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SUSCEPTIBILITY OF MAMMALIAN, AVIAN, FISH, AND MOSQUITO CELL LINES TO RABIES VIRUS INFECTION

[1]L. SEGANTI, [1]*F. SUPERTI, [1]S. BIANCHI, [1]N. ORSI, [2]M. DIVIZIA, [2]A. PANÀ

[1]Istituto di Microbiologia, Facoltà di Medicina, Università "La Sapienza", Roma and [2]Dipartimento di Sanità Pubblica, Facoltà di Medicina, Università Tor Vergata, 00100 Roma, Italy

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Summary. — The relationship between plasma membrane receptor organization and cell susceptibility *in vitro* was investigated in mammalian, avian, fish, and arthropod cell lines infected with fixed rabies virus. IMR32, HeLa, CER, and EPC cells were widely susceptible to infection with CVS virus, whereas a lower level of specific viral antigens was detectable in *A. albopictus* cells. In spite of these differences, the amount of infectious virus particles bound to the various cell surfaces was similar. Competition experiments carried out with plasma membranes extracted from all the cell types and their lipidic fractions demonstrated the ability of these components to bind the virus and to prevent infection. The different cellular permissiveness to rabies infection described here did not correlate with significant differences in number or in chemical structure of the receptor binding sites, but more likely with events following virus adsorption.

Key words: *rabies virus*; *receptors*; *permissiveness*

Introduction

It is well known that in the early stages of natural infection rabies virus replicates only in the striated muscle and successively it migrates through nerve endings and synaptic junctions to the central nervous system (Iwasaki *et al.*, 1985; Tsiang, 1985).

Although rabies virus seems to be strictly neuropathogenic *in vivo*, it is able to infect a wide range of host cells *in vitro* (Wunner and Reagan, 1986). High titres of fixed virus can be obtained in the cell lines BHK21, NIL/2, CER, and in neuroblastoma cell lines of murine and human origin (Clark, 1972). The propagation of rabies virus has also been demonstrated in cells of poikilothermic vertebrates such as Tokai gecko, side necked turtle (Clark, 1981), lizard (Atanasiu *et al.*, 1973), snakes of the families of *Crotalidae* and *Vi-*

*Present address: Laboratorio di Ultrastrutture, Istituto Superiore di Sanità.

peridae (Clark, 1981) and from fathead minnow (Solis and Mora, 1970). Moreover Reagan and Wunner (1985) were able to detect rabies virus specific antigens in Singh's *Aedes albopictus* clone C6/36 cells although the yield of infectious virus was extremely low.

Whether rabies virus uses a common receptor-mediated pathway for viral entry both *in vivo* and *in vitro* or recognizes more than one type of receptor on host cells is not known. It has been suggested that acetylcholine receptors may serve as cellular specific binding sites for rabies virus on differentiated myotubes *in vitro* and at neuromuscular junctions *in vivo* to facilitate uptake and transfer of virus to the CNS (Lentz *et al.*, 1982). However, not all cells susceptible to rabies virus infection *in vitro* express acetylcholine receptors (Reagan and Wunner, 1985), suggesting that different host cell receptors can bind rabies virus *in vitro* and *in vivo*.

The host cell plasma membrane exhibits a wide range of molecular structures which can be further rabies virus receptor candidates. The pre-eminent role of phospholipids and glycolipids is demonstrated by the following experimental data: i) human serum lipoproteins are inhibitors of viral haemagglutination (Halonen *et al.*, 1974; Suzuki *et al.*, 1977) and infectivity (Seganti *et al.*, 1983), ii) cellular L-alpha-phosphatidylserine and some highly sialilated gangliosides are involved in the binding of virus to plasma membranes (Superti *et al.*, 1984*b*; 1986), iii) phospholipase A2 or neuraminidase treatment prevents the infection of CER cells by rabies virus (Superti *et al.*, 1984*b*; 1984*a*).

