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A 65-kDa trypsin-sensible membrane cell protein as a possible receptor for dengue virus in cultured neuroblastoma cells

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Dengue virus infects primary neurons in mouse experimental model and tissue culture cells of the central nervous system (CNS). In the present work, a mouse neuroblastoma cell line (N1E-115) and a human neuroblastoma cell line (SK-N-SH), susceptible to dengue virus infection were used to study the presence of cell membrane receptor for dengue-2. By day 5 postinfection (pi), viral antigen was detected by immunofluorescence in the cytoplasm and surrounding the nucleus of N1E-115 cells, while on day 7 pi, it was also present along neural extensions. Infection of N1E-115 cells was diminished with trypsin treatment but not with neuraminidase or endoglycosidase H. Partially purified cell membrane proteins from neuroblastoma cells were analyzed by the Virus Overlay Protein Blot Assay (VOPBA), and a single band migrating at 65 kDa was detected in mouse and human neuroblastoma cells but not in C6, a non-susceptible rat glial cell line which was included as a negative control. The 65 kDa protein was eliminated only when nitrocellulose membranes were treated with trypsin. Analysis of neuronal cell infection by dengue virus provides a useful tool to understand the nature of cellular receptors and mechanisms involved in the infection of the nervous system by dengue viruses.

Keywords: neuroblastoma cells; dengue virus infection; membrane cellular receptor

Introduction

Neurotropism and neurological disease in experimental animal models and in humans are frequently caused by flaviviruses, including dengue viruses (Monath, 1989). In epidemics of dengue fever and dengue hemorrhagic fever, some alterations of the central nervous system (CNS) have been reported and dengue virus has been recovered from cerebrospinal fluid of dengue fever patients (Lum *et al*, 1996). However, the human CNS pathology caused by dengue virus infection is unknown. In experimental infection in mouse, dengue virus replicates primarily in neurons but not in glial cells nor in vascular endothelium (Monath, 1989). In mouse, the infection is age-dependent and histologic analysis shows necrosis in different brain regions of infected animals and a mild inflammatory reaction (Imbert *et al*, 1994). In addition, in infected primary cultures of mouse CNS cells the virus has been detected primarily in neurons but not in astrocytes, and by immunocytochemical analysis, dengue virus antigen has been found distributed around the nucleus, in the cytoplasm and in neuronal extensions (Imbert *et al*, 1994). The small number of infected neurons, even in long term *in vitro* cultures, and the morphology of the susceptible cells suggest that infection could be mediated by a specific receptor expressed in a subset of neurons (Imbert *et al*, 1994). This observation agrees with data reported by Hase *et al* (1990), who documented differential susceptibility of neural cells to Japanese encephalitis virus. Previously, Daughaday *et al* (1981) described that infection of adherent human monocytes with dengue virus is mediated by two mechanisms: a trypsin-sensible receptor and a trypsin-resistant immune complex receptor. More recently, Rothwell *et al* (1996) reported that a protein of 100 kDa

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present in stromal cells may be involved in the early virus-cell interaction. The present work analyzes the infection of mouse and human neuroblastoma cell lines with dengue virus, and provides some experimental evidence on the presence of a cellular membrane protein as a possible receptor for dengue virus.

Results

Susceptibility of neuroblastoma cell lines to dengue virus infection

The mouse neuroblastoma N1E-115 cell line was susceptible to dengue virus infection. The N1E-115 cell line is a clone derived from the murine neuroblastoma C1300, and it produces catecholamines when it is induced to differentiate with DMSO or dbAMPc (Kimhi *et al*, 1976). Figure 1 shows the time-course of infection at different doses of virus; the infection is dose-dependent, and 10% of infected cells were detected at the third day postinfection with a MOI of 0.1 of dengue-2. With a MOI of 10, the number of infected cells reached more than 90%, however, some cells remained uninfected during the period of experiment (10 days), in fact, with a MOI of 1 and 0.1, the maximum level of infected cells was $83 \pm 13.7\%$ and $52 \pm 4\%$, respectively. When N1E-115 cells were growing exponentially they had a neuronal appearance with extensions resembling bipolar neurons (Figure 2a). By day 5 of infection viral antigen was evenly distributed in the cytoplasm and surrounding the nucleus (Figure 2b) and on day 7 post-infection, the viral antigen was additionally present along neurites (Figure 2c, arrowhead). Additionally, the human neuroblastoma SK-N-SH was susceptible to dengue virus infection and showed a similar pattern of infection as the N1E-115 mouse neuroblastoma cell line. However, the infection rate as judged by immunofluorescence was less than in mouse neuroblastoma. Under the same experimental conditions, a rat glial cell line (C6) was non-susceptible to dengue virus infection.

