

Isolated HE-Protein from Hemagglutinating Encephalomyelitis Virus and Bovine Coronavirus Has Receptor-Destroying and Receptor-Binding Activity

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Bovine coronavirus (BCV) and hemagglutinating encephalomyelitis virus (HEV) from swine were found to grow to high titers in MDCK I cells, a subline of Madin Darby canine kidney cells. Virus grown in these cells was used to isolate and purify the HE-protein. This protein has been shown recently to have acetyltransferase activity and to function as the receptor-destroying enzyme of BCV. Here we show that HEV contains this enzyme, too. The glycoproteins were solubilized by treatment of virions with octylglucoside. Following centrifugation through a sucrose gradient the surface proteins S and HE (hemagglutinin-esterase) were obtained in purified form. After removal of the detergent by dialysis, HE formed rosettes as shown by electron microscopy. The purified HE protein retained acetyltransferase activity and was able to function as a receptor-destroying enzyme rendering red blood cells resistant against agglutination by both coronaviruses. HE protein released from the viral membrane failed to agglutinate red blood cells. However, it was found to recognize glycoconjugates containing *N*-acetyl-9-*O*-acetylneuraminic acid as indicated by a binding assay with rat serum proteins blotted to nitrocellulose and by its ability to inhibit the hemagglutinating activity of BCV, HEV, and influenza C virus. The purified enzyme provides a useful tool for analyzing the cellular receptors for coronaviruses.

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INTRODUCTION

It has been reported recently that bovine coronavirus (BCV) contains a receptor-destroying enzyme (Vlasak *et al.*, 1988a). This is the first example of such an enzyme present on a positive-stranded RNA virus. Up to then, receptor-destroying enzymes have been known as structural components only of negative-stranded RNA viruses: influenza viruses and paramyxoviruses (Hirst, 1950). Influenza A and B viruses as well as paramyxoviruses inactivate their receptors by means of a neuraminidase which releases terminal sialic acid from glycoconjugates (Klenk *et al.*, 1955). The receptor-destroying enzyme of influenza C virus, on the other hand, cleaves not a glycosidic linkage but rather an ester linkage. It has been identified as an acetyltransferase (Herrler *et al.*, 1985c), which is able to release acetate from various synthetic substrates (Vlasak *et al.*, 1987; Schauer *et al.*, 1988). The receptor-destroying activity, however, is due to the release of the acetyl group from position C-9 of *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac₂) (Herrler *et al.*, 1985c). The 9-*O*-acetyl residue is crucial for the ability of influenza C virus to recognize a glycoprotein as a receptor (Herrler *et al.*, 1985c; Rogers *et al.*, 1986).

The intriguing observation of a sequence similarity between the glycoprotein HEF of influenza C virus and a protein sequence derived from an open reading frame within the genome of MHV-A59 (mouse hepatitis virus) (Luytjes *et al.*, 1988) led to the finding that BCV has the same type of receptor-destroying enzyme as influenza C virus (Vlasak *et al.*, 1988a). The acetyltransferase activity of BCV has been shown to be a function of the glycoprotein HE (Vlasak *et al.*, 1988b), the amino acid sequence of which has been derived from the nucleotide sequence of the cloned mRNA (Parker *et al.*, 1989). Interestingly, this protein is present only on some coronaviruses, while others, such as avian infectious bronchitis virus, porcine transmissible gastroenteritis virus, and feline infectious peritonitis virus, are lacking a corresponding protein (Spaan *et al.*, 1988). The genome of MHV-A59 contains an open reading frame coding for an HE protein, which is, however, not expressed in infected cells (Luytjes *et al.*, 1988; Shieh *et al.*, 1989). Other murine coronaviruses contain such a protein (Sugiyama and Amano, 1980; Shieh *et al.*, 1989) and the HE protein of the strain MHV-JHM has been shown to have acetyltransferase activity (Yokomori *et al.*, 1989; Pfeleiderer *et al.*, 1990).

