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ROLE OF SIALIC ACID IN CELL RECEPTORS FOR VESICULAR STOMATITIS VIRUS

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Summary. — The role of sialic acid in the receptors for vesicular stomatitis virus (VSV) was investigated by treating chick embryo related (CER) cells and goose erythrocytes with neuraminidases from different sources and studying the effects produced on both infection and haemagglutination. The virus attachment to CER cells was reduced by their treatment with *C. perfringens* and *A. ureafaciens* neuraminidases and the susceptibility to viral infection of desialylated CER cells was recovered by coating with gangliosides immediately after enzymatic treatment. By contrast, enzymatic treatment of goose erythrocytes enhanced their agglutinability by VSV. The experiments carried out to test the ability of sialoglycolipids (gangliosides) and asialoglycolipids (cerebrosides) to inhibit virus attachment to CER cells or to goose erythrocytes showed that only gangliosides were able to inhibit VSV infection and haemagglutination.

Key words: vesicular stomatitis virus; sialic acid; receptors

Introduction

VSV can infect a wide variety of cells (Clark, 1979) and is able to agglutinate goose and human erythrocytes (Cohen and Summers, 1974). As to the chemical nature of cellular receptors involved in the interaction with virus, the participation of both lipid (Seganti *et al.*, 1983; Schlegel *et al.*, 1983; Superti *et al.*, 1984) and carbohydrate components (Seganti *et al.*, 1982; Sinibaldi *et al.*, 1984) has been suggested, but the role of sialic acid in the cellular binding sites has not yet been elucidated. Schloemer and Wagner (1975) reported that N-acetylneuraminic acid is not the essential part of the cell receptors for VSV, the virus being able to attach to L cells desialylated by treatment with *V. cholerae* neuraminidase. Similar results were obtained in our previous experiments with BHK 21 cells, while treatment with the same enzyme enhanced VSV binding to goose erythrocytes (Seganti *et al.*, 1982). Finally Mastromarino *et al.* (1984) observed only a slight reduction of VSV infection in CER cells treated with limulin, a lectin from *Limulus polyphemus*, which is known to interact with N-acetylneuraminic acid (Roche and Monsigny, 1979).

In this report an attempt has been made to investigate the role of sialic acid in cellular binding sites for VSV on CER cells and goose erythrocytes. For this purpose the cells were treated with different neuraminidases capable of releasing terminal neuraminic acid from membrane glycoproteins and glycolipids and characterized by a specific action on defined ketosidic linkages. The resulting modifications in the infectivity and agglutinability of cells were then studied. Moreover, we present the results of experiments carried out to test the ability of sialoglycolipide and asialoglycolipids to inhibit viral attachment to CER cells and to goose erythrocytes.

Materials and Methods

Virus. Indiana serotype of VSV was propagated in CER cells, infected with 5 PFU/cell and incubated for 20 hr at 37 °C in Eagle's MEM containing 2 mmol/l glutamine, penicillin (100 IU/ml and streptomycin (100 μg/ml). Supernatants were then collected, centrifuged (3000 rev/min for 20 min) to remove cellular debris and stored at −70 °C. In haemagglutination (HA) experiments, concentrated preparations of virus were used. They were obtained by pelleting viral particles by centrifugation at 80,000 g for 2 hr at 4 °C in Beckman Spinco model L2 6B ultracentrifuge using a SV40 rotor.

Cells. CER cells were grown at 37 °C in Eagle's MEM containing 1.2 g of $NaHCO_3$ per litre and supplemented with 10% calf serum (Flow, U.K.), glutamine (2 mmol/l), penicillin (100 IU/ml) and streptomycin (100 μg/ml) as described by Smith *et al.* (1977). Goose red blood cells (RBC) were washed (3×) in phosphate buffered saline (PBS) pH 7.4 for 10 min at 4 °C and then suspended to a 5% concentration.

