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Transfer of an Esterase-Resistant Receptor Analog to the Surface of Influenza C Virions

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A synthetic sialic acid, *N*-acetyl-9-thioacetamidoneuraminic acid (9-ThioAcNeu5Ac), is recognized by influenza C virus as a receptor determinant but — in contrast to the natural receptor determinant, *N*-acetyl-9-*O*-acetylneuraminic acid — is resistant to inactivation by the viral acetylesterase. This sialic acid analog was used to analyze the importance of the receptor-destroying enzyme of influenza C virus in keeping the viral surface free of receptor determinants. Enzymatic transfer of 9-ThioAcNeu5Ac to the surface of influenza C virions resulted in the loss of the hemagglutinating activity. The ability to agglutinate erythrocytes was restored when the synthetic sialic acid was released from the viral surface by neuraminidase treatment. Infectivity of influenza C virus containing surface-bound 9-ThioAcNeu5Ac was reduced about 20-fold. Sedimentation analysis as well as electron microscopy indicated that virions resialylated with the esterase-resistant sialic acid analog formed virus aggregates. These results indicate that the receptor-destroying enzyme of influenza C virus is required to avoid the presence of receptor determinants on the virion surface and thus to prevent aggregate formation and a reduction of the infectious titer.

INTRODUCTION

A characteristic feature of influenza viruses is that, in addition to the receptor-binding activity, they also contain a receptor-destroying activity. With influenza A and B viruses, these activities are functions of two different surface proteins. The hemagglutinin is the receptor-binding protein. It mediates the attachment of virions to the cell surface by binding to sialic acid-containing receptors. Depending on the virus strain, *N*-acetylneuraminic acid (Neu5Ac) is the only or the preferred sialic acid that is recognized by the hemagglutinin as receptor determinant. Another surface protein, the neuraminidase, is responsible for the receptor-destroying activity. It releases terminal sialic acid from sialoglycoconjugates and thus is able to inactivate receptors for influenza A and B viruses.

Influenza C viruses are different from the type A and B viruses, because they contain only a single surface glycoprotein. This glycoprotein is designated HEF (for hemagglutinin, esterase, and fusion protein) and is responsible for both the receptor-binding and the receptor-destroying activies (Vlasak *et al.,* 1987; Herrler *et al.,* 1988). The receptor determinant recognized by the HEF protein is not *N*-acetylneuraminic acid as in the case of influenza A and B viruses, but a sialic acid containing an acetyl group at position C-9, *N*-acetyl-9-*O*-acetylneuraminic acid ($Neu5,9Ac_2$) (Rogers *et al.,* 1986). The receptor-destroying activity of influenza C virus is also different from the corresponding enzyme of influenza A and B viruses. The HEF protein has an acetylesterase rather than a neuraminidase activity. It inactivates receptors by releasing the acetyl group from position C-9 of sialic acid (Herrler *et al.,* 1985b).

The receptor-destroying activity of influenza viruses may be important for different stages of the infection. For influenza C virus, it has been shown that infection is prevented if the receptor-destroying enzyme is inactivated by esterase inhibitors (Vlasak *et al.,* 1989; Strobl and Vlasak, 1993) or if only esterase-resistant receptors are available on the cell surface (Brossmer *et al.,* 1993). These findings suggest a role of the esterase activity in the early stage of an infection. Inactivation of receptors is not required for the fusion between the viral and the cellular membranes (Herrler *et al.,* 1992; Brossmer *et al.,* 1993). Therefore, the exact role of the esterase in the entry process remains to be determined. In the case of influenza A virus, evidence has been presented suggesting a role of receptor-destroying enzyme during initiation of infection. Under certain circumstances, the neuraminidase may promote the fusion activity (Huang *et al.,* 1980,1985). Results obtained with vector-expressed hemagglutinin indicated that sialic acid on oligosaccharides near the receptor-binding site interfere with the hemadsorption activity (Ohuchi *et al.,* 1995). Therefore, influenza A viruses that contain complex oligosaccharides near the sialic acid-binding site may require the

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neuraminidase for a functional receptor-binding activity. Evidence is available also for a role of the neuraminidase at the late stage of infection. Influenza A virus lacking an active enzyme was able to initiate a replication cycle up to virion formation (Liu and Air, 1993). However, the virus particles formed large aggregates that remained associated with the cell surface (Liu *et al.,* 1995). This finding suggests that the neuraminidase of influenza viruses is required to release sialic acid from the surface of virions and cells. Release of sialic acid from the viral surface would prevent aggregate formation. Removal of sialic acid from the cellular surface would promote the release of virions from the infected cell. Whether both aspects equally contribute to the inhibitory effect or whether one is more important than the other cannot be determined by this approach.

