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Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses

(receptor-destroying enzyme/acetylesterase/hemagglutination/gangliosides)

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ABSTRACT **Human coronavirus OC43 and bovine coronavirus elute from agglutinated chicken erythrocytes when incubated at 37°C, suggesting the presence of a receptor-destroying enzyme. Moreover, bovine coronavirus exhibits an acetylesterase activity *in vitro* using bovine submaxillary mucin as substrate similar to the enzymatic activity found in influenza C viruses. Furthermore, pretreatment of erythrocytes with either influenza C virus or bovine coronavirus eliminates subsequent binding and agglutination by either coronaviruses or influenza C virus, whereas binding of influenza A virus remains intact. In addition, hemagglutination by coronaviruses can be inhibited by pretreatment of erythrocytes with *Arthrobacter ureafaciens* or *Clostridium perfringens* neuraminidase or by addition of sialic acid-containing gangliosides. These results suggest that, like influenza C viruses, human coronavirus OC43 and bovine coronavirus recognize O-acetylated sialic acid or a similar derivative as cell receptor.**

Coronaviruses have been associated with a variety of diseases in many different host species (1–3). Human coronavirus strain OC43 (HCV OC43) in general affects the respiratory tract (4), causing symptoms similar to those of rhinoviruses or influenza viruses, whereas bovine coronavirus (BCV) is the cause of enteric infections in cattle (5). Mouse hepatitis virus (MHV), a third virus that belongs to the same antigenic cluster of coronaviruses (6), may infect different organs, causing enteric, respiratory, and neurologic disease (7, 8). Recently, substantial sequence similarity was observed (W.L. and W.S., unpublished data) between an open reading frame encoded by mRNA2 of MHV strain A59 and the influenza C virus HE protein, which exhibits receptor-binding and receptor-destroying (esterase) activity (9, 10). Therefore we reexamined the possibility that coronaviruses might recognize cell receptors similar to those of influenza viruses. Earlier studies (11) revealed that treatment of erythrocytes with *Vibrio cholerae* neuraminidase (EC 3.2.1.18) did not remove cell receptors recognized by HCV OC38 and OC43, and no neuraminidase activity was found to be associated with HCV OC43 (12, 13). It was also reported that the hemagglutination pattern of avian infectious bronchitis virus and of a mouse enteric coronavirus was unstable at elevated temperature, suggesting the presence of a receptor-destroying enzyme (14, 15). In all of those instances, however, the receptor-binding and/or receptor-destroying activity was found to be different from those of influenza A virus. Recently, a plasma membrane protein with affinity for MHV strain A59 was described and its presence or absence was correlated with differences in virus susceptibility of target cells (16). However, the precise mechanism of interaction between the host cell protein and the virus was not elucidated.

Thus the published evidence does not necessarily exclude the possibility that coronaviruses recognize modified sialic acids as cell receptors, and we suggest in the present study that HCV OC43 and BCV bind to sialylated receptors similar to those recognized by influenza C viruses. Furthermore, we report that BCV possesses an esterase activity that may function as receptor-destroying enzyme.

MATERIALS AND METHODS

Viruses and Cells. HCV OC43 was obtained from the American Type Culture Collection (ATCC 759-VR). BCV was obtained from Duphar B. V. Weesp (Amsterdam). BCV was grown in Madin–Darby bovine kidney (MDBK) cells, concentrated by polyethylene glycol precipitation (17), and purified on a 20–60% sucrose step gradient as described (18). Influenza C/JHG/66 virus and influenza A/PR/8/34 virus were grown in embryonated eggs as described (19). Chicken, mouse, and rat erythrocytes in Alsever's solution were obtained from Pocono Rabbit Farm (Canadensis, PA).