Enzymes. Stock solutions of enzymes (Sigma Chemical Co.) were prepared in PBS as follows: neuraminidase from *C. perfringens* 20 U/ml; neuraminidase from *A. ureafaciens* 1 U/ml; trypsin from bovine pancreas 1.10^5 U/ml. Stock solutions of neuraminidase from *V. cholerae* (Behringwerke) and neuraminidase from influenza virus (Sigma Chemical Co.) were made up in PBS with 2 mml/l $CaCl_2$ at the concentrations of 1 U/ml and 100 U/ml, respectively.

Glycolipids. Crude mixtures of gangliosides were prepared from mouse brain material as described by Svennerholm and Fredman (1980). The individual gangliosides used in this work (GM1, GD1a and GT1b) were isolated and purified according to Ghidoni *et al.* (1980) from bovine brain. Cerebrosides type I (from bovine brain) were purchased from Sigma Chemical Co.

Immunofluorescence. Virus-infected cell monolayers were washed with PBS, pH 7.4 and fixed in absolute acetone for 5 min at −20 °C. After having been covered with anti-VSV rabbit serum for 45 min at 37 °C, the cells were washed with PBS and stained for 45 min at 37 °C with fluorescein isothiocyanate-conjugated anti-rabbit-γ-globulin antibodies (Behring Institute). Then the cells were washed again with PBS, mounted into glycerine and viewed through a fluorescence microscope. The results were expressed as the percentage of infected cells in treated and untreated cultures.

HA test. The agglutinability of treated and untreated goose RBC was tested at 4 °C using microplates as previously described by Halonen *et al.* (1974). For this purpose 25 μl of concentrated VSV were two-fold diluted in borate albumin buffer saline (BABS) pH 9; 25 μl of BABS and 50 μl of a 0.6% RBC suspension in phosphate buffer (pH 5.4) were added to each well. The agglutinability of cells was expressed as percentage of HA of untreated control erythrocytes.

Neuraminidase treatments of CER cells monolayers. CER cells grown in microtissue culture chamber/slide (Miles Laboratory) for 24 hr at 37 °C in 5% CO_2 were treated with various concentrations of different neuraminidases. After one hr incubation at 37 °C, supernatants were collected to determine the released sialic acid, according to the Aminoff procedure (1959) and cell monolayers were washed three times with Eagle's MEM to remove traces of the enzymes. Then the cells were inoculated with VSV (1.5 and 0.15 PFU/cell) and incubated for 1 hr at 37 °C. The two multiplicities of infection (MOIs) used (1.5 and 0.15 PFU/cell) had been shown to infect approximately 100% and 50% of the untreated cells. The virus inoculum was removed and the cells were washed 3 times with Eagle's MEM. The infected cultures were incubated for 8 hr at 37 °C in 5% CO_2 and the percentage of infected cells was determined by immunofluorescence (in parallel cultures).

Neuraminidase treatment of RBC. Neuraminidase treatments were carried out on goose RBC at a 5% concentration in PBS. After 1 hr incubation at 37 °C with the enzymes, samples were centrifuged at 1,000 rev/min for 10 min and sialic acid in the supernatants was determined as described before. Then RBC were newly washed three times and used for HA test.

Combined neuraminidase and trypsin treatment of RBC. Goose RBC suspended in PBS at a 5% concentration were incubated with trypsin ($1 . 10^4$ U/ml) for one hr at 25 °C. In the collected supernatants the protein content was estimated according to Lowry *et al.* (1951) and the cells were washed three times with PBS to remove traces of the enzyme. Then RBC were newly suspended in PBS at a 5% concentration and treated with neuraminidase from *C. perfringens* (1 U/ml) for 1 hr at 37 °C. After incubation, supernatants were collected to estimate the release of sialic acid and RBC washed three times with PBS were used for HA test.