We have described the use of sialic acid analogs to analyze the interaction of influenza C virus with receptors. Synthetic sialic acids containing an acetamido or a thioacetamido group at position C-9 were found to be recognized as a receptor determinant by influenza C virus (Herrler *et al.,* 1992; Brossmer *et al.,* 1993). They are, however, resistant to the action of the viral acetylesterase. By enzymatic transfer of these analogs to surface glycoconjugates, it is possible to generate receptors for influenza C virus that are resistant to the receptor-destroying enzyme. A virus that encounters such receptors is in the same situation as a virus lacking a receptor-destroying enzyme that interacts with the natural receptor determinant (Neu5,9Ac_2). Therefore, the sialic acid analogs are ideally suited to analyze the importance of the acetylesterase of influenza C virus. We have applied this approach to analyze the importance of the receptor-destroying enzyme for the inactivation of receptor determinants on the viral surface. Here we show that enzymatic transfer of sialic acid analogs to the surface glycoprotein HEF resulted in the formation of virus aggregates. The aggregate formation was paralleled by the loss of the hemagglutinating activity and a reduced infectious titer of the virus preparation.

MATERIAL AND METHODS

Virus

Strain Johannesburg/1/66 of influenza C virus was used throughout this study. Stock virus was grown in 8-day-old embryonated chicken eggs. After incubation for 3 days at 33°, the allantoic fluid was harvested, clarified by low-speed centrifugation (3700 *g,* 20 min, 4°), and stored at −80°.

Cells

Madin–Darby canine kidney (MDCK) cells were grown in minimum essential medium supplemented with 10% fetal calf serum. The cells used are designated MDCK I cells (Fuller *et al.,* 1984) and were chosen because they are permissive for influenza C virus.

Growth of virus

Confluent monolayers of MDCK I cells were infected with stock virus diluted 1:10 (about 10 $TCID_{50}$/cell). After incubation for 48 hr at 33° in medium without serum, the supernatant was clarified by centrifugation for 10 min at 3700 *g.* Influenza C virus was sedimented by ultracentrifugation at 140,000 *g* for 60 min. The virus pellet was resuspended in PBS and used for resialylation experiments.

Desialylation and resialylation of virus

A concentrated virus preparation of 40 μl (about 26,000 HA units/ml) was diluted with PBS (lacking Ca and Mg ions) to a volume of 0.8 ml. For desialylation, half of the sample was incubated with 150 mU of neuraminidase from *Vibrio cholerae* (1 U/ml). For control, the other half was incubated in the absence of enzyme. After incubation for 90 min at 37°, the enzyme was removed from the virions by centrifugation for 30 min at 86,500 *g.* Each virus pellet was resuspended in 40 μl of PBS.

For resialylation, 10 μl of the desialylated virus preparation was incubated with Galβ1,4GlcNAcα2,6-sialyltransferase and one type of CMP (cytidine monophosphate)-activated sialic acid [Neu5Ac, Neu5,9Ac_2, or *N*-acetyl-9-thioacetamidoneuraminic acid (9-ThioAc-Neu5Ac)] (Brossmer and Gross, 1994). Per microliter of virus, 2 nmol of CMP sialic acids and 5 mU of detergent-free enzyme were used. For control, 10 μl of the desialylated sample was incubated in the absence of enzyme. In another control, 10 μl of the nondesialylated sample (see above) was also incubated in the absence of enzyme. After incubation for 90 min at 37°, virions and enzyme were separated by centrifugation for 30 min at 86,500 *g.* The virus pellets were resuspended in 20 μl of PBS each. To determine the hemagglutinating activity of desialylated and resialylated virus, 10 μl of the sample was diluted with 90 μl of PBS and used for a hemagglutination assay. Somewhat less than half of the hemagglutinating acitivity was recovered after each centrifugation step. More stringent centrifugation conditions were avoided to preserve the intactness and the biological activity of the virus particles. The amount of sialic acid transferred during the resialylation reaction has not been determined as the sialic acid analogue was not available in radioactive form. However, studies with glycoproteins have indicated that 9-ThioAcNeu5Ac is transferred with efficiency similar to that of *N*-acetylneuraminic acid (Sticher *et al.,* 1991; Kunze and Brossmer, in preparation).

