A Synthetic Sialic Acid Analogue Is Recognized by Influenza C Virus as a Receptor Determinant but Is Resistant to the Receptor-destroying Enzyme*

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Synthetic sialic acid analogues varying in the substituents at position C-9 were analyzed for their ability to replace the natural receptor determinant for influenza C virus, N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac2). By incubation of erythrocytes with sialyltransferase and the CMP-activated analogues, the cell surface was modified to contain sialic acid with one of the following C-9 substituents: an azido, an amino, an acethalamido, or a hexanoylamido group. Among these, only 9-acetamido-N-acetylneuraminic acid (9-acetamido-Neu5Ac) was able to function as a receptor determinant for influenza C virus as indicated by the ability of the virus to agglutinate the modified red blood cells. In contrast to the natural receptors, 9-acetamido-Neu5Ac-containing receptors were found to be resistant against the action of sialic 9-O-acetylesterase, the viral receptor-destroying enzyme. No difference in the hemolytic activity of influenza C virus was detected when analyzed with erythrocytes containing either Neu5,9Ac2 or 9-acetamido-Neu5Ac on their surface. This finding indicates that cleavage of the receptor is not required for the viral fusion activity. The sialic acid analogues should be useful for analyzing not only the importance of the receptor-destroying enzyme of influenza C virus, but also other biological processes involving sialic acid.

A virus infection is initiated by attachment of the virus particle to cell surface receptors. Both lipids and proteins have been identified as receptors. The viral attachment protein may recognize an epitope present only on a distinct protein such as the CD4 molecule, which functions as a receptor for human immunodeficiency virus (1-3). A number of viruses attach to carbohydrate epitopes present on many different glycoproteins and glycolipids. Sialic acid is recognized as a receptor determinant by more viruses than any other determinant known. Influenzaviruses, paramyxoviruses, reoviruses, and encephalomyocarditis virus have been shown to require sialic acid as a crucial part of the cellular receptors for attachment to cell surfaces (4). Among these viruses influenza viruses, paramyxoviruses, as well as some coronaviruses are unique because they contain a receptor-destroying enzyme. The enzyme of paramyxoviruses and influenza A and B viruses is a neuraminidase which releases terminal N-acetylneuraminic acid (Neu5Ac)1 from glycoproteins and glycolipids, thereby abolishing the ability of these molecules to serve as virus receptors. Influenza C viruses inactivate their receptors by an acetylesterase which cleaves the 9-O-acetyl residue from 9-O-acetyl-N-acetylneuraminic acid (Neu5,9Ac2) (5). The 9-O-acetyl group of sialic acid is crucial for the ability of a glycoconjugate to function as a receptor for influenza C virus (6, 7). Some members of the family coronaviridae have been shown recently also to contain an acetylesterase and to recognize Neu5,9Ac2 as a receptor determinant (8-11).

Influenza C virus is an RNA virus with a lipid envelope from which surface projections are protruding. These spikes are formed by a glycoprotein, which is designated HEF to indicate that it is responsible for three activities: hemagglutinating (receptor-binding), esterase (receptor-destroying), and fusion activity (12). The fusion is believed to be due to a hydrophobic amino acid sequence, which requires for its activation the proteolytic cleavage of the glycoprotein into the subunits HEF1 and HEF2 and the exposure to low pH (13, 14). The esterase has been shown to belong to the class of serine hydrolases, which are inactivated by diisopropylfluorophosphate (15). Using this inhibitor which binds covalently to the active site of serine hydrolases, amino acid 71 has been identified as the active-site serine (16, 17). Inactivation of the esterase by diisopropylfluorophosphate or other inhibitors does not abolish the hemagglutination activity (15, 17). From this finding it has been concluded that the active site of the enzyme and the receptor-binding site of HEF are located on different epitopes.

We have analyzed several synthetic sialic acid analogues for their ability to function as receptor determinants for influenza C virus. After enzymatic transfer to surface glycoproteins, 9-acetamido-N-acetylneuraminic acid was able to mediate the binding of influenza C virus to erythrocytes. The synthetic receptor determinant was, however, resistant to the action of the viral acetylesterase. This compound is, therefore, not a suitable sialic acid analogue.

