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The receptor-destroying enzyme of influenza C virus is neuraminate-O-acetylesterase

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The nature of the receptor-destroying enzyme (RDE) of influenza C virus has been elucidated by analyzing its effect on the haemagglutination inhibitors rat alpha$_1$-macroglobulin (RMG) and bovine submandibulary mucin (BSM), respectively. The inhibitory activity of both compounds is abolished by incubation with influenza C virus. After inactivation, RMG and BSM were found to contain reduced amounts of N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac$_2$) and increased amounts of N-acetylneuraminic acid (Neu5Ac). H.p.l.c. analysis revealed that purified Neu5,9Ac$_2$ is converted to Neu5Ac by incubation with influenza C virus. These results demonstrate that RDE of influenza C virus is neuraminate-O-acetylesterase [N-acyl-9(4)-O-acetylneuraminate O-acetylhydrolase (EC 3.1.1.53)]. The data also indicate that haemagglutination-inhibition (HI) by RMG and BSM and most likely virus attachment to cell surfaces involves binding of influenza C virus to Neu5,9Ac$_2$.

Key words: influenza C virus/receptor-destroying enzyme/virus receptor/sialic acid/neuraminate-O-acetylesterase

Introduction

Though the presence of a receptor-destroying enzyme (RDE) on influenza C virus has been demonstrated more than 30 years ago (Hirst, 1950), the specificity of this enzyme as well as the nature of the cellular receptor for influenza C virus have remained puzzling ever since. This is in striking contrast to influenza A and B viruses and most paramyxoviruses where it has been clearly established that the receptor is sialic acid and that RDE is neuraminidase (Klenk *et al.*, 1955; Gottschalk, 1957). Viral neuraminidases were found to be unable to destroy the receptor for influenza C virus, and RDE of influenza C virus did not inactivate the receptor for the other myxoviruses (Hirst, 1950; Kendal, 1975). Mainly based on these observations it was assumed that receptor and RDE of influenza C virus are different from sialic acid and neuraminidase, respectively.

Recently we reported that bacterial neuraminidases are able to destroy the influenza C receptor on chicken erythrocytes. The same enzymes were found to inactivate a potent haemagglutination inhibitor present in rat serum which was identified as alpha$_1$-macroglobulin (RMG) (Herrler *et al.*, 1985a). These observations suggested that sialic acid is involved in binding of influenza C virus to erythrocytes. The carbohydrate portion of RMG was found to consist primarily of a complex type of N-linked oligosaccharides with a biantennary structure terminated by sialic acid. The neutral sugar residues were shown to be unaffected by RDE of influenza C virus and sialic acid was not released (Herrler *et al.*, 1985b).

We have now analyzed the effect of influenza C virus on sialic acids of RMG and bovine submandibulary mucin (BSM), another haemagglutination-inhibitor. Evidence was obtained indicating that RDE of influenza C virus is neuraminate O-acetylesterase, which releases O-acetyl groups from Neu5,9Ac$_2$.

Results

Effect of influenza C virus on sialic acids of BSM and RMG

Upon screening various sialic acid-containing compounds, BSM was found to be a potent inhibitor of haemagglutination by influenza C virus. As shown in Table I, the inhibitory activity of BSM was sensitive to the action of neuraminidase from *Clostridium perfringens*, indicating that sialic acid is essential for the HI-activity of BSM, as recently reported for RMG (Herrler *et al.*, 1985b). Incubation of BSM with influenza C virus also caused a drastic reduction of the inhibitory activity. To determine the effect of influenza C virus on BSM at a molecular level, sialic acids were released by neuraminidase treatment and analyzed by t.l.c. As shown in Figure 1, a major and a minor band could be discriminated that co-migrated with standards of Neu5,9Ac$_2$ and Neu5Ac, respectively. Both of these derivatives of neuraminic acid have previously been shown to account for the major fraction of sialic acids on BSM (Buscher *et al.*, 1974). Samples of BSM, the HI-activity of which was reduced by in-

Table I. Effect of neuraminidase and RDE of influenza C virus on the ability of BSM to inhibit haemagglutination by influenza C virus

Treatment	HI-titer (HI-units/ml)	Percentage
None	512	100
Neuraminidase	96	18
RDE	12	2