Hemagglutination assay

The hemagglutinating activity of influenza C virus was determined in microtiter plates as previously described

(Herrler *et al.,* 1985a). The reciprocal value of the highest virus dilution causing complete agglutination of chicken erythrocytes was used as a measure of the hemagglutinating activity expressed in HA units/ml.

Western blotting

Viral proteins were separated by SDS–polyacrylamide gel electrophoresis under nonreducing conditions and blotted to nitrocellulose using a semidry Western blot method as modified by Schultze *et al.* (1991a). After blocking nonspecific binding sites by incubation with 1% bovine serum albumin in PBS, the immobilized influenza C glycoprotein HEF was detected with a monospecific rabbit antiserum. After incubation with biotinylated donkey anti-rabbit IgG and streptavidin–biotinylated horseradish peroxidase complex, the bound immunocomplexes were visualized by ECL.

Lectin blots

Viral proteins were immobilized on nitrocellulose as for Western blots. The blotted viral proteins were incubated for 1 hr with digoxigenin-labeled lectin from *Sambucus nigra* that specifically reacts with α2,6-linked sialic acid. For detection of bound lectin, the blots were incubated for 60 min at room temperature with anti-digoxigenin Fab fragments conjugated to alkaline phosphatase and then immersed in a solution of 37.5 μl of 5-bromo-4-chloro-3-indolyl phosphate 4-toluidine salt and 50 μl of nitro blue tetrazolium chloride in 10 ml of Tris–HCl, pH 9.5, 50 m*M* $MgCl_2$, 100 m*M* NaCl. The reaction was stopped by rinsing the blots with water.

Esterase activity

The enzyme activity of the influenza C glycoprotein HEF was determined with *p*-nitrophenyl acetate as substrate (Schultze *et al.,* 1991b).

Sedimentation of resialylated virus

After resialylation, 10 μl of the virus preparation (320 HA units/ml) was diluted with 700 μl of PBS and centrifuged at 4° for 10 min at 220, 1400, or 5700 *g.* The supernatants were carefully removed and centrifuged for 30 min at 125,000 *g.* All pellets (from both the low-speed and the high-speed centrifugations) were resuspended in 10 μl of PBS each, mixed with 10 μl of sample buffer, and analyzed by Western blots.

Electron microscopy

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

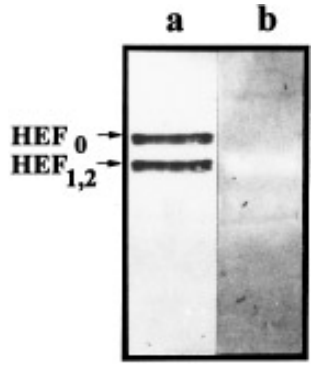

FIG. 1. Reactivity of the influenza C glycoprotein HEF with the agglutinin from *Sambucus nigra.* Purified virus gown in MDCK cells (lane a) or embryonated chicken eggs (lane b) were subjected to SDS–polyacrylamide gel electrophoresis under nonreducing conditions and electroblotted to nitrocellulose. The immobilized proteins were analyzed for reactivity with the lectin that specifically recognizes α2,6-linked sialic acid.