We describe the isolation and purification of HE from BCV and from porcine hemagglutinating encephalomyelitis virus (HEV). The activities of the purified glycoprotein are reported.

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MATERIALS AND METHODS

Viruses and cells

Strain L-9 of BCV was obtained from Dr. Rott (Giesen, FRG). Strain NT-9 of HEV was provided by Dr. Hess (Koblenz, FRG). MDCK I cells, a subline of Madin-Darby canine kidney cells, were maintained as described previously (Herrler *et al.*, 1988a).

Growth and purification of virus

BCV and HEV were grown in MDCK I cells as reported recently (Schultze *et al.*, 1990). Virus was harvested from the supernatant 48 hr p.i. After clarification of the medium by low-speed centrifugation (3000 rpm, 10 min), virus was sedimented by ultracentrifugation at 25,000 rpm for 1 hr in a SW 28 rotor. The pellet was resuspended in PBS and layered on a sucrose gradient (5–50% w/w in PBS). After centrifugation in a SW 55 Ti rotor at 35,000 rpm for 40 min, the virus band was collected, diluted with PBS, and sedimented under the same centrifugation conditions. The virus pellet was resuspended in PBS and used for (i) analysis by polyacrylamide gel electrophoresis; (ii) determination of the esterase activity; (iii) hemagglutination and hemagglutination-inhibition assays; and (iv) purification of the viral glycoproteins.

Isolation and purification of viral glycoproteins

Purified coronavirus suspended in 800 μ l PBS was incubated in the presence of 1% *n*-octylglucopyranoside for 7 min at room temperature. After centrifugation for 10 min at 12,000 *g*, the supernatant was further incubated for 20 min at 4°. Following centrifugation for 30 min at 30,000 rpm in a TLA 100.3 rotor, the supernatant was layered onto a sucrose gradient (10–30% w/w, on a cushion of 0.5 ml 60% sucrose) in PBS containing 1% octylglucoside. After centrifugation for 18 hr at 42,000 rpm in a SW55.1 rotor, fractions of 0.5 ml were collected from the bottom of the tube. The samples were analyzed by SDS–polyacrylamide gel electrophoresis and assayed for acetylcholinesterase activity. For hemagglutination and hemagglutination-inhibition assays, as well as for treatment of cell surface receptors, the fractions were dialyzed against PBS/H₂O (1:4). If the enzyme activity should be preserved for longer periods, the purified acetylcholinesterase was stored at –20° in the presence of 5 mg bovine serum albumin per milliliter.

Hemagglutination and hemagglutination-inhibition assays

Hemagglutination assays were performed as described previously (Herrler *et al.*, 1985a). The hemag-

glutination titer indicates the reciprocal value of the maximum dilution that caused complete agglutination. Hemagglutination-inhibition assays were performed as described previously (Herrler *et al.*, 1985b).

Assay for acetylcholinesterase activity

The esterase activity of purified virions or proteins was determined by incubation with 200 μ g *p*-nitrophenyl acetate (PNPA) in 1 ml PBS at room temperature. The substrate was dissolved in 1/50 vol ethanol. Using a kinetics program, the release of acetate was monitored by determining the optical density at 405 nm for 10 min at intervals of 2 min. The background level of substrate incubated in the absence of esterase was subtracted from the samples. The amount of esterase which cleaves 1 μ mol of *p*-nitrophenyl acetate in 1 min at room temperature was defined as 1 unit of enzyme.

Labeling with [³H]diisopropylfluorophosphate ([³H]DFP)

Labeling of esterases with [³H]DFP was performed as described previously (Herrler *et al.*, 1988b). Purified virions or proteins in 50 μ l of PBS were incubated with 5 μ l of ³H-labeled DFP (3.5 Ci/mmol) on ice. After 30 min the samples were prepared for electrophoresis.

SDS–polyacrylamide gel electrophoresis

Analysis of proteins by SDS–polyacrylamide gel electrophoresis was performed as described previously (Herrler *et al.*, 1988a).