1The abbreviations used are: Neu5Ac, N-acetylneuraminic acid; Neu5,9Ac2 or 9-O-Ac-Neu5Ac, N-acetyl-9-O-acetylneuraminic acid; 9-N-Ac-Neu5Ac, N-acetyl-9-acetamido-neuraminic acid; MDS, 2-(N-morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography.

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useful for studying the importance of the receptor-destroying enzyme. The approach described here is also applicable to other biological systems and should provide a valuable tool to study the importance of sialic acids.

MATERIALS AND METHODS

Viruses—Strain Johannesbur/1/66 of influenza C virus was grown in embryonated chicken eggs as described previously (5). Influenza A and B strains were grown as reported (18).

Resialylation of Erythrocytes—Erythrocytes were resialylated as described previously (10, 19). The values of sialic acid incorporation (see in Fig. 2) were obtained by incubating 40 μl of packed cells from 1-day-old chicken for 1 h at 37 °C with 1 million of Galβ1,4GalNCα2,6-sialyltransferase (from rat liver, 8 units/mg) and 20, 40, 80, or 160 pmol of CMP-Neu5α8C9, or 100, 200, 400, or 800 pmol of CMP-9-acetamido-N-acetylenuraminic acid.

To determine the amount of sialic acid incorporated into surface glycoconjugates, trace amounts of radioactively labeled CMP-sialic acids were added to the samples. CMP-Neu6,9Ac3 was not available in radioactively labeled form. As shown in Table I, the kinetic data for the transfer of Neu5Ac and Neu5,9Ac2 by Galβ1,4GalNCα2,6-sialyltransferase are very similar. Therefore, resialylated CMP-Neu5Ac was used as a tracer to estimate the incorporation of 9-O-acetylated sialic acid. Following resialylation, cells were washed three times with PBS. Half of the sample was used for an HA-assay with influenza C virus; the other half of the cells were permeabilized by addition of saponin to a final concentration of 0.1%. After centrifugation for 2 min at 3000 × g, the sediment was suspended in PBS and the radioactivity was determined in a scintillation counter.

Enzyme Treatment of Erythrocytes—Erythrocytes from 1-day-old chicken were modified to contain either 9-O-acetyl-N-acetylenuraminic acid or 9-acetamido-N-acetylenuraminic acid on their surface (see above). Aliquots containing 1 ml of a 1% erythrocyte suspension were incubated with purified acetylenurase from influenza C virus (12) at 37 °C. At the times indicated the cells were washed twice with PBS. The erythrocytes were suspended in PBS at a concentration of 1% and used to determine the HA-titer of allantoic fluid containing influenza C virus.

Hemagglutination Assay—The hemagglutination assays were performed in microtiter plates as described previously (20). The hemagglutinating activity (HA-units/ml) is expressed as the reciprocal value of the highest dilution causing complete agglutination of erythrocytes.

Hemolysis Assay—The ability of influenza C virus to cause hemolysis was determined with erythrocytes from 1-day-old chicken, which had been modified to contain either 9-O-acetyl-Neu5Ac or 9-acetamido-Neu5Ac. Aliquots containing 10 μl of packed cells were incubated with 200 μl of a virus suspension, which was obtained by diluting allantoic fluid from infected eggs with PBS. Prior to use the virus had undergone three cycles of freezing and thawing. After 15 min at 4 °C, 1 ml of cold PBS was added and the cells were sedimented. Following removal of unadsorbed virus, the erythrocytes were incubated at 37 °C in a buffer containing 0.125 M NaCl, 0.02 M MES, pH 5.2. After 30 min, the cells were sedimented and the optical density of the supernatant was determined at 530 nm.