RESULTS

Detection of α2,6-linked sialic acid on native and resialylated influenza C virions

Our approach for the analysis of the influenza C esterase required the transfer of sialic acid analogs to viral surface components. The only enzyme that was commercially available for this purpose was Galβ1,4GlcNAcα2,6-sialyltransferase. As this enzyme attaches sialic acid to galactose in an α2,6-linkage, we analyzed whether this type of sialic acid is present on the virion surface. In previous studies it was found that influenza C virus — in contrast to influenza A and B viruses — contains surface-bound sialic acid (Meier-Ewert *et al.,* 1978; Nakamura *et al.,* 1979). However, the type of linkage has not been determined. Purified virions were subjected to SDS–polyacrylamide gel electrophoresis and then transferred to nitrocellulose. The immobilized proteins were analyzed for reactivity with the agglutinin from *S. nigra,* a lectin that specifically recognizes α2,6-linked sialic acid. As shown in Fig. 1, strong labeling of two bands was detected with virus grown in MDCK I cells. The two bands represent the uncleaved precursor form (HEF_0) and the fusion active form of the influenza C glycoprotein that is obtained by proteolytic cleavage into the subunits HEF_1 and HEF_2 (Herrler *et al.,* 1979; Sugawara *et al.,* 1981). The two subunits are held together by disulfide bonds. As the gel shown in Fig. 1 was run under nonreducing conditions, the proteolytically activated form of the influenza C surface protein is detected as a single band ($HEF_{1,2}$). In contrast to virions grown in MDCK cells, egg-grown virus did not show any reactivity with the agglutinin from *S. nigra.* The failure of the agglutinin to detect HEF protein was not due to a lack of protein. The whitish band at the position of $HEF_{1,2}$ indicates that there was a sufficient amount of protein present on the nitrocellulose,

but this protein did not react with the agglutinin. At the position of HEF_0, no whitish band is visible, because in egg-grown virus all the glycoprotein is present in the cleaved form. The result from Fig. 1 shows that the presence of α2,6-linked sialic acid on the viral surface can vary depending on the cell type used for virus replication. As the sialic acid analogs were to be attached to surface components in an α2,6-linkage, we chose virus grown in MDCK cells for our studies, because they contain this type of sialic acid also in the native state.

The lectin reactivity assay was also used to determine the efficiency of the desialylation and resialylation of virions. Sialic acid was released from the viral surface by incubation of virus grown in MDCK cells with neuraminidase from *V. cholerae.* As shown in Fig. 2, this treatment almost completely abolished the recognition of HEF protein by the lectin (lane b). The reactivity with the lectin was restored when the desialylated virions were incubated with α2,6-sialyltransferase and CMP-activated *N*-acetylneuraminic acid (lane c). This result indicates that purified influenza C virions can be efficiently desialylated and resialylated.

Hemagglutinating activity of resialylated influenza C virus

The resialylation conditions from Fig. 2 were used to attach Neu5Ac, $Neu5,9Ac_2$, or 9-ThioAcNeu5Ac to the surface of influenza C virions. After the desialylation and resialylation reactions, virions were sedimented to remove the neuraminidase and the sialyltransferase, respectively. As shown in Table 1, desialylation did not affect the ability of influenza C virus to agglutinate erythrocytes. Resialylation with Neu5Ac or $Neu5,9Ac_2$ resulted only in a slight reduction of the hemagglutinating activity, most likely due to the loss of virus during the preceding sedimentation step. However, virions that were modified

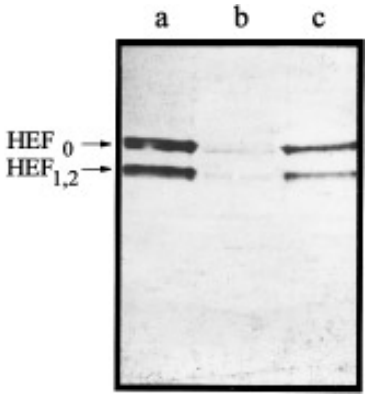

FIG. 2. Desialylation and resialylation of the influenza C glycoprotein HEF. Sialic acid was released from purified virions by neuramindase treatment (lane b). *N*-acetylneuraminic acid was attached to desialylated virions by incubation with α2,6-sialyltransferase and CMP-Neu5Ac (lane c). Control virus (lane a) was incubated in the absence of enzyme.

