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Neuraminidase Treatment of Avian Infectious Bronchitis Coronavirus Reveals a Hemagglutinating Activity That is Dependent on Sialic Acid-Containing Receptors on Erythrocytes

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The interaction of infectious bronchitis virus (IBV) with erythrocytes was analyzed. The binding activity of IBV was not sufficient to agglutinate chicken erythrocytes. However, it acquired hemagglutinating activity after treatment with neuraminidase to remove α2,3-linked *N*-acetylneuraminic acid from the surface of the virion. Pretreatment of erythrocytes with neuraminidase rendered the cells resistant to agglutination by IBV. Susceptibility to agglutination was restored by resialylation of asialo-erythrocytes to contain α2,3-linked sialic acid. These results indicate that IBV attaches to receptors on erythrocytes, the crucial determinant of which is sialic acid α2,3-linked to galactose. In contrast to other enveloped viruses with such a binding specificity (influenza viruses and paramyxoviruses) IBV lacks a receptor-destroying enzyme. © 1992 Academic Press, Inc.

There are great variations among members of the family Coronaviridae in their ability to agglutinate red blood cells. Some members such as bovine coronavirus (BCV), human coronavirus (HCV-OC43), and hemagglutinating encephalomyelitis virus (HEV) are quite potent hemagglutinating agents. Studies with BCV have shown that the S-protein is the major hemagglutinin of these viruses recognizing *N*-acetyl-9-*O*-acetylneuraminic acid ($Neu5,9Ac_2$) as a receptor determinant on cells (*1*). These coronaviruses possess another surface glycoprotein, HE, which is a less efficient hemagglutinin and functions as a receptor-destroying enzyme (*1–4*). It releases the acetyl group from position C-9 of $Neu5,9Ac_2$ similar to the HEF–protein of influenza C virus (*3, 5, 6*). Several other coronaviruses, e.g., porcine transmissible gastroenteritis virus (TGEV), feline infectious peritonitis virus (FIPV), and avian infectious bronchitis virus (IBV) lack a receptor-destroying enzyme and their ability to agglutinate red blood cells is very poor (*7–9*). The receptor determinant recognized by this group of viruses has not previously been identified.

IBV has been reported to acquire hemagglutinating activity after treatment with bacterial phosphoplipase C (*7*). However, this effect was observed only when a crude enzyme preparation was used. Therefore, the induction of the hemagglutinating activity of IBV may have been due to a contaminating enzyme rather than to phospholipase C. As we were unable to unmask the hemagglutinating activity of IBV by treatment of virus with commercially available phospholipase C, we analyzed whether neuraminidase was effective in this respect. As shown in Table 1, while untreated virus was unable to agglutinate chicken erythrocytes, high hemagglutination titers were obtained after treatment of virions with neuraminidase from *Vibrio cholerae*. The same effect was observed after incubation with the purified HN protein from Newcastle disease virus, which also has neuraminidase activity. This enzyme preferentially cleaves sialic acid in an α2,3 linkage to galactose, but is rather inefficient in releasing sialic acid from an α2,6 linkage (*10*). This result indicated that removal of α2,3-linked sialic acid from the viral surface was required for IBV to agglutinate red blood cells.

We reasoned that a possible explanation for the inhibitory effect of the surface-bound sialic acid of IBV might be that the inhibitory sugar is similar or identical to the cellular receptor determinant recognized by the virus. In this case the receptor determinant on the virion surface would compete with the receptor determinant on the cell surface for the receptor-binding site of the virus. As a consequence, the efficiency of the binding to erythrocytes would be reduced and explain the lack of hemagglutinating activity. This model implies that IBV uses α2,3-linked sialic acid as a receptor determinant for attachment to cells. To test this possibility, erythrocytes were treated with neuraminidase and analyzed for agglutination by neuraminidase-treated IBV. As shown in Table 2, the neuraminidases from *Vibrio cholerae* as well as from Newcastle disease virus rendered the cells resistant to agglutination by IBV. This result indicated that α2,3-linked sialic acid was a cru-