Competition between CER cells and glycolipids for VSV binding. CER cells grown in microtissue culture chamber/slide were infected with mixtures of gangliosides or cerebrosides and VSV. Virus-glycolipid mixtures were applied to cell monolayers either immediately or after 2 hr incubation at 25 °C. After one hr at 37 °C, viral inoculum was removed and the cells were washed three times with Eagle's MEM. The infected cultures were incubated at 37 °C in 5% CO_2 for 8 hr and the percentage of infected cells was determined by immunofluorescence.

Competition for VSV binding between RBC and glycolipids. Haemagglutination inhibition (HI) test was carried out at 4 °C using microplates. Gangliosides and cerebrosides were two-fold diluted in BABS pH 9 and 25 µl of a VSV suspension in BABS containing 4 haemagglutination units (HAU) were added to each well. After 2 hr incubation at 4 °C, 50 µl of a 0.6% suspension of goose RBC in phosphate buffer (pH 5.4) were added to each well. The inhibitory titre was calculated as the reciprocal of the highest dilution of inhibitors which caused 50% of HI.

Glycolipid coating of sialidase treated CER cells. CER cells, grown in microtissue culture chamber/slide, were treated with 10 U/ml neuraminidase from *C. perfringens* for 1 hr at 37 °C. After incubation, monolayers were washed three times with Eagle's MEM to remove traces of

Table 1. Action of neuraminidases from different sources on VSV attachment to CER cells

Neuraminidases (origin)	Concentration (U/ml)	Fluorescence (%)	NANA released (µg/ml)
None	—	100	0
V. cholerae	0.065	100	9.1
	0.125	100	12.2
	0.25	90	14.5
	0.5	80	15.5
Influenza virus	2.5	90	1
	5	90	1.6
	10	80	2
	20	70	3.6
C. perfrigens	2.5	35	15.2
	5	30	16.5
	10	10	17.5
	20	10	17.5
A. ureafaciens	0.065	80	12.5
	0.125	70	14.6
	0.25	25	16
	0.5	5	20.1

enzyme and incubated with 30 μg/ml of ganglioside mixtures or isolated gangliosides (GM1, GD1a, GT1b) or cerebrosides. After 20 min at 37 °C, the cell monolayers were washed and inoculated with VSV (1.5 PFU/cell). Virus inoculum was removed after one hr at 37 °C and the cells were washed three times with Eagle's MEM. The cultures were incubated for 8 hr at 37 °C in 5% CO_2 and the percentage of infected cells was determined by immunofluorescence.

Results

Effect of neuraminidase on VSV attachment to CER cells

Results concerning the action of neuraminidases from various sources on CER cell surface and the subsequent variation of their susceptibility to VSV infection are shown in Table 1, demonstrating the amount of sialic acid released by the enzymatic treatment and the percentage of infected cells. It can be noticed that only a small quantity of sialic acid was cleaved influenza virus neuraminidase, whereas the values of sialic acid released by the other neuraminidases were quite similar. As to the effect on viral multiplication, treatment with *V. cholerae* and influenza virus neuraminidases did not cause any significant fluorescence inhibition. In contrast, a strong reduction in the percentage of infected cells was noticed following the treatment with *C. perfringens* and *A. ureafaciens* neuraminidases. To exclude the possibility that the enzymes had some effect on permissiveness of cells follow-

Table 2. Effect of neuraminidases from various origin on the agglutinability of goose RBC by VSV

Neuraminidases (origin)	Concentration (U/ml)	HA titre (%)	NANA released (μg/ml)
None	—	100	0
V. cholerae	0.0065	100	0
	0.0125	200	1.8
	0.025	400	2.5
	0.05	600	6.4
Influenza virus	0.25	200	1.5
	0.5	200	1.7
	1	400	4.3
	2	400	4.7
C. perfringens	0.25	200	3.1
	0.5	300	4
	1	400	4.9
	2	400	6.7
A. ureafaciens	0.0065	200	1
	0.0125	400	2.8
	0.025	400	3
	0.05	500	4.1

Table 3. Action of gangliosides from mouse brain and cerebrosides on VSV infection in CER cells

Inhibitor concentration (μg/ml)	Fluorescence (%) gangliosides (a)	gangliosides (b)	cerebrosides (a)	cerebrosides (b)
—	100	100	100	100
140	50	35	100	100
700	40	20	100	100

(a) Inhibitors were added to CER cells at the time of infection.
(b) Inhibitors were preincubated with VSV for 2 hours at 25 °C before infection.

ing virus attachment, controls were used by adding the enzymes after infection. Under these conditions both treated and untreated cell cultures exhibited a similar percentage of infected cells.