These findings so far failed to demonstrate a true biological receptor specificity *in vitro* so that a nonspecific rabies virus adherence to cell surfaces has also been hypothesized (Wunner and Reagan, 1986). Moreover no data are available on the chemical components of receptors which allow the binding of rabies virus to less susceptible homeothermic cells and to poikilothermic cells. In this report additional information is provided on the early interaction between rabies virus and different phylogenetically unrelated host cells. For this purpose a comparative study has been carried out on the susceptibility of cell lines of mammalian (IMR32) (HeLa), avian (CER), fish (EPC), and arthropod (*A. albopictus*) origin to fixed rabies virus infection. This study has been performed following different approaches, i.e. by evaluating viral antigen synthesis and virus yield, by measuring the rate of attachment of the virus to the cells and by analysing the chemical components involved in the binding.

Materials and Methods

Virus. The CVS (Challenge Virus Strain) fixed rabies virus was propagated in BHK21 cells. Monolayers were infected with 5PFU/cell for 72 hr at 37 °C in Eagle's MEM containing 0.2% BSA (Bovine Serum Albumin), 2 mmol/l glutamine, penicillin (100 U.I./ml), and streptomycin (100 μg/ml). After infection supernatants were collected, centrifuged (5 000 rev/min for 10 min) to remove cellular debris and stored at — 70 °C.

Cells. IMR32 (human neuroblastoma cells), HeLa S3 (epithelioid carcinoma of human cervix), and CER (Chicken Embryo Related) cells were grown as monolayers at 37 °C in Eagle's MEM

containing 1.2 g of $NaHCO_3$ per litre and supplemented with 2 mmol/l glutamine and 10% foetal calf serum for human cells or newborn calf serum for avian cells. EPC (*Epithelioma Papulosum Cyprini*) cells were grown at 26 °C in Eagle's MEM containing 1.2 g of $NaHCO_3$ per litre and supplemented with 20% foetal calf serum and 2 mmol/l glutamine. *Aedes albopictus* (*mosquito larvae*) cells were propagated at 26 °C in Mitsuhashi-Maramorosch medium supplemented with 20% foetal calf serum.

Monolayer infection. Monolayers were incubated with rabies virus (1.5 PFU/cell or 15 PFU/cell) at 33 °C or 37 °C and various parameters of the infection of the different cell lines were monitored for 72 hr. The percentage of infected cells was measured by direct immunofluorescence and the production of infectious virus was determined by plaque assay titration of supernatants from the infected cells. Plaque forming units/ml were tested 5 days after infection in CER cells and stained with neutral red 1 : 5000.

Preparation of cell membranes. Cell membranes were prepared according to a modified procedure of Krah and Crowell (1982). Cells were pelletted (900 g × 15 min) and washed three times with 0.02 mol/l phosphate buffer containing 0.14 mol/l NaCl, pH 7.0 (PBS). The pellet was suspended in hypotonic buffer (0.02 mol/l phosphate pH 7.0) at a concentration of 4×10^7 cells/ml and allowed to swell for 15 min. Cells were disrupted with 30 strokes in a 15 ml glass Dounce homogenizer equipped with a tight-fitting "B" pestle. Cell breakage was monitored microscopically. The homogenate was centrifuged (900 g × 5 min) to remove nuclei and remaining cells. The supernatant fluid was centrifuged at 70 000 g for 60 min in a Beckman ultracentrifuge (L2-65B) equipped with a SW40 rotor. The pelletted membranes were washed in PBS, resuspended in PBS at a final concentration of 100 μg/ml lipids and stored at −50 °C.

Extraction of protein and lipid components from the cell membrane preparations. Protein components were obtained from membranes by extraction with organic solvents according to the method proposed by Shore and Shore (1967). Proteins obtained were dissolved in PBS at a final concentration of 50 μg/ml. Lipids were extracted with a mixture of chloroform and methanol 1 : 1 (v/v) according to the technique of Sperry and Brand (1955) and suspended in PBS at a final concentration of 50 μg/ml.

Isolation of membrane total lipids, phospholipids, and glycolipids. Other experiments were performed by extracting total lipids from all membranes with chloroform and methanol 1 : 1.5 (v/v) according to the procedure proposed by Folch *et al.* (1957). Extracted lipids were dried under liquid nitrogen and to obtain phospholipids and glycolipids the dried residues were dissolved in chloroform and methanol 2 : 1 (v/v) and partitioned by adding 0.2 vol distilled water. Phospholipids (lower phase) and glycolipids (upper phase) were solubilized in PBS by sonication.