Synthesis of CMP-activated Sialic Acids—The synthesis of CMP-activated sialic acids. 9-O-acetyl-N-acetylenuraminic acid has been described recently (10). CMP-9-acetamido-Neu5Ac and the corresponding 3H-labeled compound (1.6 × 10^6 cpm/nmol) was prepared according toGroß et al. (21). CMP-9-amino-, CMP-9-hexanoylamido-, and CMP-9-azido-Neu5Ac were prepared as described previously (21); the content of free CMP was less than 5% in each case. CMP-9-H-Neu5Ac (26 Ci/mm 12502 Interaction of Influenza C Virus with Sialic Acid Analogues

Table I

| Kinetic data of rat liver Galβ1,4GalNCα2,6-sialyltransferase | Km μM | % | Relative Vmax |
|------------------------------------------------------------|------|---|---------------|
| N-acetylenuraminic acid                                   | 50   | 100*|               |
| 9-O-acetyl-N-acetylenuraminic acid                        | 40   | 115*|               |
| 9-acetamido-N-acetylenuraminic acid                       | 120*| 110*|               |
| 9-hexanoylamido-N-acetylenuraminic acid                   | 65   | 120*|               |
| 9-azido-N-acetylenuraminic acid                           | n.d. | 145*|               |
| 9-amino-N-acetylenuraminic acid                           | 720*| 90* |               |

* Values obtained from Ref. 28.
day-old chicken were used, which, in contrast to cells from was further analyzed by determining the minimal amount of cytes were resialylated in the presence of various concentra-
tions of CMP-activated sialic acids to obtain cells with differ-
ent amounts of sialic acid on the surface. Using trace amounts of about 5-10 pmol of 9-O-Ac-Neu5Ac on the surface of 5 µl of packed erythrocytes was sufficient to obtain HA titers of influenza C virus which were equal or close to the titers determined with erythrocytes from adult chicken. In the case of 9-N-Ac-Neu5Ac an amount of about 20-50 pmol was re-
quired. This result indicates that the sialic acid analogue is recognized less efficiently by influenza C virus than the natural receptor determinant with a 4-5-fold difference in the minimal concentration of sialic acid on the cell surface neces-
ary for agglutination.

Esterase Resistance of 9-N-Ac-Neu5Ac—Having found that 9-N-Ac-Neu5Ac can serve as a receptor determinant for the attachment of influenza C virus to cells we analyzed whether the sialic acid analogue is affected by the receptor-destroying enzyme of this virus. Purified sialic acids were analyzed by HPLC before and after incubation with influenza C virus. The viral esterase efficiently released the 0-acetyl group of 9-
O-Ac-Neu5Ac as indicated by the almost complete conversion of 9-O-Ac-Neu5Ac to Neu5Ac (Fig. 3, peaks B and C). On the other hand, 9-N-Ac-Neu5Ac was unaffected by the treatment with influenza C virus (Fig. 3, peak A). This result indicates that the acetyltransferase of influenza C virus can release the acetyl group from C-9 of sialic acid, if it attached via an ester linkage, but not, if it is connected by an amide linkage. We tried to confirm this result in a biological assay. For this purpose erythrocytes from 1-day-old chicken were modified to contain either 9-O-Ac-Neu5Ac or 9-N-Ac-Neu5Ac, respectively, transferred to the cell surface was quantitated. As shown in Fig. 2, the presence of about 5-10 pmol of 9-O-Ac-Neu5Ac on the surface of 5 µl of packed erythrocytes was sufficient to obtain HA titers of influenza C virus which were equal or close to the titers determined with erythrocytes from adult chicken. In the case of 9-N-Ac-Neu5Ac an amount of about 20-50 pmol was re-
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ary for agglutination.

