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INVOLVEMENT OF CARBOHYDRATES IN VESICULAR STOMATITIS VIRUS-CELL EARLY INTERACTION

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Summary. — The role of N-acetylneuraminic acid and N-acetyl-D-glucosamine containing molecules in vesicular stomatitis virus-cell interaction was studied using specific lectins (limulin and wheat germ agglutinin) and esoglycosidases (neuraminidase, β-galactosidase, α-mannosidase, α-fucosidase, β-N-acetyl-D-glucosaminidase). Lectin treatment of vesicular stomatitis virus (VSV) indicated that carbohydrates of the VSV G envelope glycoprotein were not required for virus infectivity, whereas sialic acid appeared directly involved in the attachment of virus to erythrocytes. The comparative results obtained after enzymatic digestion of cell membrane carbohydrates or their cross linking by lectins demonstrated that whereas VSV infectivity was strongly reduced by pretreatment of chick embryo cells, virus binding to erythrocytes was unaffected by such treatments. We conclude that sugar residues may participate at the host cell attachment site which differs, at least in part, from the membrane binding site of erythrocytes.

Key words: *carbohydrates*; *VSV recognition unit*; *cell membrane receptor*; *lectins*; *enzymes*

Introduction

Virus attachment to host cells is mediated by specific plasma membrane bindings sites. Several data indicate that carbohydrates are involved in the binding of viral recognition units (Kennedy, 1974; McSharry *et al.*, 1978) and cell membrane receptors (Mori *et al.*, 1962; Yasui *et al.*, 1971; Burness and Pardoe, Armstrong *et al.*, 1984).

In vesicular stomatitis virus (VSV) the viral envelope glycoprotein G is responsible for the attachment of virus to cells and for haemagglutination and haemolytic activities. Using synthetic peptides corresponding to the NH_2 terminus of VSV G protein, Schlegel (1986) demonstrated that posi-

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tively charged amino acids of this region mediate viral haemagglutination (HA) and haemolysis. However, viral binding to sensitive cells seems to be due to a different functional domain of G protein suggesting that different recognition mechanisms are involved in VSV HA and host cell attachment. The G envelope glycoprotein contains approximately 10% carbohydrate by weight arranged in two oligosaccharide chains (McSharry, 1979). Carbohydrate composition of G glycoprotein greatly affects viral infectivity and HA (Schloemer and Wagner, 1974, 1975; Gibson *et al.*, 1978). Glycoconjugates also appear involved in cell membrane receptors for VSV on both erythrocytes (Superti *et al.*, 1986; Mastromarino *et al.*, 1987) and cells supporting viral replication (Conti *et al.*, 1988).

Lectins possessing an affinity for specific carbohydrate structures (Lis and Sharon, 1973) may interact reversibly with either virions or cellular receptors and thereby influence attachment of virus. Limulin is a lectin which specifically binds to sialic acid containing glycoproteins or glycolipids (Roche and Monsigny, 1979) whereas wheat germ agglutinin (WGA) has a specific affinity for N-acetyl-D-glucosamine residues (Nagata and Burger, 1974). In order to clarify the role of N-acetylneuraminic acid (NANA) and N-acetyl-D-glucosamine (glcNAc) containing molecules in VSV-cell interaction, we treated CER and goose erythrocytes with limulin and WGA and assessed infectivity, HA and haemolysis. The importance of carbohydrates in the cell membrane receptor for VSV was further analysed by studying the effects of enzymatic modification of the cell surface on subsequent virus attachment.

Materials and Methods

Virus and cells. VSV, Indiana serotype, was propagated in CER (chicken embryo related cells) grown in Eagle's MEM supplemented with 6% foetal calf serum (FCS). For haemagglutination (HA) and haemolysis (HE) the virus was pelleted at 80,000 g for 2 hr and resuspended in bovine albumin borate saline buffer (BABS) pH 9.0.

Plaque assay. Control or lectin treated virus was incubated for 1 hr at 37 °C and then inoculated on cell monolayers. After adsorption (1 hr, 37 °C or 4 °C), the inoculum was removed by washing three times with PBS and the cells were incubated until plaques developed (48 hr, 37 °C).

