The S Protein of Bovine Coronavirus Is a Hemagglutinin Recognizing 9-O-Acetylated Sialic Acid as a Receptor Determinant

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The S protein of bovine coronavirus (BCV) has been isolated from the viral membrane and purified by gradient centrifugation. Purified S protein was identified as a viral hemagglutinin. Inactivation of the cellular receptors by sialate 9-O-acetylerase and generation of receptors by sialylation of erythrocytes with N-acetyl-9-O-acetylenuraminic acid (Neu5,9Ac2) indicate that S protein recognizes 9-O-acetylated sialic acid as a receptor determinant as has been shown previously for intact virions. The second glycoprotein of BCV, HE, which has been thought previously to be responsible for the hemagglutinating activity of BCV, is a less efficient hemagglutinin; it agglutinates mouse and rat erythrocytes, but in contrast to S protein, it is unable to agglutinate chicken erythrocytes, which contain a lower level of Neu5,9Ac2 on their surface. S protein is proposed to be responsible for the primary attachment of virus to cell surface receptors. The potential of S protein as a probe for the detection of Neu5,9Ac2-containing glycoconjugates is demonstrated.

Many viruses are able to agglutinate erythrocytes. The interactions of the viral hemagglutinins with the surface of erythrocytes have served as a useful model system for analysis of the surface receptors for several viruses, e.g., influenza viruses, paramyxoviruses, and encephalomyocarditis virus (16). Some strains of coronaviruses are also known to have hemagglutinating activity. Strains which contain only two glycoproteins, M and S, are devoid of hemagglutinating activity or have only a low level of activity. In the case of infectious bronchitis virus, this low hemagglutinating activity is a function of the S protein (1). The nature of the receptor on the surface of the erythrocytes is not known. Several strains of coronaviruses are quite potent hemagglutinating agents: bovine coronavirus (BCV), hemagglutinating encephalomyelitis virus (HEV), human coronavirus OC-43, turkey coronavirus, and some murine coronaviruses. Characteristic for this group of viruses is the presence of an additional glycoprotein, HE, which has a structural and functional relationship to the glycoprotein HEF of influenza C virus. Both of these glycoproteins can act as receptor-destroying enzymes by virtue of a sialate 9-O-acetylerase activity (6, 10, 21, 24, 25, 26). The influenza C glycoprotein is also a hemagglutinin (5, 6, 24) which recognizes N-acetyl-9-O-acetylenuraminic acid (Neu5,9Ac2) as a receptor determinant for attachment to cells (19). Hemagglutination by BCV, HEV, and OC-43 is also dependent on Neu5,9Ac2-containing receptors (20, 26), though they differ from influenza C virus in binding efficiency (20). The hemagglutinating activity of BCV has been assigned to the HE protein. The evidence is based on the finding that treatment of BCV with bromelin results in the proteolysis of S protein, whereas HE as well as the hemagglutinating activity of the virus particles remain unaffected (12). In addition, some monoclonal antibodies directed against HE prevent BCV from agglutinating erythrocytes (17).

Here we report the isolation of the S protein of BCV. The purified glycoprotein was able to agglutinate erythrocytes recognizing Neu5,9Ac2 as a receptor determinant. From our results, it is evident that BCV has two hemagglutinins with an affinity for 9-O-acetylated sialic acid: the S protein and the HE protein. The S protein, which has no esterase activity, is much more efficient than the HE protein in agglutinating erythrocytes.

(This work was conducted by Beate Schultze in partial fulfillment of the requirements for the Dr.rer.physiol. degree from the Institut für Virologie.)

MATERIALS AND METHODS

Viruses and cells. Strain L-9 of BCV was obtained from R. Rott (Giessen, Germany). MDCK I cells, a subline of Madin-Darby canine kidney cells, were maintained as described previously (6).