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TABLE 1

INDUCTION OF THE HEMAGGLUTINATING ACTIVITY OF IBV BY NEURAMINIDASE TREATMENT

Pretreatment of virus	Hemagglutinating activity (HA units/ml)
None	<2
VC-neuraminidase	256
NDV-neuraminidase	256

Note. IBV was grown in embryonated eggs as described (*9*). Following sedimentation of the virus by ultracentrifugation for 90 min at 54,000*g*, the virus was suspended in PBS. After incubation with neuraminidase from *Vibrio cholerae* (VC; 200 mU/ml) or Newcastle disease virus (NDV; 3.5 U/ml), IBV was purified by sucrose gradient centrifugation (*4*). The final viral pellet was suspended in 100 μl of PBS and used for hemagglutination assays with chicken erythrocytes (*6*). The bacterial neuraminidase was purchased from Behring-Werke (Marburg, Germany). The viral enzyme was isolated by detergent (octylglucoside) treatment of purified egg-grown NDV followed by sucrose gradient centrifugation (*4*). Fractions containing neuraminidase were collected and dialyzed to remove sucrose and detergent.

cial component of the erythrocyte receptors for IBV. To confirm this finding, neuraminidase-treated cells were resialylated by incubation with Galβ1,3GalNAc α2,3sialyltransferase, and CMP-Neu5Ac. Following attachment of sialic acid to the surface of erythrocytes in an α2,3 linkage, the cells were agglutinated by both NDV and IBV (Table 3). The former virus is known to recognize α2,3-linked sialic acid as a receptor determinant for attachment to cells (*11*). Our results indicated that IBV had the same requirements for agglutination of erythrocytes as NDV.

All viruses that have been reported so far to use sialic acid for attachment to cells have found a way to keep the viral surface free of the receptor determinant.

TABLE 2

INACTIVATION OF ERYTHROCYTE RECEPTORS FOR IBV BY NEURAMINIDASE

Pretreatment of cells	Hemagglutinating activity of neuraminidase-treated IBV (HA units/ml)
None	512
VC-neuraminidase	<2
NDV-neuraminidase	<2

Note. Erythrocytes from 1-day-old chickens (300 μl, 10% suspension in PBS) were incubated in the absence or presence of neuraminidase from *Vibrio cholerae* (VC, 23 mU) or Newcastle disease virus (NDV, 350 mU) for 90 min at 37°C. Cells were washed and used to determine the hemagglutination titer of IBV which had been pretreated with neuraminidase from NDV to induce the hemagglutinating activity (see Table 1).

TABLE 3

RESTORATION OF THE RECEPTORS FOR IBV BY RESIALYLATION OF ASIALO-ERYTHROCYTES

Erythrocytes	Hemagglutinating activity (HA-units/ml) IBV	NDV
Control	512	256
Asialo	<2	<2
Resialylated, Galα2,3Neu5Ac	256	64

Note. A 10% suspension of chicken erythrocytes was incubated with neuraminidase from *Vibrio cholerae* (40 mU/ml) for 30 min at 37°C. Asialo cells were washed and suspended in PBS to a final concentration of 27.5% in a total volume of 52 μl. Resialylation was accomplished by incubation with sialyltransferase (8 mU; from Boehringer-Mannheim, Germany) and 250 nmol of CMP-activated *N*-acetylneuraminic acid (Neu5Ac). After 2 hr at 37°C, cells were washed and used as a 1% suspension (in PBS) to determine the hemagglutinating activity of IBV and Newcastle disease virus (NDV). The HA activity of IBV had been induced by pretreatment with NDV-neuraminidase (see Table 1). In order to protect the cells from lysis by the detergent present in the sialyltransferase preparation, fixed erythrocytes were used for this experiment (0.1% glutaraldehyde, 60 min).