Effect of neuraminidases on goose RBC agglutinability by VSV

Goose RBC suspended in PBS were submitted to the same enzymatic treatment and the effect on the agglutinability by VSV is reported in Table 2. All neuraminidases tested produced a noticeable enhancement of RBC agglutinability by VSV, reaching up to six times higher values than those obtained in the controls with untreated RBC. This effect was probably due to the drastic reduction of the negative charge of erythrocytes as already postulated (Seganti *et al.*, 1982). Another possible hypothesis was that the major membrane erythrocyte glycoprotein component, i.e. the glycophorin, did not allow the access of the neuraminidase to the glycolipid compounds which are present in a low percentage in RBC membranes (Gahmberg, 1981). To check this, RBC were treated with trypsin (1×10^4 U/ml) and successively with neuraminidase from *C. perfringens* (1 U/ml), which had already shown its capacity to produce a marked reduction in VSV infectivity. It was noticed that treatment with trypsin alone produced a four-fold enhancement of

Table 4. Action of gangliosides from mouse brain and cerebrosides on VSV haemagglutination

Inhibitors (3,500 μg/ml)	Haemagglutination inhibition titre
none	0
gangliosides	48
cerebrosides	0

Table 5. Effect of ganglioside and cerebroside coating of sialidase-treated CER cells on VSV infection

CER cell treatment	Concentration	Fluorescence %
—	—	100
neuraminidase from *C. perfrigens*	10 U/ml	20
neuraminidase from *C. perfringens* + gangliosides	10 U/ml 30 μg/ml	80
neuraminidase from *C. perfringens* + GM1	10 U/ml 30 μg/ml	80
neuraminidase from *C. perfringens* + GD1a	10 U/ml 30 μg/ml	60
neuraminidase from *C. perfringens* + GT1b	10 U/ml 30 μg/ml	70
neuraminidase from *C. perfringens* + cerebrosides	10 U/ml 30 μg/ml	20

agglutinability and that this result was further increased by the subsequent action of neuraminidase.

Competitive binding between susceptible cells and glycolipids for VSV

In order to obtain additional information on the role of sialic acid in VSV binding to CER cells and to goose RBC, experiments were performed in which gangliosides and cerebrosides could compete for VSV attachment. CER cells were infected with mixtures of glycolipids and virus to the inhibitory effect of sialoglycolipids (gangliosides) and asialoglycolipids (cerebrosides). Virus-glycolipid mixture were either applied to CER cells immediately after being prepared or after 2 hr incubation at 25 °C (Table 3). Gangliosides were found to inhibit VSV infectivity (80% inhibition was achieved in preincubated ganglioside-virus mixtures at a 0.7 mg/ml concentration) whereas with cerebrosides no effect was observed. In these experiments, controls were included consisting of CER cells incubated with glycolipids one

hr before and one hr after the viral infection. All these controls gave results similar to those obtained with untreated CER cells.

The ability of the same glycolipids to inhibit VSV haemagglutination was also tested by pre-incubation with virus for 2 hr before the addition of RBC suspension. The results obtained showed no inhibition by cerebrosides, while gangliosides inhibited VSV haemagglutination up to the concentration of 140 μg/ml (Table 4).