Growth and purification of virus. BCV was grown in MDCK I cells as reported recently (20). Virus was harvested from the supernatant of infected MDCK I cells 48 h postinfection. After clarification of the medium by low-speed centrifugation (2,000 × g, 10 min), virus was sedimented by ultracentrifugation at 112,000 × g for 1 h. The pellet was resuspended in phosphate-buffered saline (PBS) and layered on a sucrose gradient (5 to 50% [wt/wt] in PBS). After centrifugation at 148,000 × g 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 purification of the viral glycoproteins.

Isolation and purification of viral glycoproteins. Viral glycoproteins were isolated by treatment with n-octylglucopyranoside and purified by sucrose-gradient centrifugation as described recently (21).

Hemagglutination assay. Hemagglutination assays were performed as described previously (9), using either chicken or rat erythrocytes. The hemagglutination titer (HA titer) indicates the reciprocal value of the maximum dilution that caused complete agglutination.

SDS-polyacrylamide gel electrophoresis. Analysis of proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described previously (6).

Assay for acetylerase activity. The esterase activity of
purified virions or proteins was determined by incubation with 200 μg of p-nitrophenyl acetate in 1 ml of PBS at room temperature. The substrate was dissolved in a 1/50 volume of ethanol. Using a kinetics program, the release of acetate was monitored by determining the optical density at 405 nm for 10 min. The amount of esterase which cleaves 1 μmol of p-nitrophenyl acetate in 1 min at room temperature was defined as 1 U of enzyme.

Electron microscopy. For negative staining, samples were applied to copper grids, stained with phosphotungstate, and examined in a Zeiss Electron-Microskop 110.

Incubation of erythrocytes with acetylesterase. Samples containing 200 μl of a 10% suspension of chicken erythrocytes were incubated with 100 μl of the gradient fraction containing purified HE protein. Prior to use, the acetyl-esterase was dialyzed to remove octylglucoside. After incubation for 2 h at 37°C, the erythrocytes 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 and purified S protein.

Resialylation of erythrocytes. Synthesis of Neu5,9Ac2 and CMP-Neu5,9Ac2 and preparation of sia1yltransferase have been described previously (20). Cells were sia1ylated essentially as described by Paulson and Rogers (18), using rat liver Gaβ1,4GlcNAc α-2,6-sialyltransferase. A 10% suspension of human erythrocytes was prepared in a buffer containing 0.1 M NaCl, 20 mM CaCl2, and 50 mM morpholinoethane-sulfonic acid (MES), pH 6.5. Samples containing 0.1 ml of the erythrocyte suspension were incubated with 50 μM of neuraminidase from Vibrio cholerae. After 30 min at 37°C, the cells were washed twice with PBS and resuspended with 50 μl of PBS–1% bovine serum albumin. Following addition of 2 μl of Gaβ1,4GlcNAc α-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 and S protein.

Binding assay for the detection of Neu5,9Ac2-containing glycoproteins. Rat serum proteins were separated by SDS-polyacrylamide gel electrophoresis under nonreducing conditions and blotted on a nitrocellulose sheet. The electrophoresis and blotting were performed as described by Kyhse-Andersen (30). The system was modified by lowering the pH values of the two buffers at the anode site to 9.0 and 7.4, respectively. Nonspecific binding sites were blocked with 10% nonfat dry milk in PBS overnight at 4°C. The nitrocellulose strips were washed three times for 5 min with PBS–0.1% Tween and incubated for 1 h at 37°C with PBS, acetylesterase of BCV (200 μU), or 0.1 N NaOH. All subsequent steps were performed at 4°C. Following three washes with PBS-Tween, the nitrocellulose was incubated for 1 h with BCV, S protein, or dioisopropylfluorophosphate (DFP)-treated HE protein. After being washed with PBS-Tween, strips were incubated with rabbit antiserum directed against BCV (1:1,000 dilution). The nitrocellulose was then washed three times and then incubated for 1 h with horseradish peroxidase complexes (1 h), and washed again. Bound BCV, HE protein, or S protein was detected by incubation of the nitrocellulose with PBS, 4-chloro-1-naphthol, and H2O2 (500:100:1).