Removal of Receptors. Five percent erythrocyte suspensions in phosphate-buffered saline (PBS) were incubated with influenza C virus, BCV, 200 milliunits of *Clostridium perfringens* neuraminidase per ml (type IV, Sigma; EC 3.2.1.18) or 200 milliunits of *Arthrobacter ureafaciens* neuraminidase per ml (Boehringer Mannheim; EC 3.2.1.18). All incubations were performed at pH 7.2 and 37°C. Incubation time was usually 1 hr, except when BCV was used, for which incubation time was 3 hr. After incubation, cells were washed four times with PBS and finally resuspended in the same buffer to 0.5% (vol/vol). Control incubations were done with PBS.

Binding Assays. Hemagglutination and hemagglutination-inhibition assays were done in V-shaped microtiter plates (Flow Laboratories) as described (20). Bovine brain gangliosides (type III) were obtained from Sigma.

Acetylesterase Assay. Virus preparations were suspended in 50 μl of PBS, mixed with 250 μl of bovine submaxillary mucin (type I, Sigma; 25 mg/ml), and incubated at 37°C. At different times the presence of acetate was determined by using a test kit (Boehringer Mannheim). A polyethylene glycol precipitate (17) from a culture supernatant of mock-infected MDBK cells was used as control.

RESULTS

Preliminary tests were performed to select erythrocytes that were agglutinated by all viruses used in the study. As described earlier, HCV OC43 and BCV were found to agglutinate mouse and rat erythrocytes (11, 21). Since avian erythrocytes were routinely used in the laboratory for agglutination by influenza A and C viruses we tested these cells

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Abbreviations: HCV OC43, human coronavirus OC43; BCV, bovine coronavirus; MHV, mouse hepatitis virus; MDBK, Madin–Darby bovine kidney.

and found that BCV and HCV OC43 also agglutinated these erythrocytes. Therefore all experiments were done with avian erythrocytes.

Hemagglutination titers were determined after incubation for 1 hr at 4°C. If incubation was continued at room temperature or at 37°C we observed elution of HCV OC43 and BCV, which started in wells containing the highest concentration of virus. This phenomenon has been described in earlier studies on HCV OC43 (11) and had been used for partial purification of this virus (22). Since elution is observed regularly with viruses containing a receptor-destroying enzyme (23–25), we proceeded to test BCV for a similar activity. Specifically, the sequence similarity between an open reading frame in the mRNA2 of MHV A59 and the influenza C virus HE protein (W.L. and W.S., unpublished data) led us to test for the presence of an influenza C virus-like activity. The influenza C virus receptor-destroying activity is a sialate *O*-acetylesterase (EC 3.1.1.53) (10), which can be measured *in vitro* by using synthetic (9) and natural (10) esterase substrates such as *p*-nitrophenylacetate and bovine submaxillary mucin, respectively. We tested for the presence of acetylesterase activity in BCV, using bovine submaxillary mucin as substrate (Fig. 1). Purified preparations of BCV and influenza C virus released comparable amounts of acetate over time. Control preparations of mock-infected MDBK cells or of influenza A virus did not hydrolyze the *O*-acetylated sialic acids in bovine submaxillary mucin. This experiment suggests the presence of an enzyme in BCV with specificities similar to those of the influenza C virus HE protein.

We then asked whether this esterase activity associated with BCV eliminates virus receptors similar to those destroyed by the influenza C virus HE protein. For further experiments we selected avian erythrocytes derived from chicken strain Rhode Island Red sex-linked chromosome X, which gave the most rapid elution patterns for HCV OC43 and BCV at 37°C. Suspensions of erythrocytes were incubated with concentrated BCV or influenza C/JHG/66 virus at 37°C. Cells then were washed four times with PBS, resuspended to a 0.5% (vol/vol) suspension, and used for hemagglutination assays. Cells showed a hemagglutination titer of ≈512 for all viruses tested when incubated with PBS (Fig. 2*A*). Treatment of erythrocytes with BCV resulted in the complete loss of agglutination by HCV OC43, BCV, and influenza C/JHG/66 virus, but no effect on agglutination by influenza A/PR/8/34 virus was observed (Fig. 2*B*). This