Effect of glycolipid coating of neuraminidase-treated CER cells

On the basis of all the results reported, showing the participation of membrane sialylated compounds in VSV-cell interaction, a series of experiments was carried out treating CER cells with 10 U/ml of neuraminidase from *C. perfingens* and successively incubating the same cells with ganglioside mixtures or individual gangliosides (GM1, GD1a, GT1b) or cerebrosides. This procedure allowed the coating of desialylated cells with glycolipids, according to Markwell *et al.* (1981). The results reported in Table 5 show that the addition of gangliosides to desialylated cells produced an almost complete recovery of susceptibility to VSV infection, while no recovery was obtained with cerebrosides.

Discussion

It is well known that neuraminidases react primarily with carbohydrates, releasing terminal neuraminic acid from membrane glycoproteins and glycolipids and that the action of neuraminidase from different sources can vary on the basis of their specificity. *V. cholerae* neuraminidase hydrolyses both 2—6' and 2—3' ketosidic linkages (Gottschalk and Drzeniek, 1972); neuraminidase from influenza virus hydrolyses 2—3' ketosidic linkage between N-acetylneuraminic acid and N-acetylglucosamine (Gottschalk and Drzeniek, 1972); neuraminidase from *C. perfringens* hydrolyses GM1 and GM2 gangliosides when these glycolipids are monomeric, whereas this action is sterically hindered when gangliosides are aggregated (Rauvala, 1979); neuraminidase from *A. ureafaciens* hydrolyses 2—3', 2—6' and 2—8' linkages of the N-acetylneuraminic acid residues with a high specific action on ganglioside GM1 (even in absence of surfactant) (Sugano *et al.*, 1978).

Cell treatment with neuraminidase from *V. cholerae* and from influenza virus caused only a slight inhibition of VSV attachment to CER cells, whereas a low per cent of infected cells was observed after the action of *C. perfringens* and *A. ureafaciens* neuraminidases. It must be underlined that these last enzymes have a specific action on GM1 ganglioside which is believed to be resistant to various neuraminidases of viral, bacterial or mammalian origin (Sugano *et al.*, 1978; Rauvala, 1979). *V. cholerae* and influenza virus neuraminidases do not release sialic acid from GM1 by reason of the steric hindrance of the terminal carbohydrate structure of this ganglioside (Rauvala, 1979; Suzuki *et al.*, 1980).

The effective role of GM1 and GM1-like structures in VSV binding to CER cells was verified by coating *C. perfringens* neuraminidase treated cells with

different purified glycolipids and by testing the recovery of their susceptibility to VSV infection. Data obtained demonstrated the inability of cerebrosides to restore this susceptibility, whereas after coating with GM1, GT1b and GD1a the percentage of recovery was 80, 70 and 60 respectively. In fact, none of these sialoglycolipids showed an exclusive role in VSV-CER cell interaction, but all these data confirm the finding that VSV binding to CER cells takes place on receptor sites where sialoglycoconjugate structures are involved.

As to the interaction of VSV with goose RBC, it was shown that the agglutinability of these erythrocytes was not decreased by the neuraminidases tested. The virus binding was even enhanced by the enzyme treatment, probably as a consequence of the reduction of the cell surface net negative charge due to acidic oligosaccharide anionic residues located on glycophorin (Nicolson, 1973). The hypothesis of a possible unmasking of sialoglycolipid receptors was not supported by the results of experiments carried out by treating with neuraminidase trypsin-treated RBC. In fact, even in this case, an increase in the agglutinability of RBC by VSV was observed.

It can be concluded the neuraminidase treatment of host cells reduces their sensitivity to VSV infection and that the spatial arrangement of sialic acid molecules in cell receptors is essential. Host cell receptors appear quite different from determinants involved in RBC receptors for VSV, in which N-acetylneuraminic acid is not essential for virus binding. Taking into consideration the fact that erythrocytes do not support viral replication and that their interaction with VSV is clearly restricted to attachment, it may be suggested that sialic acid on CER cells is necessary for tight binding of virus particles in order to allow their penetration.

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