Bromelain treatment. BCV was treated with bromelain as described by Comps et al. (3) for influenza viruses. Purified virus suspended in TE buffer (0.1 M Tris-HCl, 1 mM EDTA, pH 7.2) was incubated at 37°C in the presence of bromelain and dithiothreitol (final concentrations, 1.3 mg/ml and 5 mM, respectively). After 30 min, the virus was sedimented by centrifugation for 30 min at 30,000 rpm in a TLA 100.3 rotor. The virus pellet was suspended in PBS, and aliquots were used to analyze the proteins by SDS-polyacrylamide gel electrophoresis, to determine the HA titer, and to measure the esterase activity.

RESULTS

Purified S protein is a hemagglutinin. In an attempt to isolate and characterize the acetyl-esterase of BCV and HEV, the viral glycoproteins were released from the membranes by treatment of purified virions with octylglucoside and centrifuged through a sucrose gradient. By using this method, the esterase of BCV was obtained in purified form (21). While HE was recovered from fraction 6, the second glycoprotein, S, was detected in fraction 3. Figure 1 shows an analysis of both fractions by SDS-polyacrylamide gel electrophoresis. In each fraction, only one protein is visible, with no indications of any cross-contamination. HE shows the characteristic behavior of a disulfide-linked dimer of about 130 kDa, which upon reduction migrates as a 65-kDa protein. S protein is unaffected by the treatment with reducing agents. The sucrose gradient fractions were analyzed for esterase activity and, following removal of the detergent by dialysis, for hemagglutinating activity. As shown in Fig. 2, the peak of the esterase activity was found in fraction 6, which contained HE (Fig. 1). Analysis of the gradient fractions for hemagglutinating activity revealed a minor peak in the top fractions (fractions 8 and 9) containing the viral lipids. From the lower part of the gradient, only fraction 3 was able to agglutinate chicken erythrocytes, accounting for more than 99% of the hemagglutinating activity. The fraction containing the esterase activity was completely devoid of hemagglutinating activity. This result indicates that S protein may function as a hemagglutinin.

Agglutination of erythrocytes requires binding of the hemagglutinating agent to the surface of at least two cells. In the case of virus particles, multiple binding sites can be established by the attachment of proteins present on the viral surface in a high copy number. Purified membrane proteins tend to form rosettes after removal of the detergent. Hemagglutinin molecules present in such rosettes have the potential for
multivalent binding and may be able to agglutinate erythrocytes as has been shown for the HA protein of influenza A virus (15). Many rosettes were also detected when the gradient fraction containing S protein was analyzed by electron microscopy (Fig. 3).

S protein recognizes Neu5,9Ac2 as a receptor determinant. The result from Fig. 2 raised the question of whether the hemagglutination caused by purified S protein was related to the agglutinating activity of intact virions. Therefore, BCV and purified S protein were analyzed for their ability to agglutinate erythrocytes from different sources. As shown in Table 1, no difference in agglutination behavior was observed. With use of erythrocytes from an adult chicken, high HA titers were obtained with both intact virions and purified S protein. On the other hand, erythrocytes from a 1-day-old chicken and human erythrocytes were resistant to agglutination by both agents. This result indicates that purified S protein may attach to receptors on erythrocytes similar to those recognized by intact particles of BCV.

BCV is known to require Neu5,9Ac2-containing receptors for agglutination of erythrocytes (20, 26). The importance of Neu5,9Ac2 for the agglutination of erythrocytes by isolated S protein was analyzed by pretreating chicken erythrocytes with sialate 9-O-acetyleraserase. Purified HE protein from BCV was used for this purpose. Solubilized HE retains the esterase activity. However, despite the formation of rosettes, the purified protein lacks hemagglutinating activity (21). Therefore, it does not interfere in subsequent hemagglutination assays. Incubation with acetylesterase was found to render the erythrocytes resistant to agglutination by both BCV and purified S protein (data not shown). This result indicates that Neu5,9Ac2 is an essential component of the receptors not only for BCV but also for the isolated S protein.