Incubation of erythrocytes with acetylcholinesterase

Samples containing 200 μ l of a 10% suspension of chicken erythrocytes were incubated with 100 μ l of the gradient fraction containing purified HE protein (see above). Prior to use the acetylcholinesterase was dialyzed to remove octylglucoside. After incubation for 2 hr at 37°, the red blood cells were washed twice with PBS and suspended in 4 ml of the same buffer. These erythrocytes and control cells, which had been incubated with PBS, were used to determine the HA titer of BCV, HEV, and influenza C virus.

Solid-phase assay for virus binding

Different dilutions of rat serum (1:100, 1:500, and 1:1000 in PBS) in 2 μ l were applied to nitrocellulose and air-dried. Excess protein-binding sites were blocked with 10% nonfat dry milk in PBS overnight at 4°. The nitrocellulose strips were washed three times for 5 min with PBS/0.1% Tween and incubated for 1 hr at 37° with PBS, purified acetylcholinesterase from influenza C virus (170 mU), or 0.1 *N* NaOH. All subsequent steps

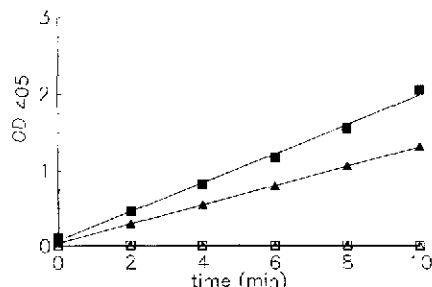


Fig. 1. Esterase activity of purified BCV (triangles) and HEV (squares). Closed symbols: untreated virus; open symbols: virus pretreated with DFP. The release of acetate from paranitrophenylacetate was determined by measuring the optical density at 405 nm.

were done at 4°. Following three washes with PBS/Tween, the nitrocellulose was incubated for 1 hr with BCV, HE protein from BCV (untreated or pretreated with DFP), or influenza A virus (WSN). After being washed with PBS/Tween, strips were incubated with rabbit antiserum directed against BCV (dilution 1:1000) or against influenza A virus (PR8, dilution 1:200). The nitrocellulose was again washed three times and then incubated for 1 hr with biotinylated anti-rabbit immunoglobulins from donkey. After being washed, the strips were incubated with streptavidin-biotinylated horseradish peroxidase complex (1 hr) and washed again. Bound BCV, HE protein, or WSN was detected by incubation of the nitrocellulose with PBS, 4-chloro-1-naphthol, and H₂O₂ (500:100:1).

Electron microscopy

For negative staining, samples were applied to Piloform-coated copper grids, stained with 2% uranyl acetate, and examined in a Siemens-Elmiskop 101.

Materials

n-Octylglucopyranoside was purchased from Sigma (Deisenhofen, FRG), [³H]DFP and the immunological reagents from Amersham (Braunschweig, FRG).

RESULTS

Growth of BCV and HEV in MDCK I cells

MDCK I cells are a subline of Madin-Darby canine kidney cells which differs from other sublines in both functional and morphological characteristics (Richardson *et al.*, 1981). Strain Johannesburg/1/66 of influenza C virus was found to grow to high titers in this cell line, while another subline—MDCK II—was resistant to infection because of a lack of cell surface receptors (Herrler and Klenk, 1987; Szepanski *et al.*, 1989). Due to the similarity of the erythrocyte receptors for BCV

