titers of influenza C virus were already obtained, when 250 pmol CMP-Neu5,9Ac<sub>2</sub> were used for the sialylation of red blood cells. Hemagglutination by BCV was only detectable with

TABLE 4

Comparison of the efficiency of HEV, BCV and influenza C virus (JHB/1/66) to utilize Neu5,9Ac<sub>2</sub> as a receptor determinant for agglutination of human red blood cells

Erythrocytes	Hemagglutination titer (HA-units/ml)		
	HEV	BCV	JHB/1/66
Chicken, untreated	256	256	512
Human, untreated	< 2	< 2	< 2
Human, resialylated with CMP-Neu5,9Ac <sub>2</sub>			
2 nmol	256	256	512
1 nmol	32	16	512
0.5 nmol	< 2	< 2	512
0.25 nmol	< 2	< 2	512
0.1 nmol	< 2	< 2	< 2

cells incubated in the presence of 1 nmol of activated sialic acid, optimal titers requiring 2 nmol CMP-Neu5,9Ac<sub>2</sub> for the resialylation reaction. This result indicates that HEV and BCV require the same minimal concentration of 9-*O*-acetylated sialic acid on the cell surface for agglutination of erythrocytes. Strain JHB/1/66 of influenza C virus, on the other hand, is more efficient in recognizing Neu5,9Ac<sub>2</sub>-containing receptors on red blood cells than the two coronaviruses tested.

## Discussion

Our results demonstrate that agglutination of erythrocytes by HEV requires the presence of Neu5,9Ac<sub>2</sub>-containing receptors on the cell surface as has been shown previously for BCV and influenza C virus (Rogers et al., 1986; Vlasak et al., 1988a): (i) Following incubation with sialate *O*-acetyltransferase red blood cells are resistant against the hemagglutinating activity of these viruses; (ii) Virus receptors can be generated on resistant erythrocytes by attachment of Neu5,9Ac<sub>2</sub> to cell surface glycoproteins. Using the resialylation method we were able to compare the viruses with respect to their efficiency in recognizing Neu5,9Ac<sub>2</sub>-containing receptors. Strain JHB/1/66 of influenza C virus requires less 9-*O*-acetylated sialic acid on the surface of erythrocytes for hemagglutination than the two coronaviruses. This finding was confirmed by the results with two hemagglutination-inhibitors. With both bovine submaxillary mucin and rat serum 16-fold lower inhibitory titers were obtained for HEV and BCV than for influenza C virus (not shown). Neu5,9Ac<sub>2</sub> of the bovine mucin is present on *O*-linked oligosaccharides (Gottschalk et al., 1972), while 9-*O*-acetylated sialic acid of rat  $\alpha$ 1-macroglobulin, the major inhibitory compound of rat serum (Herrler et al., 1985a; Kitame et al., 1985), is primarily found on *N*-linked biantennary oligosaccharides (Herrler et al., 1985b,c). Considering the difference in the carbohydrate structure between the two inhibitors, we



conclude that strain JHB/1/66 of influenza virus has an increased affinity for Neu5,9Ac<sub>2</sub> compared to HEV and BCV and that the neighboring sugars are not of major importance for this interaction. So far different strains of influenza C virus have not been compared with respect to their affinity for Neu5,9Ac<sub>2</sub>. A mutant of strain JHB/1/66, however, was isolated which is more efficient in recognizing Neu5,9Ac<sub>2</sub>-containing receptors than the wild type (Szepanski et al., 1989). This mutant has a broader cell tropism compared to the parent virus. It will be interesting to find out whether mutants with an increased affinity for cellular receptors can be obtained from coronaviruses and how such a mutation affects the tropism and the pathogenicity of the virus.

So far three coronaviruses have been shown to use Neu5,9Ac<sub>2</sub>-containing receptors for agglutination of erythrocytes: BCV, HEV and HCV-OC43. These coronaviruses contain a glycoprotein, HE, which has been implicated with both hemagglutinating (King et al., 1985) and esterase activity (Vlasak et al., 1988b). A corresponding protein is also present on turkey coronavirus (Dea et al., 1989) and some murine coronaviruses (Sugiyama and Amano, 1980), which are also potent hemagglutinating agents. We suppose that the latter viruses also use Neu5,9Ac<sub>2</sub> as a receptor determinant for attachment to cells.

Other coronaviruses lack an HE-protein. These viruses include infectious bronchitis virus (IBV), transmissible gastroenteritis virus (TGEV), and feline infectious peritonitis virus. Their attachment to cells is mediated by the S-protein (Cavanagh, 1981). The nature of the receptors recognized by these viruses is not known. Both TGEV and IBV have a low hemagglutinating activity, which is sensitive to pretreatment of erythrocytes with neuraminidase (Bingham et al., 1975; Noda et al., 1988). Using the technique of resialylation it should be possible to demonstrate whether coronaviruses lacking an HE-protein require sialic acid on the cell surface for attachment to cells and, if so, whether they recognize Neu5Ac- or Neu5,9Ac<sub>2</sub>-containing receptors.

An HE-protein is also lacking on strain A59 of mouse hepatitis virus (MHV), though an open reading frame for such a protein is present on the genome of this virus (Luytjes et al., 1988). HE-protein and the corresponding mRNA were detected only with the related strains MHV-JHM and MHV-S but not with A59 (Shieh et al., 1989; Pfliederer et al., submitted data). MHV-A59 has been reported to use a 100–110 kDa protein as a receptor (Boyle et al., 1987). Future work has to show whether such a protein is recognized only by mouse hepatitis virus, or whether it serves also, in addition to the Neu5,9Ac<sub>2</sub>-containing glycoconjugates, as a receptor for HEV and BCV.

### **Acknowledgement**

The technical assistance of Birgit Döll and Ursula Rose is gratefully acknowledged. This work was supported by Deutsche Forschungsgemeinschaft (Forschergruppe Kl 238-1/1).

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(Received 3 January 1990; revision received 20 February 1990)