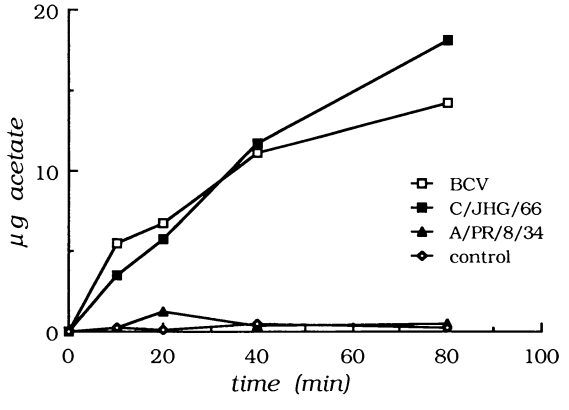

FIG. 1. Release of acetate from bovine submaxillary mucin by purified BCV and influenza C/JHG/66 virus (C/JHG/66). BCV (5 μg), C/JHG/66 (1.6 μg), and influenza A/PR/8/34 virus (5 μg) were incubated at 37°C with 25 mg of bovine submaxillary mucin in 300 μl of PBS for the times indicated. Acetate release was monitored by a test kit (Boehringer Mannheim). For the control, a supernatant of mock-infected cells was precipitated with 10% polyethylene glycol and NaCl (23.3 mg/ml) and used in the assay (20 μg).

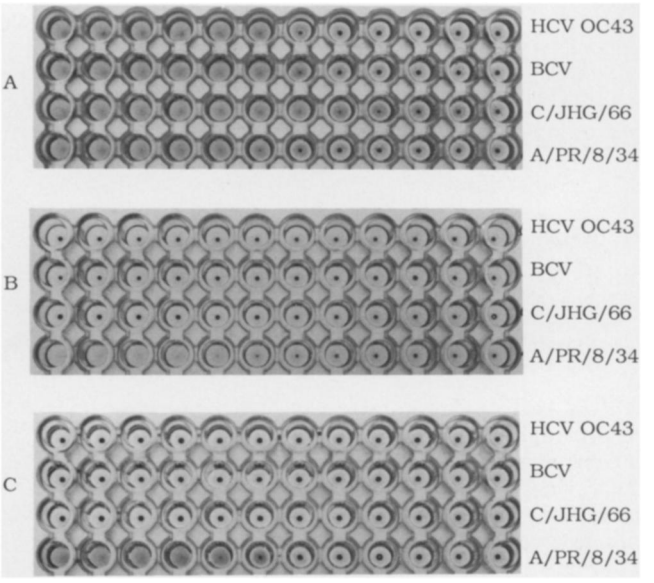

FIG. 2. Removal of cell receptors by treatment with BCV. Hemagglutination was performed at 4°C using chicken erythrocytes and at serial 1:2 dilutions (left to right) human coronavirus OC43, BCV, influenza C/JHG/66 virus, or influenza A/PR/8/34 virus. (*A*) Untreated erythrocytes. (*B*) One-half milliliter of erythrocytes (5%) was preincubated with purified BCV (420 μg) for 3 hr at 37°C, washed, resuspended to 0.5%, and used in the assay. (*C*) One-half milliliter of erythrocytes (5%) was pretreated for 1 hr at 37°C with concentrated influenza C/JHG/66 virus (250 μg), washed, resuspended, and used as in *B*.

suggested that the receptor-destroying activity of BCV affects viral cell receptors recognized by another coronavirus (HCV OC43) as well as by influenza C virus. In contrast, receptors for influenza A virus were not altered by the BCV enzyme. The analogous experiment using the influenza C virus receptor-destroying enzyme is shown in Fig. 2*C*. Treatment of erythrocytes with influenza C/JHG/66 virus not only removed the receptors for influenza C virus but also those for HCV OC43 and BCV. In contrast, hemagglutination by influenza A/PR/8/34 was essentially unchanged, confirming earlier reports (23, 24). Since the receptor determinants for influenza C viruses are 9-*O*-acetylated sialic acids (10, 26), we suggest that coronaviruses recognize similar structures on cell surfaces.