and influenza C virus, we tried to grow BCV in MDCK I cells. Strain L-9 of BCV, which had been grown previously in bovine cell cultures (Storz and Rott, 1981), was able to replicate in MDCK I cells without adaptation. Hemagglutination titers of 256 HAU/ml were determined in the supernatant 48 hr p.i. The same growth kinetics was observed with strain NT-9 of HEV, which had been grown previously in porcine cell cultures (Hess and Bachmann, 1978). These titers are equally high as or higher than those reported for BCV and HEV in other cell lines. Both MDCK-grown coronaviruses were found to have acetyl esterase activity. Comparing virus suspensions with the same HA titer, the ability of both viruses to release acetate from *p*-nitrophenyl acetate was similar, HEV being somewhat more active than BCV (Fig. 1). As expected from previous studies with influenza C virus and BCV (Muchmore and Varki, 1987; Vlasak *et al.*, 1988b), the esterase activity of the MDCK-grown coronaviruses was abolished after treatment with DFP. This compound inhibits serine esterases and proteases by covalently attaching to the active-site serine.

Up to the time of virus harvest no cytopathic changes were detectable in the monolayer of MDCK I cells infected with either BCV or HEV. Virions released into the medium were purified by centrifugation into a sucrose gradient and analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2 for HEV, following Coomassie staining of the gel, the major bands visible are the known structural polypeptides: the nucleocapsid protein N, the membrane protein M, the surface protein S, and the hemagglutinin-esterase protein HE. The latter polypeptide is detected under nonreducing conditions as a disulfide-linked dimer, (HE)₂. Several other coronaviruses require two rounds of gradient centrifugation for a satisfying degree of purification. Using the MDCK cell system a single gradient

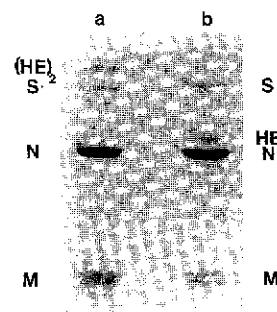


Fig. 2. SDS-PAGE of purified HEV stained with Coomassie brilliant blue. Viral proteins were analyzed under nonreducing conditions (lane a) and under reducing conditions (lane b). The proteins have the following apparent molecular weights: M, 23 kDa; N, 50 kDa; HE, 85 kDa; S, 100 kDa; (HE)₂, 140 kDa.

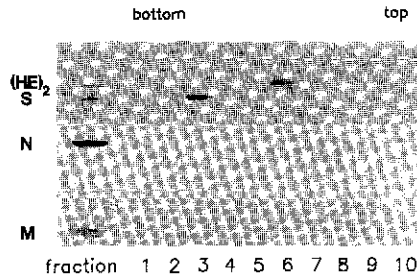


Fig. 3. Separation of the HEV glycoproteins by octylglucoside (OG) treatment and sucrose gradient centrifugation. The viral polypeptides N and M were sedimented by centrifugation after OG treatment and the supernatant, containing S and HE, was loaded on a sucrose gradient. After centrifugation, 10 fractions were collected from the bottom of the tube, analyzed by SDS-PAGE, and stained with Coomassie brilliant blue. On the left the polypeptide pattern of whole virions is shown.

centrifugation step is sufficient to obtain both BCV and HEV in purified form.

Isolation and purification of the acetylcholinesterase of BCV

The acetylcholinesterase was isolated from coronaviruses by a modification of the procedure used for the purification of the influenza C glycoprotein (Herrler *et al.*, 1988a). Purified HEV or BCV were treated with octylglucoside to solubilize the components of the lipid envelope. The viral polypeptides N and M were sedimented by centrifugation (not shown). The glycoproteins remaining in the supernatant (S and HE) were centrifuged into a sucrose gradient containing detergent. To determine the location of the viral proteins, the gradient fractions were analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 3 for HEV, the peak of the HE protein was detected in fraction 6. IgG was found to have the same sedimentation behavior (not shown). The S-protein was well separated from HE with the majority being present in fraction 3. An identical separation was obtained with the glycoproteins of BCV (compare Figs. 4 and 5). The clear separation of both coronavirus glycoproteins was also evident when the individual fractions were analyzed for acetylcholinesterase activity using *p*-nitrophenyl acetate as a substrate (Fig. 4). The peak fraction of the esterase activity of both BCV and HEV coincided with the HE-protein (fraction 6); no enzymatic release of acetate was detectable with fraction 3. The purification procedure preserved not only the acetylcholinesterase activity of HE, but also the sensitivity of this enzyme to treatment with DFP, which binds covalently to serine hydrolases. As shown in Fig. 5, upon incubation with [³H]DFP, purified HE becomes radioactively labeled. Figure 5 also