TABLE 1

Hemagglutinating Activity of Influenza C Virus after Desialylation and Resialylation with Neu5Ac, $Neu5,9Ac_2$, or 9-ThioAcNeu5Ac

	HA activity (HA units/ml)	
Treatment of virus	Before desialylation	Desialylated[a]
None	128	64
Desialylated	128	64
Resialylated		
Neu5Ac	64	16
$Neu5,9Ac_2$	64	16
9-ThioAcNeu5Ac	<2	16

[a] After desialylation and resialylation of virus, the HA activity was determined (left column). These virus preparations were neuraminidase-treated to release sialic acid (right column).

to contain the sialic acid analog 9-ThioAcNeu5Ac were devoid of HA activity. The inability of these virions to agglutinate erythrocytes is not due to an insufficient amount of protein, because in Western blots the virus preparation containing the synthetic sialic acid was found to contain HEF protein in an amount comparable to the virions that had been modified to contain Neu5Ac or $Neu5,9Ac_2$ (not shown). The lack of HA activity is due to the attachment of the sialic acid analog to the viral surface, because neuraminidase treatment of these virions restored the ability to agglutinate erythrocytes (Table 1). The lower titer (16 compared to 64 HA units/ml) is explained by losses during the sedimentation after the incubation with enzyme; virions containing either Neu5Ac or $Neu5,9Ac_2$ also had an HA activity of 16 HAU/ml after neuraminidase treatment (Table 1). This result indicates that attachment of a receptor determinant that is resistant to the receptor-destroying enzyme results in the loss of the HA activity of influenza C virus. The finding that virions that had been modified to contain the natural receptor determinant, $Neu5,9Ac_2$, were able to agglutinate erythrocytes suggests that the esterase activity of HEF has released the 9-*O*-acetyl group and thus inactivated the receptor activity. The esterase activity of influenza C virus was not affected by the different treatments. Using *p*-nitrophenyl acetate as substrate, no difference in the enzyme activity was found between control virus and virus that was desialylated and resialylated with either of the three sialic acids mentioned above (not shown).

Aggregate formation of virions containing 9-ThioAcNeu5Ac

In order to explain the loss of HA activity by influenza C virus after resialylation with 9-ThioAcNeu5Ac, we analyzed whether the attachment of the sialic acid analog to the viral surface resulted in the formation of aggregates. For this purpose virus that had been resialylated with either Neu5Ac or 9-ThioAcNeu5Ac was subjected to low-speed centrifuga-

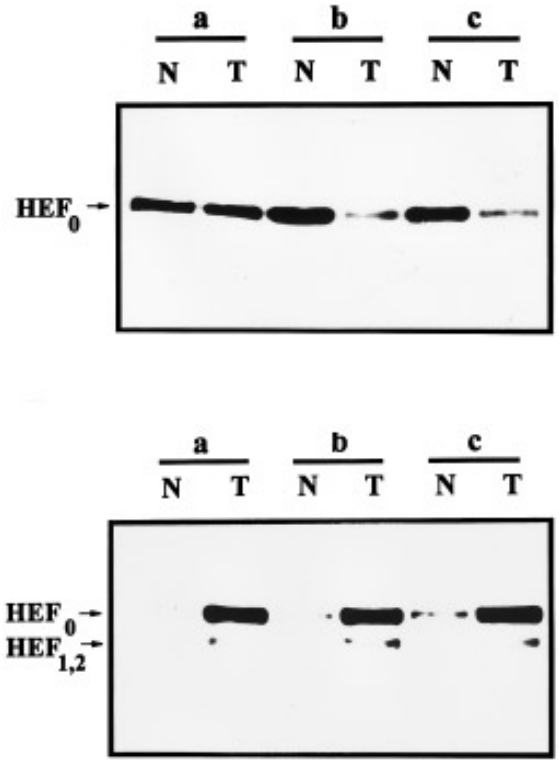

FIG. 3. Sedimentation analysis of influenza C virions that had been resialylated to contain Neu5Ac (lanes designated N) or the sialic acid analog 9-ThioAcNeu5Ac (lanes designated T). After resialylation virions were sedimented at 220 *g* (lanes a), 1400 *g* (lanes b), or 5700 *g* (lanes c). The pellets were analyzed by Western blot using a rabbit antiserum directed against the influenza C glycoprotein HEF (bottom). Virions remaining in the supernatant (top) were pelleted by high-speed centrifugation prior to Western blot analysis.

