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A sialic acid analogue acting as a receptor determinant for binding but not for infection by influenza C virus

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We describe a synthetic sialic acid analogue, 9-thioacetamido-*N*-acetylneuraminic acid (9-thioacetamido-Neu5Ac), which is recognized by the receptor-binding activity of influenza C virus, but is resistant to the receptor-destroying enzyme (acetylesterase) of this virus. Following transfer of the analogue to the surface of receptor-negative cells, influenza C virus is able to attach to these cells, but is unable to infect the cells. This result suggests that inactivation of virus receptors by the receptor-destroying enzyme is essential for initiation of infection Because of their unique properties such analogues promise to be powerful chemotherapeutic agents.

Influenza C virus; *N*-Acetyl-9-*O*-acetylneuraminic acid, Sialic acid analogue; Receptor determinant

1. INTRODUCTION

Influenza C virus usually infects the upper respiratory tract of man and causes a mild disease characterized by fever and long-lasting nasal discharge [1], but lower respiratory infections have also been reported [2]. On a molecular level, influenza C virus is characterized by having only one surface glycoprotein (HEF), which is responsible for three biological activities: receptor-binding, receptor-inactivation, and fusion activity (reviewed in [3]). Virus attachment to the cell surface is accomplished by the ability of HEF to recognize receptors the crucial determinant of which is *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac$_2$) (Fig. 1) [4,5]. The receptor-destroying activity of influenza C virus has been identified as an acetylesterase [6]. The enzyme inactivates the cellular receptors for this virus by releasing the 9-*O*-acetyl group of sialic acid. The viral esterase may be important for several stages of the virus infection. Some data suggest that the enzyme is required for a step following attachment to the surface receptors [7]. It has also been speculated that the acetylesterase may be required for virus maturation or for inactivating competitive inhibitors, which are present in the mucus covering the respiratory tract [3].

Recently, we have shown that a synthetic sialic acid analogue, 9-acetamido-*N*-acetylneuraminic acid (Fig. 1) [8], can be used by influenza C virus as a receptor determinant for attachment to the cell surface [9]. Erythrocytes containing the analogue were agglutinated to the same titer as cells containing *N*-acetyl-9-*O*-acetylneuraminic acid, the natural receptor determinant. However, the sialic acid analogue is not a substrate for the receptor-destroying enzyme; the amide linkage at C-9 of sialic acid is resistant to the action of the acetylesterase [9]. Because of these characteristics, the analogue or variants of it can be used to analyze the importance of the receptor-destroying enzyme for the infection of cultured cells.

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2. EXPERIMENTAL

2.1. *Virus*

Strain Johannesburg/1/66 of influenza C virus was grown in embryonated chicken eggs as described previously [6].

2.2. *Cells*

MDCK II cells were grown as described previously [5] For virus infection, confluent monolayers in 35 mm plastic petri dishes were incubated with 0.2 ml of stock virus diluted with PBS to obtain an m o.i. of about 10 $TCID_{50}$/cell. After an adsorption time of 1 h at room temperature, cells were washed with PBS and incubated with minimal essential medium at 33°C. Two days post-infection the yield of virus released into the supernatant was determined by HA titration [10]

2.3. *Synthesis of sialic acid analogues*

9-Thioacetamido-9-deoxy-*N*-acetylneuraminic acid was obtained by thioacetylation of 9-amino-9-deoxy-*N*-acetylneuraminic acid The detailed synthesis will be described elsewhere (manuscript in preparation) Conversion to the CMP-activated compound was accomplished as described previously [8].

2.4 *Resialylation of cells*

Erythrocytes from one-day-old chicken and MDCK II cells were treated with neuraminidase and resialylated as described previously [10,11].

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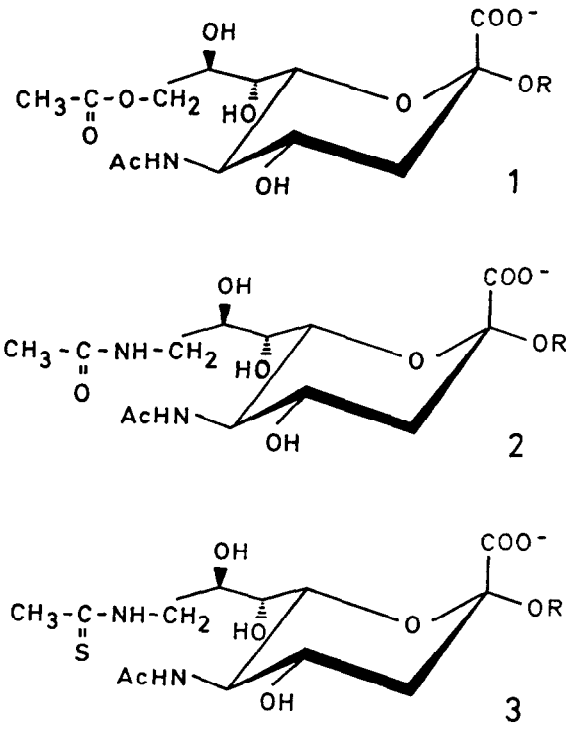

Fig. 1. Structures of 9-*O*-acetyl-*N*-acetylneuraminic acid (**1**), 9-acetamido-*N*-acetylneuraminic acid (**2**), and 9-thioacetamido-*N*-acetylneuraminic acid (**3**) R, following sugar in the oligosaccharide chain.