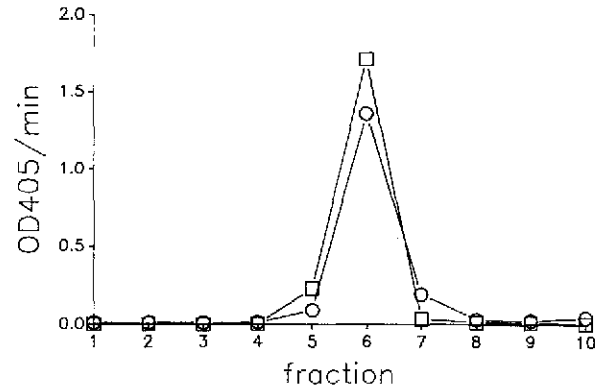


Fig. 4. Esterase activity of sucrose gradient fractions after centrifugation of the glycoproteins HE and S from BCV (circles) and HEV (squares). Compare Fig. 3 for a polypeptide analysis of the gradient fractions.

illustrates why the initial treatment of virus with detergent was performed in two steps with intermittent centrifugation. If the first centrifugation was omitted, part of the M-protein remained in the supernatant which during gradient centrifugation cosedimented with HE (compare lanes e and f). In order to analyze the effect of purified HE-protein on cells, it was necessary to remove octylglucoside. The gradient fraction containing HE was dialyzed and analyzed by electron microscopy. Removal of the detergent resulted in rosette formation of the glycoprotein (shown in Fig. 6 for BCV-HE) as has been reported for other viral surface glycoproteins.

Analysis of the purified HE-protein for receptor-binding and receptor-destroying activity

The purified HE protein was analyzed for its ability to function as a receptor-destroying enzyme. As shown in

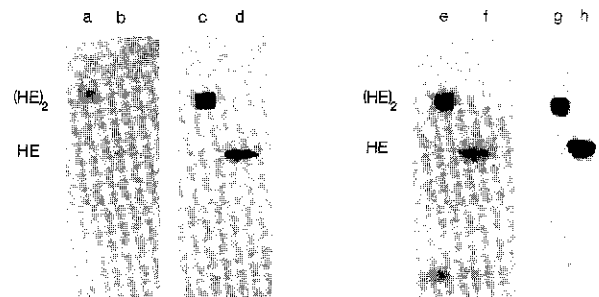


Fig. 5. SDS-PAGE of purified HE-protein from BCV (lanes a-d) and HEV (lanes e-h) labeled with [³H]DFP. The samples were analyzed under nonreducing conditions (lanes a, c, e, and g) and under reducing conditions (lanes b, d, f, and h) and visualized with Coomassie brilliant blue (lanes a, b, e, and f) or by fluorography (lanes c, d, g, and h). During the isolation of the BCV proteins, M and N were removed by two centrifugation steps as described under Materials and Methods. The HEV proteins, which were subjected to only one centrifugation step, still contain some M protein, which is visible at the bottom of the gel.

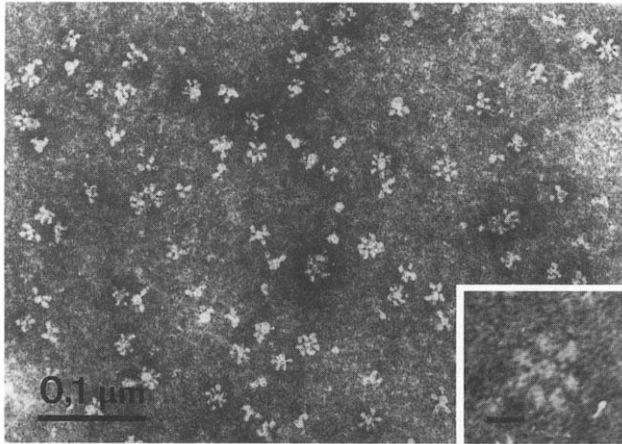


FIG. 6. Electron micrograph of purified HE from BCV after removal of octylglucoside by dialysis. Magnification: $\times 110,000$. The inset at the lower right corner shows a rosette at a magnification of 450,000. The bar in the inset indicates 10 nm.