Reoviruses, polyomavirus, and encephalomyocarditis virus are nonenveloped viruses without glycoconjugates containing sialic acid. Enveloped viruses contain both glycoproteins and glycolipids. However, some of these viruses possess a receptor-destroying enzyme, which is responsible for the lack of sialic acid on the surface (*10*): a neuraminidase in the case of influenza A and B viruses and paramyxoviruses, an acetylesterase in the case of influenza C virus and several coronaviruses. IBV is the first enveloped virus reported to recognize α2,3-linked sialic acid which lacks a receptor-destroying enzyme.

There are several ways to explain the masking effect of sialic acid on the hemagglutinating activity of IBV: (i) The inhibitory sialic acid of a virion might interact with the viral binding protein of another virus particle, resulting in the formation of virus aggregates; (ii) The sialic acid and the viral attachment protein may be part of the same virion, e.g., the interaction between neighboring S-proteins; (iii) The sialic acid molecule may be part of a cellular component, which is bound by the virion and acts as an inhibitor of the viral hemagglutinating activity. The first possibility can be excluded because there is no indication by electron microscopic analysis or by gradient centrifugation that coronaviruses without receptor-destroying enzyme have a greater tendency to form aggregates than do coronaviruses with receptor-

destroying enzyme. We were also unable to detect any difference in this respect between untreated and neuraminidase-treated IBV (not shown). Future work has to show whether the inhibition is due to the interaction between viral components of the same virus particle or due to the interaction between the viral binding protein and a cellular component containing sialic acid.

Our findings raise the question about the importance of a viral binding activity that is masked by the receptor determinant present on the virion surface. The lack of hemagglutinating activity does not imply that IBV is unable to attach to the receptors on erythrocytes. Conditions for the agglutination of cells by a virus, (i.e., the bridging of many erythrocytes) are more stringent than those for the attachment of a single virion to the surface of a single cell. Thus, despite the lack of hemagglutinating activity, untreated IBV might be able to use the sialic acid-binding activity for attachment to cells and for initiation of an infection. Alternatively, the sialic acid-binding activity may mediate a primary attachment, facilitating the interaction with a second type of receptor which might be necessary for a closer contact between virus and cell and/or for fusion between viral and cellular membranes. The latter possibility is especially intriguing, because it combines two receptor-recognition strategies that have been reported for the MHV–BCV serogroup of coronaviruses. In the case of BCV, 9-*O*-acetylated sialic acid has been shown to function as a receptor determinant for the initiation of infection (*12*). For strain A59 of MHV, a murine member of the carcinoembryonic antigen family of glycoproteins has been identified as a receptor (*13, 14*). It should be noted that the sialic acid binding activity of IBV has been analyzed so far only with erythrocytes. Future work with cultured cells will be necessary to determine whether sialic acid is involved in the initiation of infection. These studies will be performed with transmissible gastroenteritis virus (TGEV), another coronavirus lacking a receptor-destroying enzyme. Like IBV, TGEV is a poor hemagglutinin and from our preliminary experiments we expect that both viruses have the same agglutination characteristics. However, TGEV grows more readily in cell culture than IBV and, therefore, is more suitable for analyzing the role of sialic acid in virus infection.

The family *Coronaviridae* comprises members with a receptor-destroying enzyme (e.g., BCV, HEV, and HCV–OC43) and members that lack such an enzyme (e.g., IBV, TGEV, and FIPV). The former group of viruses are known to utilize 9-*O*-acetylated sialic acid present on the surface of erythrocytes for the agglutination of cells (*3, 6*). Our results obtained with IBV demonstrate that a coronavirus, which lacks a receptor-destroying enzyme, can also recognize sialic acid though of a different subtype (Neu5Ac versus Neu5,9Ac_2). Now we have the unique situation that viruses of the same family recognize a common sugar as receptor determinant for attachment to cells, but only some members of the family contain a receptor-destroying enzyme. Thus, either some coronaviruses have acquired the corresponding gene or the others have lost it during evolution. It has been speculated that coronaviruses such as BCV have acquired the esterase gene by a nonhomologous recombination between an ancestral coronavirus and influenza C virus (*15*). Certainly the family *Coronaviridae* is a promising model for evolutionary studies of the acquisition or loss of a receptor-destroying enzyme by viruses.

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