To confirm the presence of sialic acid as a receptor component for BCV and HCV OC43, erythrocytes were also treated with bacterial neuraminidases. Treatment with *A. ureafaciens* neuraminidase again resulted in a complete loss of agglutination by HCV OC43, BCV, and influenza C/JHG/66 virus (Fig. 3*B*). In addition, agglutination by influenza A/PR/8/34 virus was reduced by a factor of ≈32 due to the action of the neuraminidase. Treatment of erythrocytes with *C. perfringens* neuraminidase gave a similar pattern (Fig. 3*C*). Agglutination of treated cells was completely lost for HCV OC43 and influenza C/JHG/66 virus and was reduced for BCV and, as previously shown (27), influenza A/PR/8/34 virus. Since the latter neuraminidase removed receptors for HCV OC43 and influenza C/JHG/66 virus more efficiently than those for BCV, we suggest that there are strain-specific differences in the interaction between coronaviruses and cell receptors.

Agglutination of erythrocytes by coronaviruses was also inhibited in the presence of sialic acid-containing gangliosides. Eight hemagglutinin units of coronaviruses were mixed with gangliosides and inhibition occurred at concentrations that also inhibited hemagglutination by influenza A and C viruses (Table 1).

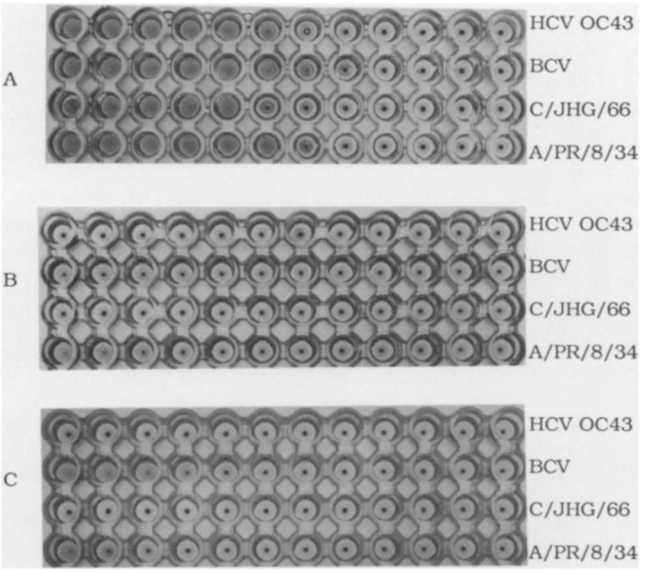

FIG. 3. Removal of coronavirus receptors by treatment of cells with bacterial neuraminidases. Hemagglutination was performed at 4°C using chicken erythrocytes and at serial 1:2 dilutions human coronavirus (OC43), BCV, influenza C/JHG/66 virus, or influenza A/PR/8/34 virus. (*A*) Untreated cells. (*B*) One milliliter of erythrocytes (5%) was treated with *A. ureafaciens* (200 milliunits/ml) for 1 hr at 37°C. Cells then were washed, resuspended, and used for hemagglutination. (*C*) As in *B* except for treatment using *C. perfringens* neuraminidase (200 milliunits/ml).

DISCUSSION

Results presented in this study suggest that BCV possesses an influenza C virus-like esterase activity. When purified BCV was incubated with bovine submaxillary mucin as substrate, acetate was released at a rate comparable to that obtained following treatment with purified influenza C virus. Since the acetylesterase of influenza C virus catalyzes removal of its viral cell receptors (10, 23–25), an analogous activity present in BCV may play a similar role as receptor-destroying enzyme. Whether this activity is coded for by the virus and whether one of the surface glycoproteins (6, 28, 29) possesses esterase activity remain to be determined by biochemical approaches.