tion. As shown in Fig. 3 (lanes a), after sedimentation for 10 min at 220 *g,* some of the virus containing 9-ThioAcNeu5Ac (lanes T) could be recovered from the pellet fraction, whereas virus containing Neu5Ac (lanes N) was detectable only in the supernatant. Centrifugation at 1400 *g* (lanes b) or 5700 *g* (lanes c) resulted in the almost complete disappearance of virus containing the sialic acid analog from the supernatant fractions. On the other hand, the majority of the virus containing Neu5Ac was still found in the supernatant fractions. In order to determine whether the change in the sedimentation behavior is due to aggregate formation, the resialylated samples were analyzed by electron microscopy. As shown in Fig. 4, large aggregates were detected in the virus preparations containing surface-bound 9-ThioAcNeu5Ac; comparable aggregates were not found in the virus sample that had been resialylated with Neu5Ac. This result shows that attachment of the sialic acid analog to the surface of influenza C virus resulted in the generation of receptors that caused the virions to bind to each other.

Effect of resialylation with 9-ThioAcNeu5Ac on the infectivity of influenza C virus

The results presented above show that resialylation with 9-ThioAcNeu5Ac resulted in aggregate formation and in the loss of the HA activity. To determine how the aggregate formation affected the infectious titer of the virus preparation, an infectivity assay was performed. As shown in Table 2, virus containing 9-ThioAcNeu5Ac had a more than 10-fold reduced infectivity (2.1×10^4 $TCID_{50}$/ml) compared to virus that had been resialylated with Neu5Ac (5.2×10^5 $TCID_{50}$/ml). This result indicates that the presence of the sialic acid analog on the viral surface drastically reduces the infectivity of the influenza C virus preparation.

DISCUSSION

Sialic acid is the terminal sugar of many oligosaccharides present on glycoproteins and glycolipids. Therefore, it may be considered as an attractive receptor determinant for viruses. However, the recognition of sialic acid will result not only in the binding to receptors on the target cell. A number of extracellular sialoglycoconjugates have been reported to be competitive inhibitors of influenza A and B viruses, e.g., mucins and serum glycoproteins (reviewed by Gottschalk *et al.,* 1972). As glycosylation of viral components is completely dependent on cellular enzymes, sialic acid is present not only on cellular glycoconjugates, but also on viral glycoproteins or glycolipids. Therefore, virions that use sialic acid as a receptor determinant are expected to bind to each other, resulting in the formation of aggregates. In order to avoid the negative effect of competitive inhibitors and of aggregate formation, viruses recognizing sialic acid may have acquired a receptor-destroying enzyme. The ability to inactivate their own receptors is characteristic for influenza A and B viruses and paramyxoviruses that contain a neuraminidase (Drzeniek, 1972) as well as for influenza C virus and some coronaviruses that contain an acetylesterase (Herrler *et al.,* 1991). For influenza A viruses, it has been reported that abrogation of the neuraminidase activity by antibodies (Compans *et al.,* 1969), inhibitors (Palese and Compans, 1976), or mutations (Palese *et al.,* 1974; Shibata *et al.,* 1993) resulted in the formation of virus aggregates that remained associated with the surface of the infected cell or were even endocytosed. From these studies it has been concluded that the receptor-destroying enzyme is required for prevention of virus aggregates and for the release from the infected cell. The contribution of each of these two aspects to the inhibitory effect has not been determined.

We have used sialic acid analogs to analyze the interaction between influenza C virus and cellular surface receptors. Virus binding to the cells can be mediated not only by receptors containing the natural receptor determinant Neu5,9Ac_2, but also by receptors containing synthetic sialic acids with an acetamido or a thioacetamido group at position C-9 (Herrler *et al.,* 1992; Brossmer *et al.,* 1993). Thus, the receptor binding activity of the influenza C glycoprotein HEF does not differentiate as to