Table 1. Agglutination of erythrocytes by BCV and purified S protein of BCV

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>HA titer (HA units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S protein of BCV</td>
</tr>
<tr>
<td>Chicken</td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>1.024</td>
</tr>
<tr>
<td>1 day old</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Human</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

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Resialylation of erythrocytes with naturally occurring
sialic acids has been used to characterize the receptors for several hemagglutinating viruses. The same approach was applied to characterize the receptors for the S protein. Human asialo erythrocytes were modified to contain either Neu5Ac or Neu5,9Ac₂ on the cell surface. As shown in Table 2, both BCV and purified S protein were able to agglutinate the cells following resialylation with Neu5,9Ac₂. On the other hand, erythrocytes containing Neu5Ac were resistant to the actions of both agents. These results demonstrate that S protein has the same binding characteristics as intact virions and therefore is a true hemagglutinin of BCV.

S protein is a more efficient hemagglutinin than is HE. There is some discrepancy between our finding and the report by King et al. (12) which correlated the hemagglutinating activity of BCV with the HE protein. The evidence of these authors is based on the analysis of virus treated with bromelain. This protease removed the S protein from the virions, whereas the HE protein as well as the hemagglutinating activity were found to be unaffected. Reproducing these experiments, we confirmed that bromelain selectively removes the S protein from BCV (Fig. 4). The protease has no effect on HE, as shown not only by the protein pattern but also by comparison of the esterase activities of treated and untreated virus (Table 3). For hemagglutination titration, King and coworkers had used mouse erythrocytes, which are known to contain a high level of Neu5,9Ac₂, accounting for more than 50% of the total sialic acid on the cell surface (22). For convenience, we used rat erythrocytes, which contain similar levels of 9-0-acetylated sialic acid (22). As shown in Table 3, rat erythrocytes were indeed agglutinated to the same titer by both control virus and bromelain-treated virus. However, with use of chicken erythrocytes, HA titers could be determined only for control virus. After treatment with bromelain, BCV was unable to agglutinate chicken erythrocytes (Table 3), which have been shown to contain a lower level of 9-0-acetylated sialic acid than do mouse and rat erythrocytes (8). This result indicates that both S and HE proteins have hemagglutinating activity; S is more powerful hemagglutinin than HE, because it can agglutinate cells with a lower content of Neu5,9Ac₂.

We reexamined the hemagglutinating activity of gradient-purified HE protein by using rat erythrocytes rather than chicken cells. As shown in Table 4, HE was indeed able to agglutinate rat erythrocytes. The HA titer determined for the undiluted HE fraction was comparable to the titer obtained by a 1:100 dilution of the S-containing fraction. As S and HE proteins are present in comparable amounts in the original gradient fractions (Fig. 1, lanes b, c, e, and f), the result from Table 4 confirms that S protein is more efficient than HE protein in agglutinating rat erythrocytes. We also analyzed whether S and HE proteins recognize the same type of receptors on rat erythrocytes. After pretreatment of the cells with purified acetyl esterase, the HA titer was reduced by more than 90% for BCV as well as for S and HE proteins. The hemagglutinating activity of S protein appeared to be somewhat more sensitive to esterase treatment than that of HE protein. This result indicates that both S and HE proteins use 9-0-acetylated sialic acid as the major receptor determinant for agglutination of erythrocytes. Subtle differences in the receptor specificity cannot be excluded at the present time.