Affinity of Influenza C Virus for 9-N-Ac-Neu5Ac—The ability of 9-N-Ac-Neu5Ac to serve as a receptor determinant was further analyzed by determining the minimal amount of the analogue required on the cell surface for agglutination by influenza C virus. For this purpose red blood cells from 1-
day-old chicken were used, which, in contrast to cells from adult chicken, are resistant to agglutination by influenza C virus because of a lack of 9-O-Ac-Neu5Ac (22). The erythro-
cytes were resialylated in the presence of various concentra-
tions of CMP-activated sialic acids to obtain cells with differ-
ent amounts of sialic acid on the surface. Using trace amounts of 1H-labeled CMP-sialic acids, the actual amount of 9-O-Ac-Neu5Ac and 9-N-Ac-Neu5Ac, respectively, transferred to the cell surface was quantitated. As shown in Fig. 2, the presence of about 5-10 pmol of 9-O-Ac-Neu5Ac on the surface of 5 µl of packed erythrocytes was sufficient to obtain HA titers of influenza C virus which were equal or close to the titers determined with erythrocytes from adult chicken. In the case of 9-N-Ac-Neu5Ac an amount of about 20-50 pmol was re-
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Effect of 9-N-Ac-Neu5Ac on the Fusion Activity—The results presented above show that the receptor-binding and the receptor-destroying activity of influenza C virus respond to 9-N-Ac-Neu5Ac in a different manner. The sialic acid ana-
logue is recognized as a receptor determinant for attachment to cells, but it is not a substrate for the acetyltransferase. It was therefore of interest to know, whether or not the third activity of the influenza C glycoprotein HEF is affected by 9-N-Ac-Neu5Ac. The ability of HEF to induce the fusion of the viral membrane with cellular membranes at an acidic pH was determined in a hemolysis assay. Chicken red blood cells were modified to contain 9-O-Ac-Neu5Ac or 9-N-Ac-Neu5Ac, re-

| Virus     | Native | Asialo | Resialylated (sialic acid α2,6Gal) | HA activity (HA units/ml) |
|-----------|--------|--------|----------------------------------|--------------------------|
| A/PR/8    | 1024   | 0      | 128                              | 0                        |
| A/X-31    | 32     | 0      | 64                               | 0                        |
| B/Eng     | 16     | 0      | 128                              | 0                        |
| C/JHB/1/66| 0      | 0      | 0                                | 64                       |

**TABLE II** Comparison of the ability of influenza viruses to agglutinate human erythrocytes containing various sialic acids and sialic acid analogues

**Fig. 2.** Comparison of the ability of 9-O-Ac-Neu5Ac and 9-N-Ac-Neu5Ac to serve as receptor determinants for influenza C virus. Asialoerythrocytes from 1-day-old chicken were resialylated to contain different amounts of either 9-O-Ac-Neu5Ac (open circles) or 9-N-Ac-Neu5Ac (filled circles) on the surface. The modified red blood cells were used to determine the hemagglutination titer of allantoic fluid containing influenza C virus.

**Fig. 3.** Effect of the acetyesterase of influenza C virus on 9-O-Ac-Neu5Ac and 9-N-Ac-Neu5Ac. Sialic acids were analyzed by HPLC at 200 nm as described previously (21), before (upper chromatogram) and after (lower chromatogram) incubation with influenza C virus. The assay mixture (100 µl) consisted of 0.1 M phosphate buffer, pH 7.5, 1 mM of the respective substrate, and 1 milliunit of acetyltransferase. Samples were incubated at 37 °C for 20 h. Left, analysis of 9-N-Ac-Neu5Ac (peak A); right, analysis of 9-O-Ac-Neu5Ac (peak B); peak C represents Neu5Ac.
Sialic acids play an important role in various biological processes. Sialic acid analogues promise to be valuable tools in analyzing the interactions between glycoconjugates involved in these processes (27–30). We have used analogues with different substituents at the position C-9 of sialic acid to study the three activities of the influenza C glycoprotein HEF.

With respect to the natural receptor determinant, Neu5,9Ac2, it was previously not known whether the 9-O-acetyl group itself is recognized by the receptor binding site of HEF or whether its function is the substitution of the free hydroxyl group at position C-9 of Neu5Ac to increase the hydrophobicity of the sialic acid molecule. Several Neu5Ac derivatives with different substituents at position C-9 were included in our analysis. The amino group introduces a positive charge; the acetamido group is similar to the naturally occurring substituent, the 9-O-acetyl group; the hexanoylamido group is an example of a space-filling hydrophobic substituent; the azido group is a small-sized photolabile residue. None of these analogues was able to mimic a receptor for the influenza A and B viruses tested. In the case of influenza C virus, only that analogue was accepted as a receptor determinant, which resembled Neu5,9Ac2 most closely. It can be concluded, therefore, that the structure of the substituent is critical for the recognition of sialic acid by influenza C virus. It will be of future interest to analyze other analogues with substituents which closely resemble an acetyl group.