3. RESULTS AND DISCUSSION

As we have shown recently, replacement of the 9-*O*-acetyl group of sialic acid by an acetamide group does not abolish the ability of the molecule to serve as a receptor determinant for influenza C virus [9]. We analyzed whether the receptor-binding activity of the virus also recognizes a sialic acid molecule containing a thioacetamido group at position 9. The analogue 9-thioacetamido-9-deoxy-*N*-acetylneuraminic acid (9-thioacetamido-Neu5Ac) (Fig. 1) was transferred to the surface of erythrocytes which were resistant to agglutination by influenza C virus. Following resialylation, erythrocytes containing the analogue were agglutinated by influenza C virus as efficiently as cells which had been sialylated to contain 9-*O*-acetylated sialic acid (Table I). The amount of CMP-9-thioacetamido-Neu5Ac required for positive results was higher compared to Neu5,9Ac$_2$, but somewhat lower than in the case of 9-acetamido-Neu5Ac. This result indicates that influenza C virus can use the 9-thioacetamido compound as a *receptor determinant for attachment to cells.*

Table I

Ability of influenza C virus to agglutinate erythrocytes containing either the natural receptor determinant, Neu5,9Ac$_2$, or a sialic acid analogue (9-thioacetamido-*N*-acetylneuraminic acid or 9-acetamido-*N*-acetylneuraminic acid, respectively) on the surface

CMP-sialic acid (nmole)	HA activity (HA U/ml)		
	Neu5,9Ac$_2$	9-thioacetamido-Neu5Ac	9-acetamido-Neu5Ac
0.25	<2	<2	<2
0.5	256	<2	<2
1.0	256	32	<2
2.0	256	64	64
4.0	256	256	256

Sialic acids were transferred to the surface of neuraminidase-treated erythrocytes from one-day-old chicken by incubation with 2.5 mU Galβ1,4GlcNAc α2,6-sialyltransferase and the amount of CMP-activated sialic acid indicated above. Resialylated cells were used to determine the HA titer of allantoic fluid containing influenza C virus.

In order to analyze whether the analogue can serve as a receptor determinant also on cultured cells, 9-thioacetamido-Neu5Ac acid was attached to the surface of MDCK II cells. These cells are resistant to infection by influenza C virus due to a lack of receptors. After generation of receptors by incorporation of exogenous gangliosides or by enzymatic transfer of 9-*O*-acetylated sialic acid, the cells become susceptible to infection [5,12]. As shown in Table II, low virus yields were obtained already when 2 nmol of CMP-9-*O*-acetyl-*N*-acetylneuraminic acid were used for the resialylation of MDCK II cells. After infection of cells that had been sialylated in the presence of 10 nmol of CMP-Neu5,9Ac$_2$, a titer of 256 HA U/ml was determined in the supernatant which is comparable to the amount of virus released by permissive MDCK I cells. On the other hand, influenza C virus was unable to infect cells containing the thioacetamido analogue, even if 50 nmol were used during the sialylation reaction. This result indicates that the analogue cannot serve as a *receptor determinant for the initiation of infection.* From the result with erythrocytes (Table I), it is expected that influenza C virus is able to attach to MDCK II cells containing the analogue. In fact, binding of virus to cell surface proteins resialylated to contain 9-thioacetamido-NeuAc can be demonstrated by overlay assays (manuscript in preparation). Therefore, it is not the attachment but a subsequent step of the infectious cycle which prevents influenza C virus from using the analogue as a receptor determinant for infection of cells. As the analogue is resistant to inactivation by the viral acetylesterase, it is likely that the receptor-destroying enzyme of this virus plays an important role at the post-adsorption stage of the infection. This conclusion was also drawn from studies with esterase inhibitors [7]. The inactivation of the virus receptors is not an absolute requirement for the fusion activity of influenza C virus, because erythrocytes containing 9-acetamido-Neu5Ac are efficiently hemolyzed though this receptor determinant cannot be inactivated by the viral acetylesterase [9]. Studies with esterase inhibitors suggested that the enzyme is required for the fusion activity only, if low amounts of virus are used for the hemolysis assay [13]. A possible role for the receptor-destroying enzyme in the initiation of infection

Table II

Ability of influenza C virus to infect MDCK II cells modified to contain either the natural receptor determinant, Neu5,9Ac$_2$, or a sialic acid analogue, 9-thioacetamido-*N*-acetylneuraminic acid, on the surface

CMP-sialic acid (nmole)	HA activity (HA U/ml)	
	Neu5,9Ac$_2$	9-thioacetamido-Neu5Ac
2	16	<2
10	256	<2
50	256	<2

Sialic acids were transferred to the surface of neuraminidase-treated MDCK II cells by incubation with 1 mU Galβ1,4GlcNAc α2,6-sialyltransferase and the amount of CMP-activated sialic acid indicated above. Resialylated cells were infected with influenza C virus and two days post-infection the yield of virus released into the supernatant was determined by HA titration

might be to facilitate the transfer from primary attachment sites to secondary attachment sites. For example, a transfer from glycoproteins to glycolipids would result in a closer contact between the viral and the cellular membrane which may be favorable for the subsequent fusion event. Secondary attachment sites might also be cellular glycoproteins of coated pits, which are endocytosed and therefore suitable for directing the bound virus particle to the acidic compartment which is required to trigger the fusion between influenza viruses and cellular membranes.

Whatever the role of the receptor-destroying enzyme may be, the results obtained demonstrate that synthetic sialic acid analogues can effectively interfere with initiation of infection by viruses which contain a receptor-destroying enzyme. Preliminary studies have shown that polymers containing 9-thioacetamido-Neu5Ac inhibit virus infection. Because of their inhibitory activity the analogues promise to be powerful chemotherapeutic agents, which should be applicable also to other viruses with receptor-destroying enzymes.

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