S protein is a probe for detection of Neu5,9Ac₂. Because of its reactivity, S protein might be a useful analytical tool for detection of Neu5,9Ac₂ on glycoconjugates or on cell surfaces. To test this possibility, a solid-phase binding assay has been developed in which test proteins are blotted to nitrocellulose. Rat serum proteins are used because they are known to contain potent hemagglutination inhibitors for influenza C virus as well as for BCV and HEV (20, 23). This characteristic indicates that they are a rich source of 9-0-acetylated sialic acid, which has been proven by chemical

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**TABLE 2. Generation of receptors for BCV and S protein of BCV by resialylation of human erythrocytes**

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>Sialic acid attached</th>
<th>HA titer (HA units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S protein of BCV</td>
</tr>
<tr>
<td>Untreated</td>
<td>None</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Asialo</td>
<td>Neu5Ac</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>Neu5,9Ac₂</td>
<td>384</td>
</tr>
</tbody>
</table>

**TABLE 3. Effect of bromelain on the hemagglutinating and esterase activities of BCV**

<table>
<thead>
<tr>
<th>Virus</th>
<th>HA titer (HA units/ml) with chicken erythrocytes*</th>
<th>Esterase activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>2,048</td>
<td>23.2</td>
</tr>
<tr>
<td>Bromelain treated</td>
<td>&lt;2</td>
<td>22.6</td>
</tr>
</tbody>
</table>

* The HA titer with rat erythrocytes for both untreated and bromelain-treated virus was 1,024 HA units per ml.

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**FIG. 4. Effect of bromelain on the polypeptide pattern of BCV.** Control virus (U) and bromelain-treated virus (B) were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions and stained with Coomassie brilliant blue. Only the relevant portion of the gel is shown.

**TABLE 4. Comparison of the ability of BCV and its purified glycoproteins (S and HE) to agglutinate rat erythrocytes before and after treatment with purified acetyl esterase from BCV**

<table>
<thead>
<tr>
<th>Cells</th>
<th>HA titer (HA units/ml)</th>
<th>S (1:100)*</th>
<th>HE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,024</td>
<td>128</td>
<td>64</td>
</tr>
<tr>
<td>Esterase treated</td>
<td>32</td>
<td>&lt;2</td>
<td>4</td>
</tr>
</tbody>
</table>

* The gradient fraction containing S protein was diluted 1:100 before determination of the HA titer; the fraction containing HE was used undiluted.

* A 10% suspension of rat erythrocytes (200 μl) was incubated for 1 h at 37°C in the presence of 400 mU of purified acetyl esterase from BCV.
analysis in the case of rat α1-macroglobulin (7). Following SDS-polyacrylamide gel electrophoresis of rat serum, the proteins were blotted to nitrocellulose. BCV and purified S protein were analyzed for their ability to recognize any of the sera samples. As shown in Fig. 5, in both cases, binding to three major bands could be demonstrated (lanes a). A few additional bands were faintly stained. Purified HE was unable to detect any of the rat serum proteins under these conditions (not shown). However, if the esterase was inactivated by pretreatment with DFP, the same staining pattern was obtained as with BCV and S protein (Fig. 5C, lane a). To determine whether the attachment of all three agents was mediated by Neu5,9Ac2, the serum proteins blotted to nitrocellulose were incubated with either acetyl-esterase or sodium hydroxide. Under both conditions, the 9-O-acetyl residues are released from Neu5,9Ac2. Following this treatment, no binding of BCV, S protein, or DFP-treated HE protein to any of the rat proteins was detectable (lanes b and c). The result from Fig. 5 indicates that the S protein of BCV is a powerful tool for the detection of Neu5,9Ac2 on glycoconjugates.

