Action of influenza virus neuraminidase on gangliosides

Haemagglutinin inhibits viral neuraminidase

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The action of partly purified neuraminidase (NA) of influenza A virus, a mixture of detergent solubilized NA and haemagglutinin (HA) and of intact virions on gangliosides G_{T1b}, G_{D1a}, G_{D1b}, G_{M1} was studied. The viral NA transformed G_{T1b} mainly into G_{D1b} with formation of only minor amounts of G_{M1}. HA was found to inhibit the hydrolysis activity of viral NA. At the same time viral NA transformed G_{D1a} quantitatively into G_{M1} which was not hydrolyzed by the enzyme. These results suggest that the function of NA is to transfer the 'primary' receptor (such as G_{T1b}) into the proper carbohydrate sequence (G_{D1b}-like) which is proposed to serve as the minimal structure required for influenza virus reception.

Influenza virus receptor *Viral neuraminidase* *Ganglioside*

1. INTRODUCTION

Recent investigations revealed that gangliosides are implicated in influenza virus reception by EAC cells [2,3]. This conclusion was based on the following facts.

(i) NA-treated cells were resistant to viral infection, but incubation of cells with exogenous gangliosides fully restored their virus binding capacity.

(ii) Among the gangliosides tested only those with terminal galactose residues or containing the sequence NeuAc α2,3-Gal were effective as viral attachment sites.

(iii) Only G_{D1b} and G_{T1b} were able to induce virus penetration into the cell.

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Abbreviations: NA, neuraminidase; PPNA, partially purified neuraminidase; HA, haemagglutinin; IV, intact influenza A virus; SRID, single radial immunodiffusion; ganglioside abbreviations according to Svennerholm [1]

However, the interaction of influenza viruses and their two envelope glycoproteins, HA and NA, with cell surface receptors in vitro, has not been previously described. Therefore here, the activity of viral neuraminidase against individual gangliosides was examined using partly purified NA (PPNA), purified particles of influenza A virus (IV) and a mixture of solubilized HA and NA in the native ratio.

2. MATERIALS AND METHODS

2.1. *Virus and viral glycoproteins*

Influenza A virus, strain A/Leningrad/385/80 (H3N2) was used. The virus was propagated in embryonated eggs and purified as described in [4]. From 2 l of virus-containing allantoic fluid with a protein content of 34 mg/ml and a haemagglutinin titre 1:512, 40 ml of purified virus suspension was obtained (haemagglutinin titre, 1:64000; protein content, 4.8 mg/ml; haemagglutinin content as estimated by SRID [5] 1.6 mg/ml; neuraminidase activity, 0.22 U/ml).

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Both NA and HA were solubilized from purified virus using the detergent octyl-β-D-glucopyranoside [6]. After centrifugation the supernatant had the following characteristics: protein content, 0.8 mg/ml; haemagglutinin content, 0.7 mg/ml; neuraminidase activity, 0.15 U/ml; this preparation was designed as 'natural mixture of HA-NA'.

Detergent-solubilized HA-NA mixtures were separated according to [7]. The PPNA fraction showed the following characteristics: protein content, 0.75 mg/ml; neuraminidase activity, 0.28 U/ml; haemagglutinin content, less than 0.6 mg/ml.

2.2. *Analytical procedures*

Proteins were determined by a modification [8] of the method of Lowry. The HA content of viral components and whole virus was assayed by SRID [5]. The NA activity was determined according to standard methods using fetuin as substrate [9]. 1 unit of neuraminidase activity was defined as the amount of activity required to release 1 μmol NeuAc from fetuin per min.

2.3. *Gangliosides*

Gangliosides from bovine brain (G_{M1}, G_{D1a}, G_{D1b}, G_{T1b}) were purified by column chromatography using DEAE-Sephadex A-25 [10] and preparative thin-layer chromatography (TLC) [11].

2.4. *Kinetic studies of enzyme activity*

Assay of gangliosides hydrolysis by IV or NA preparations was conducted as follows: the reaction mixture (1 ml) contained 330 nmol ganglioside and 20 mU NA (in form of virus, PPNA or NA-HA) in phosphate-buffered saline (pH 7.4). When PPNA or NA-HA mixtures were used, octylglucoside (0.5%) was added. After incubation at 37°C for appropriate periods, the amount of released NeuAc was assayed by the thiobarbituric acid method [12]. To identify the hydrolysis products, the reaction mixture was evaporated under N_2, the residue was dissolved in $CHCl_3$/MeOH (2:1, v/v), and the solution subjected to TLC on a precoated silica gel 60 plate (Merck, Darmstadt), using a mixture of $CHCl_3$/MeOH/H_2O (55:45:10, v/v, containing 0.02% $CaCl_2$) [13] as developing system. NeuAc and gangliosides were detected by the resorcinol-HCl reagent. Gangliosides were quantitatively estimated by scanning the chromatograms with an Opton densitometer at 580 nm.