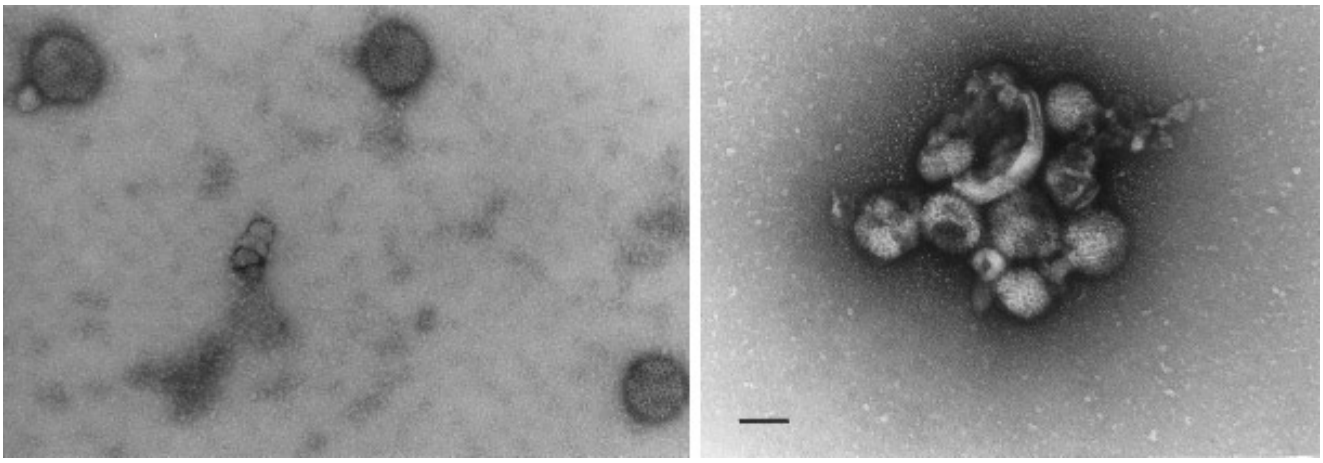

FIG. 4. Electron micrographs of influenza C virions after resialylation with Neu5Ac (left) or 9-ThioAcNeu5Ac (right). The scale bar indicates 100 nm.

whether the substituent is attached to C-9 via an ester linkage or an amide linkage. The receptor-destroying activity, however, will only cleave ester but not amide linkages (Herrler *et al.,* 1985b,1992). Therefore, the viral acetylesterase is able to inactivate the natural receptor determinant, but not the synthetic receptor determinants. By enzymatic transfer of sialic acid analogs to the influenza C glycoprotein HEF, we demonstrated that the presence of a receptor determinant on the virion surface results in aggregate formation. The binding of virus particles to each other was accompanied by the loss of the hemagglutinating activity and a more than 10-fold reduction of the infectivity titer. Thus, the presence of a receptor-destroying enzyme that inactivates potential receptors on the viral surface prevents aggregate formation and thus greatly facilitates the spread of virus infection. The efficiency of the acetylesterase of influenza C virus in clearing the viral surface from the receptor determinant is evident from the fact that no inhibitory effect was detectable when the natural receptor determinant was enzymatically transferred to the viral surface.

To what extent the receptor-destroying enzyme affects the release of virions from the surface of the infected cells remains to be established. In the case of influenza A and B viruses that recognize the most common type of sialic acid, Neu5Ac, this aspect may be of importance. $Neu5,9Ac_2$, the receptor determinant for influenza C virus, is less frequently found on sialoglycoconjugates and therefore, the number of influenza C virus receptors is expected to be lower than the number of receptors for the other influenza viruses. Therefore, the release of influenza C virus from the infected cell may be not as difficult as in the case of influenza A and B viruses.

Our results show that sialic acid analogs that were previously used to study the interaction of viruses with cellular receptors in the early stage of infection are also useful to analyze virus–cell interactions in the late stage of infection.

TABLE 2

Infectivity of Influenza C Virus after Desialylation and Resialylation with Neu5Ac or 9-ThioAcNeu5Ac

	Virus yield (HA units/ml) after infection of MDCK cells by influenza C virus resialylated with	
Virus dilution	Neu5Ac	9-ThioAcNeu5Ac
10^{-2}	128	96
10^{-3}	128	128
10^{-4}	64	<2
10^{-5}	4	<2
10^{-6}	<2	<2

Note. Different dilutions of the resialylated virus preparations were used to infect MDCK cells. Virus released into the supernatant was measured 5 days p.i. by hemagglutination titration. The HA titers are the mean values of five parallel infections.

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