DISCUSSION

We have presented evidence that the S protein of BCV is a viral hemagglutinin. The hemagglutinating activity can be demonstrated with purified glycoprotein. The interaction between S protein and erythrocytes has the same characteristics as the hemagglutinating activity of intact virus particles, as shown by (i) the type of erythrocytes that are sensitive or resistant to agglutination (Table 1), (ii) the inactivation of the receptors by treatment with acetyesterase (Table 4), and (iii) the generation of receptors by sialylation of erythrocytes with Neu5,9Ac2 (Table 2). These data indicate that the hemagglutinating activity of purified S protein is not an artifact. S protein is a genuine hemagglutinin of BCV that recognizes Neu5,9Ac2-containing receptors. Up to now, hemagglutinating activity has been correlated with the HE protein. The agglutinating activity of HE, however, is restricted to mouse and rat erythrocytes, which are very rich in Neu5,9Ac2. Adult chicken erythrocytes, which contain less 9-O-acetylated sialic acid on their surface, are agglutinated only by S protein, not by HE protein. Thus, among the two hemagglutinins of BCV, S and HE, the former is more powerful because it requires a lower amount of the receptor determinant for the hemagglutination reaction. The ability to agglutinate erythrocytes is not restricted to the S protein of BCV. HEV, a serologically related porcine coronavirus, has been shown recently to use Neu5,9Ac2 as a receptor determinant for attachment to cells (20). The surface proteins of this virus have activities similar to those of the corresponding proteins of BCV. Purified HE was found to have esterase activity but was unable to agglutinate chicken erythrocytes (21). The S protein from HEV, however, is a very efficient hemagglutinin (unpublished results). The same appears to be true for human coronavirus OC-43, which was reported many years ago to be affected by bromelain in such a way that the spike protein is lost together with the ability of the virus to agglutinate chicken erythrocytes (11). Because of the efficiency of S protein in recognizing Neu5,9Ac2-containing receptors, it is reasonable to assume that the primary attachment of these coronaviruses is mediated by S protein rather than by HE. This conclusion is in accord with the electron microscopic observation of the viral glycoproteins. They are visible as a double fringe of projections on the virion surface (4). HE protein, which is smaller in size, forms the inner layer of projections. The peplomers, which are characteristic of coronaviruses, form the outer layer of spikes and are made up of S protein. Thus, whenever a virus particle approaches a cell, the cellular receptors will first encounter S protein. For coronaviruses that lack an HE protein, such as avian infectious bronchitis virus, it has been shown that S is the attachment protein (1). From the results presented here, we propose that attachment of all coronaviruses to cell surfaces is mediated by S protein irrespective of the type of receptors recognized.

With respect to the distribution of activities among the glycoproteins, there is some similarity between BCV and influenza A viruses. The influenza virus hemagglutinin has receptor-binding and fusion activity just like the S protein of coronaviruses. The second glycoprotein, a neuraminidase, has receptor-destroying activity and is therefore comparable to HE. There are different subtypes of influenza neuraminidases, and most of them lack hemagglutinating activity. The purified neuraminidase of the N9 subtype, however, is a very potent hemagglutinin (14). The binding activity of this neuraminidase appears not to be essential for the attachment of influenza virus to the cell surface. In the case of coronaviruses, we cannot exclude the possibility that the HE protein has a supporting function in the attachment of virions to cell surfaces. The fact that coronaviruses with a strong hemagglutinating activity such as BCV, HEV, and human coronavirus OC-43 have an HE protein might be interpreted in this way. However, it is also possible that the differences in the hemagglutination efficiency reflect differences in the binding activity of S protein. In this case, viruses with a strong hemagglutinating activity may require the HE protein as a receptor-destroying enzyme. Using Neu5,9Ac2 as a receptor determinant, these viruses will attach not only to surface receptors but also to glycoconjugates which will not allow the initiation of an infection. The receptor-destroying enzyme can inactivate such false receptors and therefore raises the chances of coronaviruses infecting their target cells.

The importance of 9-O-acetylated sialic acids is not restricted to their function as receptor determinants for virus attachment. They play a role also as developmental markers.
on cells (8) and may be involved in tumor antigens (2). Recently, influenza C virus has been used as a specific probe to detect Neu5,9Ac₂. In the future, it may be preferable to use the S protein of BCV for such purposes. In contrast to the HEF protein of influenza C virus and the HE protein of coronaviruses, the S protein retains its activity after isolation from the viral membrane and can therefore be used as a purified protein. Another advantage of the S protein is its lack of an acetyl esterase activity which counteracts the binding activity. Consequently, one can avoid handling of toxic inhibitors such as DFP, to inactivate the esterase. Therefore, the S protein of BCV and related coronaviruses should be a useful tool for the analysis of 9-O-acetylated sialic acids.

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