3. RESULTS

In preliminary experiments the hydrolysis of gangliosides was evaluated by determining chromatographically the glycolipid composition after incubation of PPNA, the NA-HA mixture or IV with individual gangliosides. In these experiments G_{D1b} and G_{M1} were not hydrolysed to any significant extent. Results obtained with G_{T1b} and G_{D1a} are presented in table 1. As can be seen from the table, significant differences were observed between the three NA preparations. PPNA converted G_{T1b} during 6 h almost quantitatively into a 9:1 mixture of G_{D1b} and G_{M1}. The NA-HA

Table 1

Action of various NA preparations and IV on gangliosides G_{T1b} and G_{D1a}

Substrate	NA preparation	Incubation time (h)	Ganglioside composition after incubation (%)			
			G_{M1}	G_{D1b}	G_{D1a}	G_{T1b}
G_{T1b}	PPNA	6	9	87	nd[a]	4
	NA-HA mixture	6	7	62	nd	31
	Virus	6	traces	29	nd	71
G_{D1a}	PPNA	2	87.7	–	12.3	–
	NA-HA mixture	2	89.6	–	10.4	–
	Virus	2	23.6	–	76.4	–

[a] nd, not detected

mixture was less active: only 69% of G_{T1b} was cleaved during the same time; the ratio of G_{D1b}/G_{M1} formed was again 9:1. With IV the only cleavage product was G_{D1b} (20%) and the major part of the starting ganglioside remained unaltered.

When G_{D1a} was used as substrate the only cleavage product was G_{M1}. Again the reaction proceeds faster with PPNA and slower with the NA-HA mixture or the intact virus. The cleavage rate of G_{T1b} expressed as the increase of the G_{D1b}/G_{T1b} ratio or the amount of sialic acid released after different time intervals is shown in fig.1,2. The data clearly show that the NA-HA mixture cleaves G_{T1b} much slower than PPNA. It should be noted that when G_{T1b} was digested with the NA-HA mixture or IV the amount of released sialic acid measured directly was consistently lower than that calculated from the ganglioside composition of the incubation mixture (fig.2b,c). Such differences were not seen with PPNA (fig.2a).

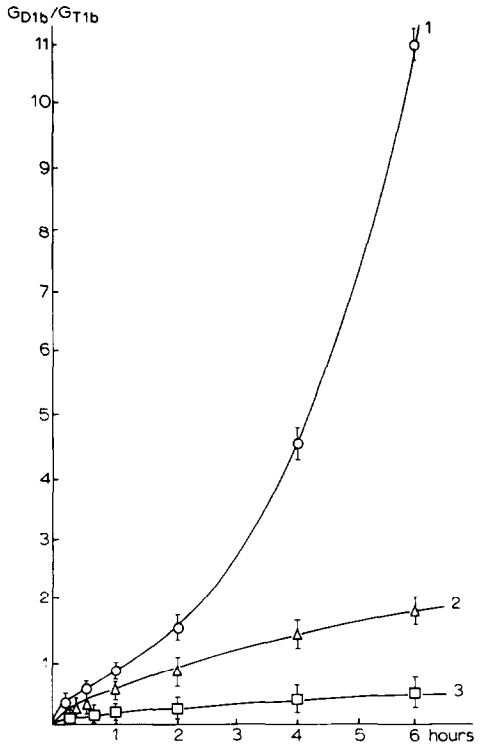

Fig.1. Hydrolysis rate of ganglioside G_{T1b} by IV and NA-preparations expressed as the G_{D1b}/G_{T1b} ratio in the incubation mixture. (1) PPNA; (2) NA-HA mixture; (3) IV.