Haemagglutination and haemolysis were carried out using 1% goose erythrocytes as previously described (Mastromarino *et al.*, 1987).

Lectin treatment of CER and erythrocytes. Confluent monolayers of CER grown in 6-well plates were washed twice in PBS and treated with WGA (in PBS pH 7.2) or limulin (in TBS, pH 7.2, 0.15 mol/l NaCl, 0.05 mol/l Tris, 0.1 mol/l $CaCl_2$) for 1 hr at 37 °C. After incubation, the cells were washed three times with PBS and assayed for VSV plaque forming ability.

Lectin treatments were carried out with a 10% suspension of goose erythrocytes washed three times in PBS. After incubation for 1 hr at 23 °C with various concentrations of lectins, the cells were washed three times with PBS and tested for HA and HE activities.

Enzyme treatment of CER and erythrocytes. Enzymatic treatments were performed on CER in suspension rather than on monolayers where glycosidases caused dislodgement of attached cells. Confluent monolayers were detached with EDTA; the cells were resuspended in Eagle's MEM, centrifuged at 170 g for 10 min and washed twice in PBS. Cells were then diluted in PBS at a concentration of 1.8×10^6 cells/ml and incubated in the presence or absence of neuraminidase (NA) for 1 hr at 37 °C. The supernatants were collected to determine sialic acid release according to the method of Aminoff (1959). Cells, washed in PBS, were then digested with β-galactosidase, α-mannosidase, α-fucosidase, β-N-acetyl-D-glucosaminidase for 1 hr at

23 °C. After washing three times in PBS, 2 ml of CER, diluted at 6×10^5 cells/ml with MEM containing 2% FCS, were mixed with 200 µl of viral dilutions and incubated in 6-well plates at 37 °C for 2 hr. The medium was then removed and infected cell monolayers were overlayed with MEM containing 1% agar.

Enzymatic treatments of goose erythrocytes were carried out using a 1% suspension washed three times in PBS. The cells were incubated in the presence or absence of enzymes for 1 hr at 37 °C (neuraminidase) or 23 °C (β-galactosidase, α-mannosidase, α-fucosidase, β-N-acetyl-D--glucosaminidase). They were collected by centrifugation and washed three times with PBS before being used for virus HA and HE studies.

Chemicals and enzymes. Sialic acid, D-mannose, L-fucose, D-galactose, and N-acetyl-D-glucosamine were purchased from Sigma. α-mannosidase (from *Canavalia ensiformis*), α-fucosidase (from beef kidney), β-galactosidase (from *Escherichia coli*), and β-N-acetyl-D-glucosaminidase (from beef kidney) were obtained by Boehringer Mannheim. Neuraminidase from *Cl. perfringens* was purchased from Sigma. Wheat germ agglutinin (from *Triticum vulgaris*) and limulin (from *Limulus polyphemus*) were from Sigma.

Results

Effect of lectin treatment of the virus

To verify the role of viral sialic acid and glcNAc on the biological activities of VSV, virus was treated with limulin or WGA before testing infectivity, HA, and HE. Results in Table 1 show that the infectivity of the virus was not affected by lectin treatments and HA activity was not modified after preincubation with WGA. On the contrary, the capacity of VSV to bind to goose erythrocytes and to fuse with them at acidic pH was greatly reduced after treatment with limulin. This effect was dose-dependent.

Effect of lectin treatment of cells

The importance of cell membrane carbohydrates in the interaction with VSV was investigated by treating CER monolayers and goose erythrocytes

Table 1. Infectivity, haemagglutination, and haemolysis of lectin-treated VSV

Lectin	Concentration (µg/ml)	Infectivity* (per cent)	HA titre**	Haemolysis** (per cent)
Limulin	0	100	1024	100
	25	98	1024	72
	100	112	384	42
	250	135	192	31
WGA	0	100	1024	ND
	25	97	1024	ND
	100	91	1024	ND
	250	93	1024	ND

* Virus (2.7×10^7 PFU/ml) was incubated with lectins for 1 hr at 37 °C, ten-fold diluted and tested for plaque forming ability on CER monolayers.