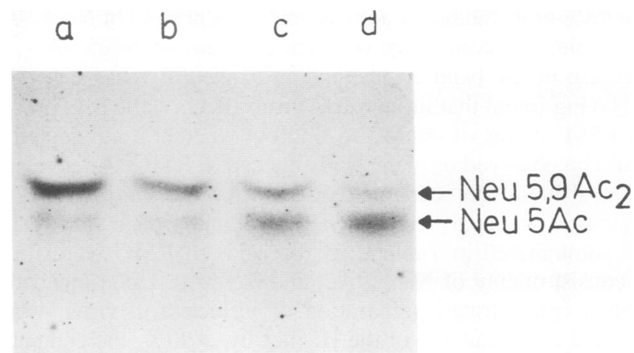

Fig. 1. T.l.c. of sialic acids isolated from BSM after treatment with influenza C virus. Samples were incubated with the following amounts of a purified preparation of influenza C virus: **a:** 0 μl; **b:** 1 μl; **c:** 10 μl; **d:** 100 μl. After sedimentation of virions by ultracentrifugation the HI-titer of the supernatants expressed as percentage of the sample containing no virus (**a:** 100%) was determined as follows: **b:** 50%; **c:** 8%; **d:** 2%. R_f values: Neu5Ac: 4.8; Neu5,9Ac$_2$: 5.3

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Table II. Sialic acids of rat alpha$_1$-macroglobulin before and after incubation with influenza C virus

Derivative of neuraminic acid	Amount (%)[a]	
	native	influenza C virus-treated
N-acetyl-9-O-acetylneuraminic acid	40%	10%
N-acetylneuraminic acid	50%	80%
N-glycolylneuraminic acid	10%	10%

[a]The figures are the combined results obtained by t.l.c., g.l.c. and h.p.l.c. of sialic acids released from RMG by treatment with neuraminidase from *A. ureafaciens*.

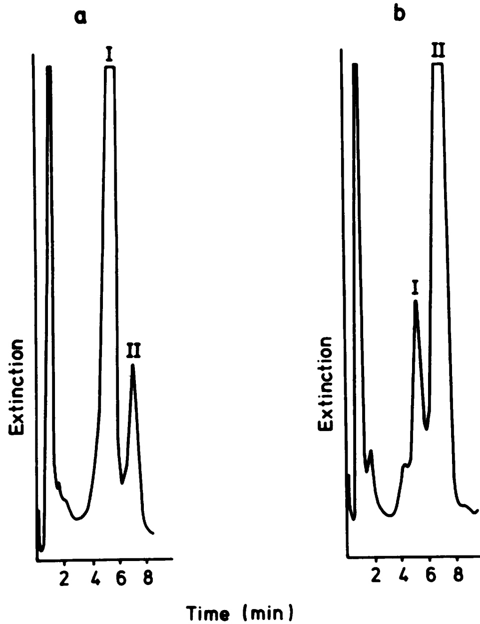

Fig. 2. Hydrolysis of the acetyl group of Neu5,9Ac$_2$ by influenza C virus. **(a)** Neu5,9Ac$_2$ incubated with influenza C virus. **(b)** Neu5,9Ac$_2$ incubated in the absence of virus. **a:** I, Neu5Ac (90%); II, Neu5,9Ac$_2$ (10%). **b:** I, Neu5Ac (18%); II, Neu5,9Ac$_2$ (82%).

cubation with increasing concentrations of influenza C virions, were analyzed in the same way and found to contain reduced amounts of Neu5,9Ac$_2$ (Figure 1, b–d). In sample d, which had lost 98% of its inhibitory activity, only a faint band of Neu5,9Ac$_2$ is visible. Concomitantly with the decrease of Neu5,9Ac$_2$, an increase of the band corresponding to Neu5Ac was observed.

Having found that influenza C virus affects sialic acid present on BSM, it was of interest to find out whether a similar effect could be observed on sialic acid attached to RMG. After release by incubation with neuraminidase from *Arthrobacter ureafaciens*, sialic acid was analyzed by t.l.c., g.l.c. and h.p.l.c. The results are summarized in Table II. Sialic acids of RMG were found to consist mainly of Neu5,9Ac$_2$ and Neu5Ac. Upon incubation with a concentrated preparation of influenza C virus, which resulted in a reduction of the HI-titer by >90%, the content of Neu5,9Ac$_2$ was reduced to ~10% and the relative amount of Neu5Ac was concomitantly increased. Thus, for both RMG and BSM, reduction of the HI-activity caused by influenza C virus is correlated with a reduction of the content of Neu5,9Ac$_2$. We conclude therefore that Neu5,9Ac$_2$ is essential for the HI-activity of both inhibitors and that influenza C virus destroys the inhibitory activity by hydrolyzing O-acetyl groups.