In earlier studies the existence of a receptor-destroying activity had been postulated for avian infectious bronchitis virus (14) and a murine enteric coronavirus (15). In both cases elution of the coronavirus from agglutinated erythrocytes was reported, but the specificity of the proposed enzyme was not defined. Similar observations were reported for HCV OC43 and BCV. However, the unstable hemagglutination/hemadsorption pattern could not be linked to the presence of a specific receptor-destroying enzyme (11, 21, 22). For further studies we screened erythrocytes of different origin for hemagglutination by coronaviruses. In addition to mouse and rat erythrocytes we found that avian erythrocytes were agglutinated by both coronaviruses tested. Elution of BCV was observed when concentrated preparations of virus were used. However, the rate of elution of BCV and OC43 varied with different batches of avian erythrocytes. Similar differences in elution can be observed with influenza A and C viruses and may be associated with the age or the strain of the animal from which the erythrocytes are derived. To test the possibility that the BCV-associated acetylesterase is indeed a receptor-destroying enzyme, we selected erythrocytes that gave the most rapid elution pattern. These cells were incubated with BCV or influenza C virus and used for hemagglutination assays. Results obtained from these experiments suggest that the BCV acetylesterase is in fact a receptor-destroying enzyme. It removed receptors for BCV as well as those for HCV OC43 and influenza C virus but not those for influenza A viruses. Moreover, we conclude that BCV and HCV OC43 recognize receptors similar to those of influenza C virus, because the sialate *O*-acetylesterase of the latter virus removed receptors for coronaviruses as well. An involvement of neuraminic acids in binding of BCV and HCV OC43 to cell surfaces is also suggested, because sialic acid-containing gangliosides compete with coronavirus receptors at concentrations comparable to those necessary for inhibition of hemagglutination by influenza C virus. Furthermore, *A. ureafaciens* and *C. perfringens* neuraminidase, which both have broad specificities for sialic acid-containing substrates (30, 31), removed receptors for HCV OC43 and BCV. Since receptors for HCV OC43 are removed more efficiently than those for BCV, it is likely that there are quantitative or qualitative differences in coronavirus receptors on erythrocytes. Differences in receptor binding have also been reported for influenza A virus receptors (32, 33), where linkages of the sialic acid to other sugars and modifications of the sialic acids contribute to differences in virus–receptor interactions. In summary, the present data strongly suggest that modified sialic acid, possibly 9-*O*-acetyl-*N*-acetylneuraminic acid or a similar derivative, is a major receptor determinant for HCV OC43 and BCV.

It will be important to examine whether modified sialic acids are the exclusive determinants of coronavirus receptors or if viral binding *in vivo* involves additional receptors lacking sialic acid. Viral growth experiments involving the removal of sialic acid from permissive tissue culture cells may shed light on this question; also, these experiments may help to define the biological significance of sialic acid-containing receptors for the *in vivo* replication of coronaviruses. As mentioned earlier, the murine coronavirus MHV A59 has been shown to bind tightly to a plasma membrane protein (16). It is possible that this protein is a glycoprotein that presents sialic acid residues in optimal spatial conformation for specific binding to the coronavirus. Alternatively, this protein may interact with MHV through another mechanism not involving sialic acids, suggesting that, as is the case with other viruses (34), more than one cell receptor determinant may be recognized.

Future experiments must be directed at determining whether only some or all of the coronaviruses possess a receptor-destroying activity. Secondly, attempts must be made to further elucidate the specificity and fine structure of sialic acid-bearing host receptors. It is possible that different cellular proteins that can appropriately present sialic acids may function as coronavirus cell receptor. Finally, it will be of interest to determine whether the presence of receptor-binding and/or receptor-destroying activities of coronaviruses can be correlated with pathogenicity *in vivo*.

Table 1. Inhibition of hemagglutination by gangliosides

Virus	Concentration of gangliosides, μg/ml
Human coronavirus (HCV OC43)	98
Bovine coronavirus (BCV)	24
Influenza virus (C/JHG/66)	49
Influenza virus (A/PR/8/34)	390

Eight hemagglutinin units of each hemagglutinating virus were mixed with varying concentrations of gangliosides. Chicken erythrocytes were added to a final concentration of 0.25% (vol/vol). The end point refers to the minium concentration of gangliosides that completely inhibits hemagglutination.

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