** VSV was treated with lectins for 1 hr at 23 °C, followed by 5 hr at 4 °C before addition of goose erythrocytes.

ND = Not determined

Table 2. Susceptibility of lectin-treated cells to VSV infection, haemagglutination and haemolysis

Lectin	Concentration (μg/ml)	Infectivity* (per cent)	HA titre**	Haemolysis** (per cent)
Limulin	0	100	32	48
	50	49	32	57
	125	28	64	62
	300	19	ND	ND
WGA	0	100	32	50
	2.5	80	32	50
	12.5	42	ND	ND
	50	18	ND	ND
	125	10	ND	ND
	250	9	ND	ND

* CER monolayers were treated for 1 hr at 37 °C with lectins before VSV infection.
** 10% suspension of goose erythrocytes was incubated with lectins for 1 hr at 23 °C before the haemagglutination and haemolysis tests.
ND = Not determined

with lectins before addition of the virus. As shown in Table 2 the capacity of VSV to replicate in cells in which membrane sialic acid and glcNAc residues were cross-linked by the lectins was strongly reduced. On the contrary, preincubation of erythrocytes with limulin produced a slight increase in the binding and fusion activity of the virus.

The effect of limulin and WGA on CER infected cells was verified to rule out the possibility that lectins interferred with metabolic cell functions necessary for viral replication. In this case different effects were observed

Table 3. Effect of limulin and WGA on CER infected cells

	Concentration (μg/ml)	Per cent infectivity	
		37 °C	4 °C
Limulin	0	100	100
	125	105	96
	300	97	60
WGA	0	100	100
	125	114	53
	250	121	35

After incubation with virus for 1 hr at 37 °C or 4 °C CER monolayers were washed with PBS and treated with lectins for 1 hr at 37 °C.

Table 4. Effect of enzyme on cell susceptibility to VSV infection, haemagglutination, and haemolysis

	Concentration (U/ml)	Infectivity* (per cent)	HA titre	Haemolysis (per cent)
Control	0	100	32	53
Neuraminidase	2	71	128	70
	4	44	384	89
	8	32	768	100
Neuraminidase +	2			
α-mannosidase +	0.95			
β-galactosidase +	36			
α-fucosidase	0.16	60	128	71
Neuraminidase +	2			
α-mannosidase +	0.95			
β-galactosidase +	36			
α-fucosidase	0.16			
β-N-acetyl-D-glucosaminidase	0.28	42	128	73

CER and goose erythrocytes were digested with NA alone or in combination with other glycosidases.

* The amounts of enzymes used were the maximal that did not produce modification of cell viability as verified by trypane blue exclusion.
Sialic acid released from membranes by action of NA was 0.018, 0.026, 0.029 μmoles for CER, respectively, and 0.030, 0.035, 0.038 μmoles for erythrocytes, respectively.

when the adsorption of virus on the cell membrane was performed at 37 °C or 4 °C (Table 3). The addition of lectins immediately after viral infection at 37 °C did not produce any inhibitory effect, demonstrating that only early events of viral infectious cycle were affected. When WGA and limulin were added to cells after VSV binding at 4 °C, a significant reduction of viral infectivity was observed (Table 3). This effect could be related to the displacement of virus from the cell surface by the lectins because of a stronger affinity of WGA and limulin for the membrane carbohydrates.

Effect of enzymatic digestion of cell membrane on the infectivity, HA, and HE

The results reported have suggested the role of sialic acid and glcNAc as a part of the cell membrane receptor for VSV. To confirm the importance of these molecules, CER and goose erythrocytes were digested with NA alone or in combination with other glycosidases and the sensitivity of modified cells to VSV was studied. The results shown in Table 4 indicated that after NA treatment of CER the infectivity of the virus was reduced in a dose-dependent manner. Digestion of neuraminidase-treated CER with α-mannosidase, β-galactosidase, and α-fucosidase produced a reduction in

virus infectivity. When β-N-acetyl-D-glucosaminidase was added to the mixture of glycosidases a significant further reduction was observed. However, the isolated purified carbohydrates (sialic acid, D-mannose, α-L-fucose, D-galactose, N-acetyl-D-glucosamine) at 50 mmol/l were ineffective in inhibiting both viral infectivity and HA (data not shown). In control experiments the addition of enzymes to infected CER monolayers did not modify virus replication.