Table 1, incubation of erythrocytes with purified acetylcsterase from either BCV or HEV rendered the cells resistant to agglutination by both coronaviruses as well as by influenza C virus. On the other hand, agglutination by influenza A virus was not affected. This result indicates that the specificity of the coronavirus esterase as a sialate 9-*O*-acetylcsterase is preserved during purification of the HE protein.

Next we analyzed whether purified HE has receptor-binding activity. As shown in Table 2, the gradient fraction containing HE was unable to agglutinate chicken red blood cells. However, it was found to have a low hemagglutination-inhibition activity; i.e., purified HE was able to prevent intact virions (BCV or HEV) from agglutinating erythrocytes. This finding suggests that, following purification, HE is still able to attach to coronavirus receptors on red blood cells. However, this interaction appears not to be sufficient for agglutination of erythrocytes. The ability of purified HE to bind to Neu5,9Ac₂-containing receptors was further analyzed

TABLE 1

INACTIVATION OF THE RECEPTORS FOR BCV, HEV, AND INFLUENZA C VIRUS (JHB/1/66) BY TREATMENT OF CHICKEN ERYTHROCYTES WITH PURIFIED ACETYLESTERASE (HE) FROM EITHER BCV OR HEV

Treatment of red blood cells	Hemagglutination titer (units/ml)			
	BCV	HEV	JHB/1/66	WSN
None	256	256	512	1024
HE-BCV	<2	<2	<2	1024
HE-HEV	<2	<2	<2	768

TABLE 2

HEMAGGLUTINATION (HA) AND HEMAGGLUTINATION-INHIBITION (HI) ACTIVITY OF ISOLATED HE PROTEIN FROM BCV AND HEV

HE protein from	HA (units/ml)	HI (units/ml)
BCV	<2	16
HEV	<2	32

by a solid-phase binding assay. Rat serum is known to be a potent hemagglutination-inhibitor of influenza C virus (Styk, 1955) as well as of BCV and HEV (Schultze *et al.*, 1990). The inhibitory activity of rat serum is mainly due to $\alpha 1$ -macroglobulin (Herrler *et al.*, 1985b; Kitame *et al.*, 1985), which has been shown to contain a substantial amount of Neu5,9Ac₂ (Herrler *et al.*, 1985c). BCV and purified HE from this virus were analyzed for their ability to bind to rat serum which had been spotted on a nitrocellulose filter. As shown in Fig. 7 (left lane), attachment of BCV to rat serum could be demonstrated. Purified HE gave a positive result in this assay only at the highest concentration of rat serum tested (compare sections designated BCV and HE). If HE was pretreated with DFP, however, to inactivate the esterase activity, binding of HE protein to rat serum was as efficient as binding of whole virus (compare sections designated BCV and HE-DFP). To find out whether attachment of BCV and HE-protein was mediated by Neu5,9Ac₂, the serum proteins blotted on the nitrocellulose were preincubated with either acetylcsterase or sodium hydroxide. Both treatments are known to release 9-*O*-acetyl residues from Neu5,9Ac₂. Preincubation of the serum proteins at alkaline pH completely abolished binding of both BCV and HE protein, while attachment of influenza A virus (strain WSN) could still be demonstrated (Fig. 7, middle lane). Pretreatment of serum proteins with acetylcsterase from influenza C virus (HEF) drastically reduced binding of BCV, HE, or DFP-treated HE protein (Fig. 7, right lane). These results indicate that purified HE protein is able to recognize Neu5,9Ac₂ as a receptor determinant for attachment to glycoproteins. Binding of HE is, however, less efficient than binding of whole virions and can be increased by inactivation of the esterase.