Competition for rabies virus binding between CER cells and membranes or membrane components. CER cells grown in microtissue chamber/slide for 24 hr at 37 °C in 5% CO_2 were infected with mixtures of inhibitors and rabies virus. The virus-inhibitor mixtures were either applied to cell monolayers immediately of after 2 hr incubation at 25 °C. After 1 hr at 37 °C, the inocula were removed and the cells were washed 3 times in Eagle's MEM. The infected cultures were incubated at 37 °C in 5% CO_2 for 24 hr and the percentage of infected cells was determined by direct immunofluorescence.

Neuraminidase treatment of cell monolayers. The different cells, grown in microtissue chamber/slide (Miles Laboratory) for 24 hr at 37 °C or 26 °C were treated with neuraminidase from *C. perfringens* (20 U/ml) (Sigma Chem. Co.) for 60 min at 37 °C. After incubation, supernatants were collected for the determination of sialic acid content and cell monolayers were washed 3 times with Eagle's MEM to remove traces of the enzyme. Cells were then infected with CVS (1.5 PFU/cell and 15 PFU/cell) for 1 hr at 37 °C. The percentage of infected cells was determined by immunofluorescence 24 hr post-infection (p.i.) for homeothermic cells and 72 hr p.i. for poikilothermic cells.

Chemical determinations. Protein concentration was determined by the method of Lowry *et al.* (1951) using serum albumin as standard. Lipid concentration was determined by the phosphovanillin procedure described by Frings *et al.* (1974) using olive oil as standard. Sialic acid was determined by the method of Svennerholm *et al.* (1980) using n-acetyl neuraminic acid as standard.

Immunofluorescence. Rabies virus infected cells, dried in acetone, were stained with fluorescein-isothiocyanate-conjugated rabbit antirabies nucleocapsid IgG (Institut Pasteur Production). The results were examined through a fluorescence microscope and expressed as the percentage of infected cells (Superti *et al.*, 1984*a*).

Table 1. Comparison of rabies virus grown in IMR32, HeLa, CER, EPC, and A. Albopictus (A.a.) cells

			IMR32	HeLa	CER	EPC	A.a.
Fluorescent cells (%)	24 hr	33 °C	55	80	90	5	10
		37 °C	55	85	90	35	10
	48 hr	33 °C	40	30	90	5	10
		37 °C	30	80	90	30	30
	72 hr	33 °C	10	15	40	0	0
		37 °C	10	30	50	30	30
Virus yield (PFU/ml)	24 hr	33 °C	1.0×10^3	1.0×10^3	4.1×10^4	1.5×10	0
		37 °C	5.0×10^3	4.1×10^3	9.5×10^4	1.0×10^2	0
	48 hr	33 °C	1.0×10^4	9.5×10^3	1.5×10^5	1.5×10	0
		37 °C	1.0×10^4	9.5×10^3	1.5×10^5	1.5×10	0
	72 hr	33 °C	5.0×10^4	4.1×10^4	1.4×10^5	1.0×10^2	0
		37 °C	5.0×10^4	4.1×10^4	1.5×10^5	1.5×10	0

Results

Multiplication of rabies virus in homeothermic and poikilothermic cell lines

The comparative study of rabies virus multiplication was performed in HeLa, CER, EPC, and *A. albopictus* cells which were chosen for their mammalian, bird, fish, and arthropod origin, respectively, and in human neuroblastoma cells (IMR32) for their neuronal origin.

Monolayers of homeothermic cell lines (IMR32, HeLa, CER) and of poikilothermic cell lines (EPC and *A. albopictus*) were infected with CVS fixed rabies virus at a multiplicity of infection of 1.5 PFU/cell and 15 PFU/cell, respectively. Infected monolayers were incubated in duplicate at 33 °C and at 37 °C. At different times p.i. (24 hr, 48 hr, 72 hr) cells were stained to assess viral antigen synthesis by immunofluorescence assay and aliquots of supernatants were withdrawn in order to verify virus yield by plaque count assay. Results reported in Table 1 show that all cells tested were susceptible

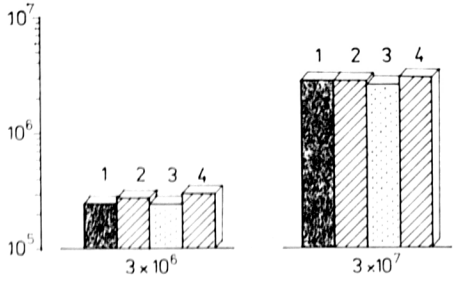

Fig. 1
Rabies virus attachment to HeLa (1), CER (2), EPC (3), and *A. albopictus* cells (4) by evaluation of unadsorbed virus after 1 hr at 0 °C.
Abscissa: rabies virus added (PFU/ml).
Ordinate: unadsorbed rabies virus (PFU/ml).