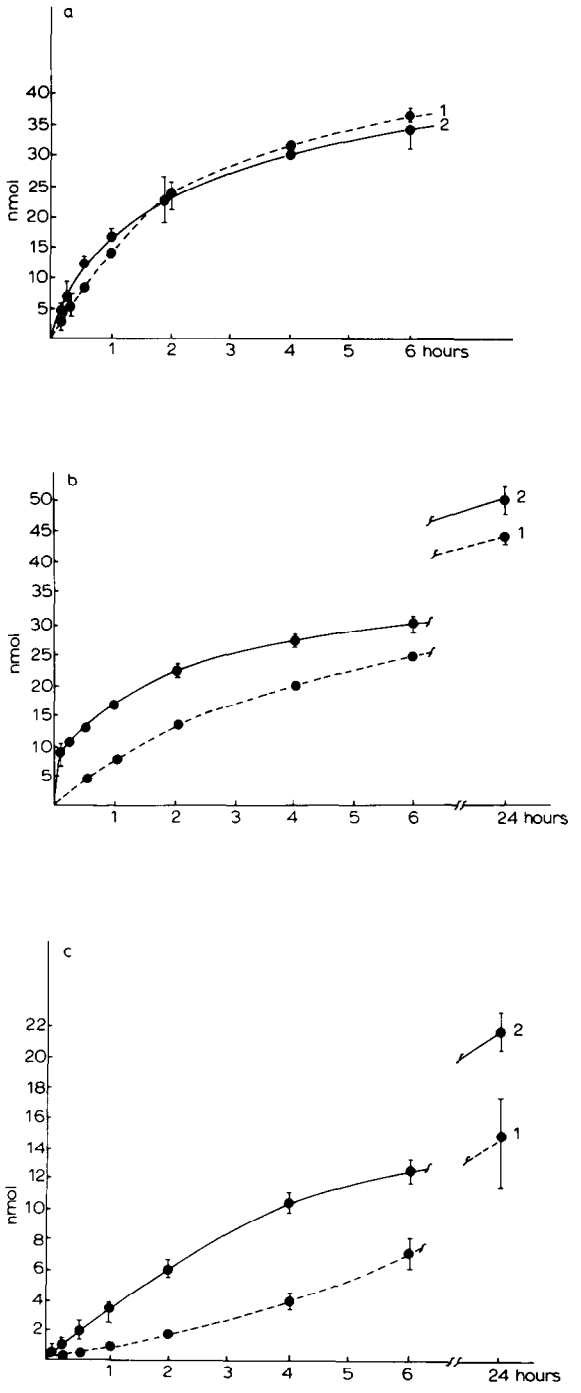

Fig.2. Rate of sialic acid release during incubation of ganglioside G_{T1b} with IV and NA-preparations. (1) Free sialic acid measured according to Warren [12]; (2) sialic acid released as calculated from the ganglioside composition of the incubation mixture. (a) PPNA; (b) NA-HA mixture; (c) IV.

4. DISCUSSION

The gangliosides investigated in the present study represent structures with sialic acid attached $\alpha(2-3)$ to terminal and internal galactoses (G_{D1a}) and $\alpha(2-8)$ to a second sialic acid residue (G_{T1b} and G_{D1b}). The data obtained are in general agreement with previous results, which revealed a marked specificity of influenza virus NA for substrates containing $\alpha(2-3)$-linked sialic acid in the terminal position of the chain, whereas $\alpha(2-8)$-linked sialic acid is poorly attacked [14,15].

The sialidase activity of the intact virus is difficult to compare with that of PPNA and the NA-HA mixture because the latter two preparations contain β-octyl-glucoside. However comparison of the activities of PPNA and NA-HA mixture is legitimate as both preparations contain about equal quantities of detergent. Such comparison shows unequivocally that PPNA hydrolyses gangliosides much faster than the NA-HA mixture (table 1, fig.2). Thus, HA appears to inhibit the hydrolysis of gangliosides G_{T1a} and G_{D1a}. The mechanism of this inhibitory effect is not clear. It should be noted that part of the sialic acid released upon NA treatment of G_{T1b} in the presence of excess HA, apparently binds to some component of the incubation mixture, whereas such binding is not observed with partly purified NA, i.e., when the HA content is very low (fig.2).

The results presented here demonstrate that the main cleavage product of G_{T1b} is G_{D1b}. These gangliosides both have been shown to induce influenza virus penetration, whereas both G_{D1a} and its cleavage product G_{M1} do not possess such ability [2,3]. It thus seems that the oligosaccharide chain of G_{D1b} is the minimal structure required for influenza virus penetration. The information presently available leads to the conclusion that the influenza virus receptor (or receptor complex) must contain exposed galactose residues [16] as well as sialic acids (review, [17]) accessible to the virus. The structure of G_{D1b} meets both these requirements. That the presence on the cell surface of a sialic acid $\alpha(2-8)$ linked to another sialic acid residue which in turn is attached to an internal galactose is of crucial importance for virus penetration is underlined by the fact that G_{M1} having a terminal galactose but only one sialic acid $\alpha(2-3)$ attached to the internal galactose does not stimulate virus penetration significantly [2]. Although the results here are still insufficient to provide a full picture of the interaction of the influenza virus with cell surface gangliosides, they demonstrate that both HA and NA are involved in a cooperative manner in the binding to and modification of the influenza virus receptor. As is well known, HA is responsible for binding of the virion to the cell surface receptor, and the cleaved form of HA is involved in the fusion of the viral and host membranes. The role of viral NA is still less clear. The enzymes function may include removal of neuraminic acid from a 'primary' receptor and thus unmasking a 'secondary' receptor for the smaller hydrophobic unit of HA which induces the fusion process. In view of the results presented here it is tempting to speculate that the primary receptor is a glycoprotein or a polysialoganglioside and the secondary one is a ganglioside of the G_{D1b} type.

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