DISCUSSION

Coronaviruses grow most readily in cells from their natural host, although adaptation to cells from other species is possible (Siddell *et al.*, 1982). In our attempts to set up a cell culture system for two hemagglutinating coronaviruses, BCV and HEV, it turned out that both viruses can be grown to high titers in MDCK I

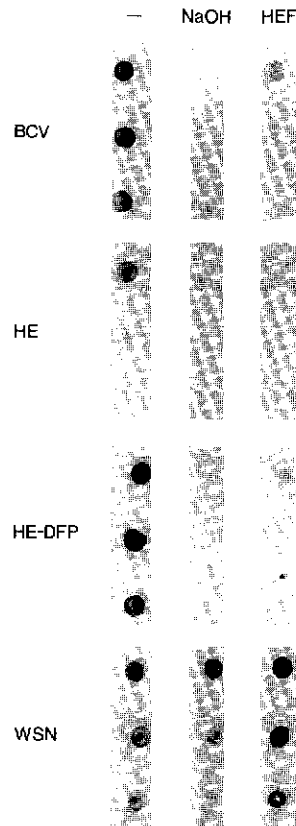


FIG. 7. Binding of BCV, HE-protein from BCV, and influenza A virus (strain WSN) to rat serum proteins. After different dilutions (1:100, 1:500, and 1:1000, from top to bottom for each binding assay) of rat serum were blotted to nitrocellulose, the serum proteins were incubated with PBS (left lane), sodium hydroxide (middle lane), or acetyl-esterase from influenza C virus (HEF, right lane). The samples were then incubated with virus (BCV, WSN) or purified HE-protein, which had been either untreated (designated HE) or pretreated with DFP (designated HE-DFP). Binding was detected by an enzyme-linked immunoassay using rabbit antiserum directed against BCV or influenza virus.

cells without prior adaptation. This subline of Madin-Darby canine kidney cells has been shown recently to be sensitive to infection by strain Johannesburg/1/66 of influenza C virus (Szepanski *et al.*, 1989). This virus initiates infection of cultured cells by attaching to Neu5,9Ac₂-containing receptors, which are a major determinant of the cell tropism of influenza C virus (Herrler and Klenk, 1987). Neu5,9Ac₂ serves also as a receptor determinant for attachment of BCV and HEV to red blood cells (Vlasak *et al.*, 1988a; Schultze *et al.*, 1990). It remains to be shown whether the same type of receptors is also used for infection of cells. Another question to be answered is whether the restricted tropism of coronaviruses is determined by the cellular receptors or at a later stage of the infectious cycle. With MHV-A59 evidence has been presented indicating a crucial role of cellular receptors for the cell tropism.

This virus strain has been reported to use a 100 to 110-kDa protein as a receptor (Boyle *et al.*, 1987). MDCK cells provide a promising system to analyze the initial stage of infection with other coronaviruses such as BCV and HEV, which appear to recognize different receptors on the cell surface. This cell line is also known for its polarized organization, which is reflected—among other characteristics—by a polarity of the virus infection. With vesicular stomatitis virus it has been shown that virus entry and release is restricted to the basolateral domain of the plasma membrane. Influenza A virus, on the other hand can infect MDCK cells from both the apical and the basolateral side, whereas budding of the virus is restricted to the apical portion of the plasma membrane (Rodriguez-Boulan and Sabatini, 1978; Fuller *et al.*, 1984). Coronaviruses are known to bud into intracellular vesicles (Tooze *et al.*, 1984). It will be interesting to analyze how viruses with this type of maturation are released by polarized cells.