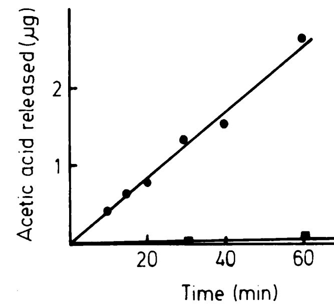

Fig. 3. Time course of the release of acetic acid from BSM by influenza C virus. ●, influenza C virus; ■, influenza A virus.

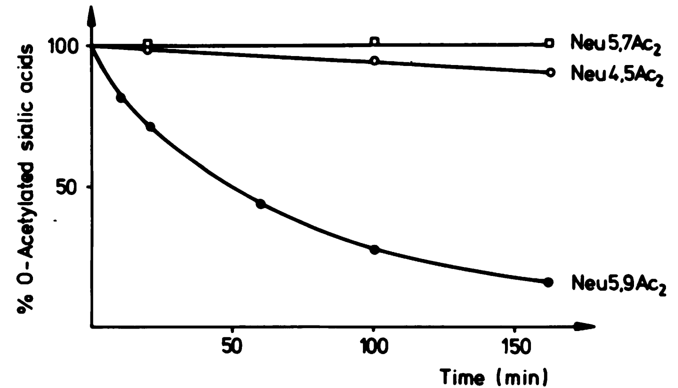

Fig. 4. Rate of hydrolysis of O-acetyl groups from different sialic acids by influenza C virus. ○, Neu4,5Ac$_2$; □, Neu5,7Ac$_2$; ●, Neu5,9Ac$_2$.

Neuraminate O-acetylesterase is associated with influenza C virus

To test whether indeed a neuraminate O-acetylesterase is present on influenza C virions, Neu5,9Ac$_2$ was incubated with a preparation of egg-grown influenza C virus and analyzed by h.p.l.c. As shown in Figure 2b the original preparation of sialic acid consisted mainly of Neu5,9Ac$_2$. Only a small peak of Neu5Ac is visible. After incubation with influenza C virus the ratio of the two derivatives of neuraminic acid was reversed, the peak of Neu5Ac being now predominant (Figure 2a). This result confirms that an enzyme is associated with influenza C virus which according to the enzyme nomenclature is designated N-acyl-O-acetylneuraminate O-acetylhydrolase (EC 3.1.1.53). This enzyme appears to be specific for influenza C virus, because neither purified influenza A virus [strain FPV/Rostock/34 (H7N1)] grown in eggs nor the allantoic fluid of uninfected eggs were able to convert Neu5,9Ac$_2$ to Neu5Ac. That the esterase is a genuine property of influenza C virus and not of influenza A virus could also be demonstrated in an assay in which the release of acetic acid from BSM was monitored (Figure 3). The specific activity of the O-acetylesterase at pH 7.5 was determined to be 0.7 U using Neu5,9Ac$_2$ as substrate. The initial rate of hydrolysis of the O-acetyl group from Neu4,5Ac$_2$ was ~ 30 times lower than from Neu5,9Ac$_2$, whereas the O-acetyl group of Neu5,7Ac$_2$ was resistant to hydrolysis under these conditions (Figure 4). These observations indicate that the enzyme cleaves preferentially 9-O-acetyl groups.

Discussion

The results presented above demonstrate that neuraminate O-acetylesterase is associated with influenza C virus. Release of O-acetyl groups from Neu5,9Ac$_2$ was the only effect on sialic

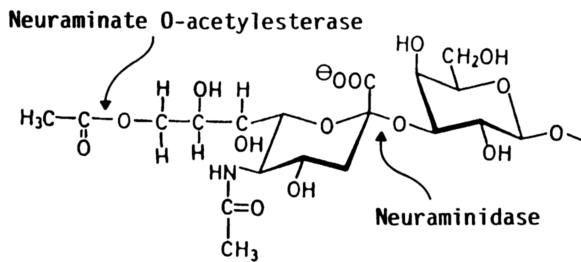

Fig. 5. The sites of action of neuraminate O-acetylesterase and neuraminidase. Sialic acid is shown to be connected with galactose via an α2-3 linkage. Neuraminidase may also cleave α2-6 linkages between sialic acid and galactose, and α2-8 linkages between two sialic acid residues.