Table 2. Inhibition of rabies virus attachment to CER cells by membranes and membrane components extracted from HeLa, CER, EPC, and Aedes albopictus cells

Cell origin	Components	Concentration (μg/ml) Proteins	Lipids	Fluorescence inhibition %
HeLa	Whole membranes	95	50	40
	Membrane proteins	100	—	0
	Membrane lipids	—	50	45
CER	Whole membranes	105	50	70
	Membrane proteins	100	—	0
	Membrane lipids	—	50	60
EPC	Whole membranes	150	50	80
	Membrane proteins	150	—	0
	Membrane lipids	—	50	70
Aedes	Whole membranes	150	50	75
	Membrane proteins	150	—	0
	Membrane lipids	—	50	80

to rabies virus infection, but viral antigen synthesis and virus yield were significantly different. Among homeothermic cells, CER were the most susceptible to virus infection and the highest production of virus was obtained 72 hr p.i. As to poikilothermic cells, when infection was performed at 37 °C it was possible to observe a 35% of infected cells in fish cell line after 24 hr and a 30% of fluorescent cells in *A. albopictus* cells after 48 hr. As to viral yield from these latter cells, we were unable to detect infecting virus particles in supernatants, while in supernatants of infected fish cells infectious virus was detected at both incubation temperatures from the 24th hour.

Rabies virus adsorption to cell monolayers

Virus binding was evaluated by measuring the rate of attachment to the cells at 0 °C. Experiments were performed in which rabies virus (1.5 PFU/cell or 15 PFU/cell) was added to HeLa, CER, EPC, and *A. albopictus* cells

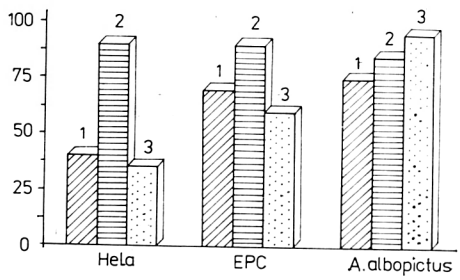

Fig. 2
Inhibition of rabies virus attachment to CER cells by lipid (1), phospholipid (2), and glycolipids (3) components (50 μg/ml) extracted from HeLa, EPC, and *A. albopictus* cell membranes.

grown in microtissue chamber/slides. After 1 hr at 0 °C the supernatants were collected to evaluate unbound virus by infecting CER cell monolayers. After removal of the unadsorbed virus the different cells were washed with cold medium and successively fresh medium containing 2% newborn calf serum was added. After 24 hr incubation at 37 °C specific viral antigen synthesis was measured in the infected cultures (Fig. 1). It can be seen that in spite of the marked differences in the permissiveness observed, the rate of attachment of rabies virus to the different cells was very similar.

Competition for rabies virus binding between CER cells and cell membranes or membrane components

A different experimental approach was followed by isolation of plasma membranes from the different cells and by extraction of their relative lipid and protein components. CER cells were infected with mixtures of virus and membranes or membrane components in order to test the ability of these fractions to bind the virus and to impede the infection. Results in Table 2 that all membrane preparations caused fluorescence inhibition. As to the effect of isolated components, protein molecules did not show any activity whereas lipid moieties always inhibited rabies virus infection. The highest values of fluorescence inhibition were obtained with lipids extracted from *A. albopictus* cell membranes.

Further experiments were performed in order to identify among plasma membrane lipid components those capable of interacting with rabies virus. Since many data previously reported on CER cell surface lipids involved in rabies virus interaction unequivocally demonstrated the participation of both phospholipids and glycolipids (Superti *et al.*, 1984*b*; 1986), these components were extracted from the membrane lipids of HeLa, EPC, and *A. albopictus* cells.