NA treatment of erythrocytes (Table 4) greatly increased their sensitivity to viral HA and HE. Removal of carbohydrates from NA-treated erythrocytes did not produce modification in binding and fusion activities of VSV suggesting that these molecules are not involved in virus membrane receptors of red blood cells. This is supported by the observation that periodate pretreatment (1 mmol/l) of goose erythrocytes did not produce any effect on their sensitivity to viral induced HA and HE (data not shown).

Discussion

Conflicting results have been reported concerning the role of viral carbohydrates in the process of VSV attachment to cells. Schloemer and Wagner (1974, 1975) showed that after NA digestion HA and infectivity of VSV were strongly reduced. On the contrary, Cartwright and Brown (1977) could not demonstrate any effect of DNA digestion on viral infectivity. Furthermore, the small amount of carbohydrate-free virions produced in the presence of tunicamycin were as infectious about as normal virions (Gibson *et al.*, 1978).

In our experiments we used lectins with affinity for specific carbohydrates to study the role of VSV sialic acid and N-acetyl-D-glucosamine in the interaction between virus and cell membranes. Results obtained suggest that the viral carbohydrates are not required for infectivity, since pretreatment of VSV with limulin and WGA did not affect virus plaque forming ability. N-acetyl-D-glucosamine residues of the envelope glycoprotein did not appear important for the binding to goose erythrocytes, since HA activity of the virus was not modified after WGA treatment. The strong reduction in the capacity of the virus to produce HA and HE after limulin treatment, on the contrary, indicate that sialic acid of the G protein of VSV can be directly involved in the attachment of virus to erythrocytes. A similar hypothesis has been suggested by McSharry *et al.* (1978) who demonstrated that virions grown in HKCC cells possessed a higher sialic acid content which correlated with a higher specific HA activity. Comparison of VSV receptors on goose erythrocytes and CER using both lectins and enzymes indicate that carbohydrates are differently involved in the two systems.

The presence of oligosaccharide residues on CER membrane appeared important for viral binding and replication since their cross-linking by the lectins or removal by enzymatic digestion produced a reduction in viral infectivity. Active carbohydrate residues removed by glycosidase treatment are probably part of surface glycolipid moiety since proteins and glycoproteins extracted from cell membranes were unable to affect VSV infectivity

(Conti *et al.*, 1988) or HA (Mastromarino *et al.*, 1987). However, it was not possible to ascertain whether these sugars represent a part of the cell membrane receptor for the virus, or if their role is indirect, i.e. maintaining a suitable steric conformation of other cell surface components directly involved in the binding with VSV. It has been reported that phospholipids and gangliosides (Viti *et al.*, 1987; Conti *et al.*, 1988) extracted from the membrane of sensitive cells are capable of interfering with the attachment of VSV to cell surface receptors and that desialilated gangliosides lose their activity. However, up to now no receptor molecules bearing a fundamental carbohydrate sequence have been isolated.

The involvement of erythrocyte membrane carbohydrates as VSV receptors seems unlikely. The strong increase in binding and fusion of VSV to limulin and NA-treated erythrocytes could be due to a decrease of the electrostatic repulsive forces between virus particles and the cell membrane. This is supported by the observation that polycation DEAE-dextran increased the binding of VSV to the cell membrane, probably by neutralizing the negative charge on the cell surface (Bailey *et al.*, 1984). In conclusion, the data reported here suggest that VSV recognition units for CER and erythrocyte membrane receptors are related to discrete regions of the G glycoprotein and that host cell receptors differ, at least in part, from the membrane binding sites of erythrocytes.

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