acids of RMG detectable after incubation with influenza C virus. The neutral sugar residues have recently been shown to be unaffected by this treatment. As reduction of the HI-activity of BSM by incubation with influenza C virus was also correlated with the conversion of Neu5,9Ac$_2$ to Neu5Ac, it is evident that inactivation of the haemagglutination-inhibitors BSM and RMG is due to neuraminate O-acetylesterase activity associated with influenza C virus. It can be inferred that this enzyme is also responsible for the inactivation of the receptors for influenza C virus on chicken erythrocytes and therefore constitutes the receptor-destroying enzyme of this virus. Neu5,9Ac$_2$ was shown to be the preferred substrate for the acetylesterase of influenza C virus. The presence of the 9-O-acetyl group on sialic acid appears also to be a specific requirement for the inhibitory activity of RMG. This is supported by the observation that alpha$_2$-macroglobulin from horse serum, which contains Neu4,5Ac$_2$ (Pepper, 1968), has no HI-activity for influenza C virus (unpublished observations).

Since it is now evident that Neu5,9Ac$_2$ mediates the binding of the haemagglutination inhibitors RMG and BSM to influenza C virus, it is reasonable to conclude that Neu5,9Ac$_2$ serves also as the receptor for this virus on erythrocytes and other cells. This concept provides an explanation for previous observations, which seemed to argue against an involvement of sialic acid in the binding of influenza C virus. As Neu5,9Ac$_2$ is known to be oxidized by periodate at a greatly reduced rate compared to Neu5Ac (Haverkamp *et al.*, 1975), it can now be understood why the receptors for influenza C virus on chicken erythrocytes are more resistant to periodate treatment than the receptors for influenza A and B viruses (Ohuchi *et al.*, 1978). Similarly, the reduced rate of hydrolysis of O-acetylated sialic acids by neuraminidases (Schauer, 1982) explains the finding that the receptors for influenza C virus on chicken erythrocytes are not as easily destroyed by bacterial neuraminidases as the receptors of the other influenza viruses (Herrler *et al.*, 1985a). The resistance of the receptors for influenza C virus to the action of viral neuraminidases (Hirst, 1950; Kendal, 1975) may be due to the substrate specificity of these enzymes which has been reported to be narrow compared to bacterial neuraminidases (Corfield *et al.*, 1981).

The only viruses, which have been reported to contain RDE, are influenza viruses and some paramyxoviruses. Except for influenza C virus, RDE of all these viruses was found to be neuraminidase, an exoglycosidase also found in bacteria and animal tissue that releases terminal sialic acid residues from oligosaccharides. As mentioned above the rate of hydrolysis by various neuraminidases is determined in part by the substituents present on sialic acid, e.g. O-acetyl groups at positions C-4, C-7, or C-9. Neuraminidases differ also in their substrate specificities depending on linkage between sialic acid and the adjacent sugar residue by an α2-3, α2-6, or α2-8 bond. The available evidence indicates that in general bacterial neuraminidases have a wider substrate specificity than viral neuraminidases (for review see Corfield *et al.*, 1981). RDE of influenza C virus acts also on sialic acid as substrate. However, in contrast to the neuraminidases, it does not cleave the glycosidic linkage, but removes an acetyl group from the sugar (Figure 5). It should be noted that neuraminate O-acetylesterases have also been observed in man and other vertebrates (Shukla *et al.*, 1984). All of these enzymes release 9-O-acetyl but not 7-O-acetyl groups. Some of them also hydrolyze 4-O-acetylated sialic acids. The occurrence of acetylesterases acting on other sugars has been reported with a variety of phages (Geyer *et al.*, 1983). None of these enzymes has been purified to homogeneity.

The acetylesterase of influenza C virus should be useful for analysis of the biological role of Neu5,9Ac$_2$. Sialic acids acetylated at position C-9 have been reported to occur in various tissues of different species. Thus, sialic acids of human B lymphocytes have been shown to consist primarily of Neu5,9Ac$_2$, whereas T lymphocytes contain only Neu5Ac (Kamerling *et al.*, 1982). Neu5,9Ac$_2$ appears therefore to be a specific surface marker for B lymphocytes, and the acetylesterase of influenza C virus may help to elucidate whether this is of biological significance. Attempts to characterize the purified neuraminate O-acetylesterase are in progress. Future work should also provide direct evidence for the involvement of Neu5,9Ac$_2$ in binding of influenza C virus to cells.

Materials and methods

Virus

Strain Johannesburg/1/66 of influenza C virus was grown in embryonated eggs as described previously (Herrler *et al.*, 1979). Strain FPV/Rostock/34 (H7N1) of influenza A virus was propagated in 11-day-old embryonated eggs.