Fig. 2 shows the inhibition of rabies virus attachment to CER cells by whole lipids, phospholipids, and glycolipids extracted from HeLa, EPC, and *A. albopictus* plasma membranes. All fractions tested prevented rabies virus infection of CER cells. Whereas phospholipids extracted from different cells did not show significant differences, total lipids and glycolipids, extracted from mosquito and fish cells, were more active as compared to those extracted from human cells. To ascertain whether the activity of glycolipid fractions extracted from different cell membranes had to be referred to sialilated components, additional experiments were carried out. For this purpose the surface of different cell lines was modified by treatment with *C. perfringens* neuraminidase (20 U/ml). Sialic acid removal from plasma membranes caused a dramatic reduction in the susceptibility to virus infection of HeLa, CER, and EPC cells (80%, 85%, and 95% fluorescence inhibition, respectively) but not in *A. albopictus* cells.

Discussion

Permissiveness of mammalian, avian, fish, and arthropod cells to rabies virus infection was demonstrated by specific viral antigen synthesis, although

with significant differences in virus yield. Among the tested lines, CER cells, used for isolation and assay of rabies serogroup viruses (Smith *et al.*, 1977), were the most efficient system for CVS propagation. High titres of virus were produced 48 hr p.i. and the replication was poorly influenced by the incubation temperature in a range between 33 °C and 37 °C.

Mammalian cells (IMR32 and HeLa) exhibited a similar behaviour, but with a much lower virus yield. It must be noticed that, in spite of the neurotropism of rabies virus, human neuroblastoma cells were, among homeothermic cells, the least susceptible to the infection (*in vitro*). Neuroblastoma cells, on the other hand, have been reported to be several-fold more permissive to the infection than any other cells from nervous tissue (Tsiang *et al.*, 1983). In spite of the neurotropism of rabies virus, human neuroblastoma cells were, among homeothermic cells, the least susceptible to the infection (*in vitro*).

In poikilothermic cell lines maximum viral antigen synthesis was obtained at 37 °C whereas the highest virus yield could be observed in fish cells 24 hr p.i. at 37 °C and 72 hr p.i. at 33 °C. On the contrary, no virus was detectable in mosquito cell supernatants at both temperatures. The response of *A. albopictus* cells to rabies virus infection was comparable to that observed for vesicular stomatitis virus infection (Seganti *et al.*, 1986) and in agreement with Reagan and Wunner (1985), who suggested that *Aedes albopictus* (SAA) clone C6/36 could support a persistent infection by rabies virus. Because of the different behaviour of infected cell lines, a different rate of virus attachment to cell surfaces might be expected. The results obtained, however, demonstrated that the amount of rabies virus particles absorbed at 0 °C to the binding sites of different cell surfaces was comparable.

It is well known that rabies virus attachment to CER cells is mediated by specific phospholipid (Superti *et al.*, 1984*b*) and glycolipid (Superti *et al.*, 1984*a*; 1986) receptors and for this reason the chemical nature of binding sites of HeLa, EPC, and *A. albopictus* cells was investigated. Protein molecules extracted from all cell membranes did not show any inhibition activity, whereas lipid components obtained with different procedures always competed with CER cells for rabies virus binding. The highest activity was shown by total lipids from EPC and Aedes cells. As to phospholipids, no noticeable differences were observed and, independently from their origin, these compounds prevented rabies virus infection. The most significant difference was observed with glycolipid fraction extracted from mosquito cells which caused 95% fluorescence inhibition.

The results with neuraminidase treatment, in agreement to preceding reports (Superti *et al.*, 1984*a*; 1986), indicate the presence of glycosphyngolipids among active components of the glycolipid fraction in HeLa, CER and EPC cells and demonstrate that in mosquito cell line rabies virus attachment is independent from sialic acid residues. As to *Aedes albopictus* cells, it has also been reported that clones lacking sialic acid on their plasma membranes are susceptible to rabies virus infection (Reagan and Wunner, 1985). These data suggest that in mosquito cells different chemical components can act

as viral receptors; among these molecules a central role is probably played by other carbohydrates, as previously reported for vesicular stomatitis virus infection of arthropod cells (Seganti *et al.*, 1986).

In conclusion, the different permissiveness of homeothermic and poikilothermic cells to rabies virus infection *in vitro* does not appear to depend on quantitative or qualitative differences in receptorial structures, but on events following virus attachment to host cells.

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