The amount and the purity of both BCV and HEV grown in MDCK I cells were sufficient to attempt the isolation of the viral glycoproteins. Using the protocol described it was possible to get a complete separation of the coronavirus proteins HE, S, and M. A similar method using Triton X-100 rather than octylglucoside had been used previously to purify the glycoproteins of HEV (Callebaut and Pensaert, 1980). As Triton X-100 cannot be removed by dialysis, these authors used butanol precipitation to obtain detergent-free glycoprotein. HE prepared in this way was unable to agglutinate red blood cells. At that time the esterase activity as well as the ability of this virus to recognize Neu5,9Ac₂ as a receptor determinant was unknown. Using octylglucoside as a detergent we obtained both HE and S in purified form. The isolated S-protein was also subject to a biochemical and functional analysis (manuscript in preparation). The sedimentation behavior of the isolated HE protein was similar to that of IgG. Considering the molecular weight of the monomeric glycoprotein (Parker *et al.*, 1989), a dimeric structure is suggested for the purified HE protein. Dimers of HE are also detected following polyacrylamide gel electrophoresis under nonreducing conditions (see Fig. 2), indicating that the two monomers are held together by disulfide bonds. We cannot, however, exclude the possibility that the functional protein is embedded in the lipid envelope as a multimeric structure, e.g., a tetramer, which is dissociated into dimers upon detergent treatment and/or sucrose gradient centrifugation. For the G-protein of vesicular stomatitis virus it has been reported that gradient centrifugation results in the dissociation of the trimeric glycoprotein into monomers (Doms *et al.*, 1987).

Of the two functions which have been assigned to

the HE-protein (hemagglutinin and esterase) the esterase could be detected easily in the purified protein. The acetylerase activity was evident both in the color test with the synthetic substrate PNPA (Fig. 4) and in the ability of HE to inactivate the erythrocyte receptors for BCV, HEV, and influenza C virus (Table 1). The hemagglutinin function of HE could not be demonstrated with the purified glycoprotein. However, HE was still able to recognize Neu5,9Ac₂-containing receptors as indicated by the binding-assay with rat serum proteins (Fig. 7) and by the low hemagglutination-inhibition activity of the purified glycoprotein (Table 2). This interaction was, however, not sufficient for agglutination of erythrocytes. This situation is reminiscent of influenza C virus, the purified glycoprotein (HEF) of which also has esterase and hemagglutination-inhibition but no hemagglutinating activity (Herrler *et al.*, 1988a). The purified hemagglutinin of influenza A viruses, on the other hand, has been shown to form rosettes which are able to agglutinate red blood cells (Laver and Valentine, 1969). The failure of purified HE protein to cause hemagglutination is not due to a lack of rosette formation (Fig. 6). The receptor-destroying activity of HE counteracts the receptor-binding activity. This is evident from the result of the solid phase binding assay. Binding of BCV-HE was found to be more efficient after inactivation of the esterase by DFP (Fig. 7). The binding assay has not been applied to HEV-HE, but because of the similarity of both HE proteins in other tests (see Figs. 1–5 and Tables 1 and 2) it is likely that the results obtained with BCV-HE are also valid for HEV-HE. DFP-treated HE was still unable to agglutinate red blood cells. There was, however, some interaction between erythrocytes and the purified coronavirus glycoprotein. Erythrocytes incubated with DFP-treated HE did not pour, when the microtiter plate was placed in a semivertical position (not shown), which is in contrast to control cells. The lack of HA activity in the purified HE protein might be due to a conformational change during the purification procedure, which affects the receptor-binding activity more than the receptor-destroying activity. Another possibility is that the individual glycoproteins have only a low affinity for Neu5,9Ac₂-containing receptors. In this case the multivalent attachment of HE-proteins present in the viral membrane may increase the binding activity and enable agglutination of erythrocytes. In any case, the purified acetylsterases of BCV and HEV are useful analytical tools to study the importance of Neu5,9Ac₂ in general, and specifically the cellular receptors for coronaviruses.

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