Several viruses have been reported to recognize sialic acid preferentially in a certain linkage type, e.g. α2,3-linked to galactose (23). The importance of the regioselective linkage for the recognition of a receptor determinant is also known in the case of influenza C virus. 9-0-Ac-Neu5Ac is a receptor determinant, whereas the isomeric sialic acid acetylated at position C-4 is not. The former type of sialic acid is present on rat α1-macroglobulin, a potent inhibitor of the influenza C hemagglutinin (24, 25). The equine counterpart of this serum protein, which contains 4-O-Ac-Neu5Ac, has no inhibitory activity. Our results indicate that influenza C virus also has a preference for an ester linkage compared to an amide linkage. The difference is reflected in a 4–5-fold difference in the minimal concentration of surface-bound sialic acid required for agglutination of erythrocytes. However, if there is a sufficient amount of 9-N-Ac-Neu5Ac present on the cell surface, the virus attachment appears indistinguishable from the binding to receptors containing the natural receptor determinant. This is indicated not only by the HA titers obtained but also by the fact that the hemolysis by influenza C virus at low pH occurred to the same extent.

While 9-N-Ac-Neu5Ac is accepted as a receptor determinant by the receptor binding activity of HEF, it is not a suitable substrate for the acetylase activity of this glycoprotein. Both a biochemical (HPLC analysis) and a biological assay indicated that the 9-N-acetyl group is unaffected by the viral esterase, whereas the 9-O-acetyl residue is cleaved very efficiently. The acetylase of influenza C virus belongs to the class of serine hydrolases comprising both esterases and proteases (15). Although ester and amide linkages differ in their stability, both groups of enzymes are assumed to work by the same mechanism. The cleavage is accomplished by a charge relay system involving a catalytic triad which is composed of a serine, a histidine, and an aspartic acid (26). In the case of the influenza C glycoprotein, serine 71 has been shown to be the active-site serine (16, 17). The histidine and the aspartic acid involved in the catalytic reaction have not yet been identified. At present we do not know whether the resistance of 9-N-Ac-Neu5Ac against the acetylase activity of HEF is related to the increased stability of an amide linkage compared to an ester linkage. The resistance might also, at least in part, be due to a reduced affinity for the analogue. In the case of the receptor-binding activity the decreased affinity may be compensated by an increased number of receptors resulting in an efficient attachment of the virus particle to a cell. The enzymatic cleavage of a chemical bond which involves one molecule each of enzyme and substrate may not allow for such a compensation.

The unique properties of 9-N-Ac-Neu5Ac allow to dissect the function of the receptor-binding and the receptor-destroying activity. Inactivation of the influenza C esterase by diisopropylfluorophosphate and isocoumarins has been reported to reduce the infectivity (15, 17). As virus attachment to cells is not affected by the inhibitors, it has been proposed that the receptor-destroying enzyme is necessary for virus entry into
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A step in the infectious cycle following the virus adsorption to the cell surface is the fusion between the viral and cellular membranes. Our results indicate that a noncleavable receptor determinant not only allows attachment but also hemolysis of erythrocytes. Therefore, the fusion activity does not require the inactivation of the receptors. Either the esterase is involved in another step of virus entry or the inhibitors mentioned above have an indirect effect which is not related to the inactivation of the enzyme.

The results presented here show that 9-N-Ac-Neu5Ac is a valuable tool for analyzing the importance of the receptor-destroying enzyme. Applying this approach to cultured cells should provide an answer to the question, whether cleavage of the receptor is required for influenza C virus to infect a cell. The sialic acid analogue will also be used to obtain information about the role of the receptor-destroying enzyme in virus maturation.

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