Virus purification

Virus was purified by zonal centrifugation as described by Chucholowius and Rott (1972).

Assay for HI-activity

HI-activity of RMG and BSM was determined as described previously (Herrler *et al.*, 1985b).

Isolation of sialic acids from RMG and BSM following incubation with influenza C virus

Samples of RMG were incubated in the presence or absence of purified influenza C virus as described by Herrler *et al.* (1985b). After sedimentation of influenza C virions by ultracentrifugation, the supernatant was assayed for HI-activity and dialysed against distilled water. Samples were dried in a vacuum centrifuge and dissolved in 200 μl of 10 mM sodium acetate, pH 5.0. Each sample was incubated in the presence of 10 mU of neuraminidase from *A. ureafaciens* for 3 h at 37°C. Incubation was followed by dialysis against 30 ml of distilled water. The water was changed twice at intervals of 12 h. The dialysates were applied on anion-exchange columns of Dowex 50, 2 × 8 (6 ml). Sialic acids were eluted with 1 M formic acid, followed by lyophilization.

To samples containing 5 mg of BSM (Boehringer-Mannheim, FRG) in 200 μl of phosphate-buffered saline (PBS), 100 μl of the same buffer was added containing various amounts of a purified preparation of influenza C virus with a HA-titer of ~4000 HA-units/ml (0, 1, 10 and 100 μl, respectively). After incubation for 90 min at 37°C, virions were removed by ultracentrifugation for 30 min at 100 000*g*. The supernatant of each sample was assayed for HI-activity and dialyzed against distilled water. After addition of NH_4HCO_3 to a concentration of 0.1 M, samples were incubated for 2 h at 37°C in the presence of 50 mU of neuraminidase from *C. perfringens*. Samples were dried in a vacuum centrifuge. Sialic acids of the dried residue were dissolved in 10 μl of a mixture of propanol and water (7:3) and analyzed by t.l.c.

Rate of hydrolysis of O-acetyl groups from different sialic acids by influenza C virus

Samples (50 μl) containing 1 mM sialic acid in 50 mM Tris-HCl buffer, pH 7.5, were mixed with 50 μl of a purified preparation of influenza C virus (~2000 HA-units/ml) diluted 1:50 with 50 mM Tris-HCl buffer and incubated at 37°C. After different time intervals samples were analyzed by h.p.l.c.

Hydrolysis of the O-acetyl group of Neu5,9Ac$_2$ by influenza C virus

A sample (20 µl) containing 1 mM Neu5,9Ac$_2$ in 50 mM Tris-HCl buffer, pH 7.5, was mixed with 20 µl of a purified preparation of influenza C virus (~2000 HA-units/ml) and incubated at 37°C for 15 min. The incubation mixtures were directly analyzed by h.p.l.c.

Assay of acetic acid released from BSM by incubation with influenza C virus

Samples containing 5 mg of BSM in 200 µl of PBS were incubated in the presence of 10 µl of a purified preparation of egg-grown influenza A virus (strain FPV/Rostock/34) or influenza C virus (strain Johannesburg/1/66). Both virus preparations had an optical density of 0.75 at 280 nm. After various times at 37°C, acetic acid released was determined enzymatically using a commercial test kit (Boehringer-Mannheim, FRG). A sample of BSM incubated with influenza C virus at 4°C served as a control. The value of the control sample was subtracted from the samples incubated at 37°C.

High-performance liquid chromatography (h.p.l.c.)

Sialic acids were analyzed by h.p.l.c. on an anion-exchange resin (Aminex A-29) as described by Shukla and Schauer (1982). Sialic acid was eluted isocratically by 0.75 mM sodium sulfate at a flow-rate of 0.5 ml/min and 15 bar. The eluate was monitored at 200 nm.

Thin-layer chromatography (t.l.c.)

T.l.c. of sialic acids of RMG was performed on cellulose plates which were prerun in 0.1 M HCl and dried under a current of air at room temperature for 30 min. Sialic acids were chromatographed using the solvent system n-butanol-n-propanol-0.1 M HCl (1:2:1, v/v). T.l.c. of sialic acids of BSM was performed on plates of silica gel 60 (Merck, Darmstadt, FRG) using the solvent system n-propanol-water (7:3, v/v). Bands of sialic acids were visualized with a resorcinol/Cu^{2+} spray reagent (Schauer, 1978).

Gas-liquid chromatography (GLC)

G.l.c. was performed as described by Schauer *et al.* (1984).

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