Effect of the enzymatic treatment of N1E-115 on the dengue virus infection

Cultures of mouse neuroblastoma cells (N1E-115) were treated in separated experiments with: (a) trypsin, (b) neuraminidase, and (c) an endoglycosidase H. Table 1 shows that by days 5 and 7, only the

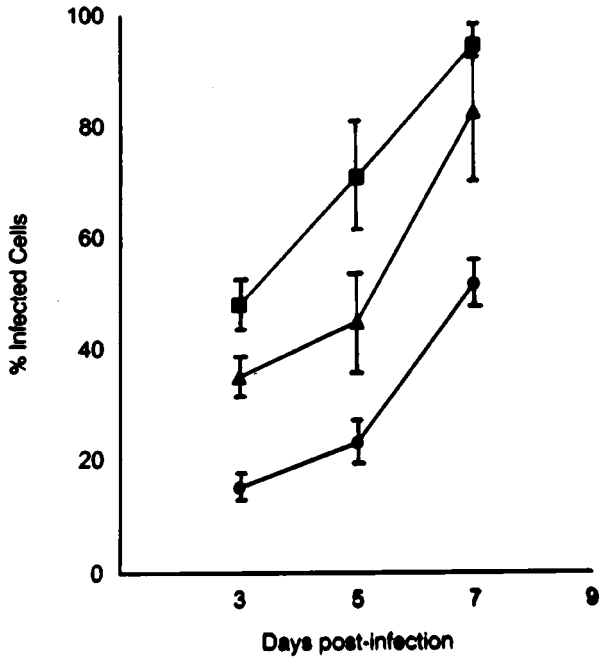

Figure 1 Time-course infection of N1E-115 cells with dengue virus. Cultures of N1E-115 in exponential growth phase were infected with 0.1 p.f.u./cell (●), 1(▲) and 10 (■); percentage of infected cells was determined by an indirect immunofluorescent assay at days 3, 5 and 7 post-infection by counting 100 cells (40×). The Figure shows the results of four independent experiments. Bar=standard deviation (s.d.).

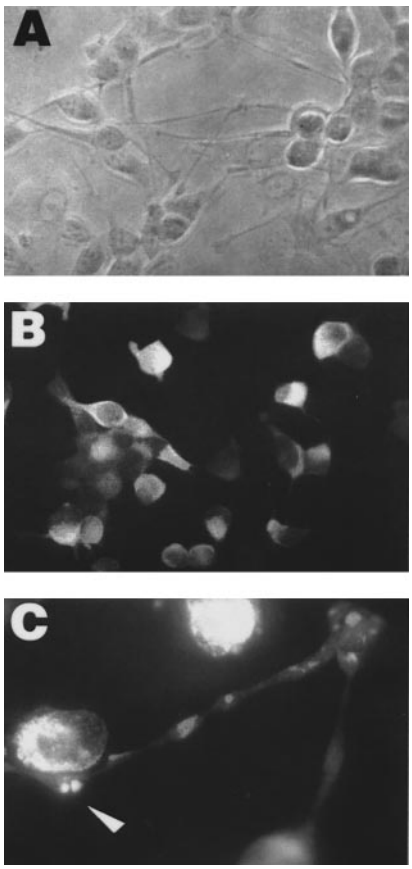

Figure 2 Cultures of neuroblastoma cells N1E-115 infected with dengue virus. (**A**) Phase contrast of non-infected cells (40×); (**B**) Direct immunofluorescence (polyclonal antibodies against dengue virus-FITC) of infected cells (Den-2, MOI=0.1) by day 5 (40×), and (**C**) Den-2, MOI=0.1, by day 7 (64×). The arrowhead on Figure **C** shows viral antigen in neural extensions.

cells treated with trypsin showed a significant decrease in the number of infected cells as compared with non-treated infected cells. The effect of trypsin was not attributable to a decrease in cellular viability, since numbers of viable cells were similar in both experiments, nor to a residual effect of the trypsin on virus infectivity.

Virus attachment to a cellular membrane protein by the Virus Overlay Protein Blot Assay (VOPBA)
Membrane proteins from susceptible mouse (N1E-115) and human (SK-N-SH) neuroblastoma cells, and from a non-susceptible rat glial cell line (C6) were analyzed by the VOPBA as described by Williams *et al* (1991) with some modifications. Partially purified cellular membranes were obtained with the procedure described by Takacs and Staehelin (1981). As shown in Figure 3, a single band that migrates at 65 kDa was revealed with the monoclonal antibody specific for dengue-2 (3H5); this band was observed with membrane proteins from N1E-115 and from SK-N-SH cells (lanes 3 and 8), however it was absent from membrane proteins of a non-susceptible rat glial (C6) cell line, included as a control (lane 2) or when virus (lane 1) or monoclonal antibody (lane 7) were omitted. Similar results were obtained when human polyclonal antibody was used instead of Mab 3H5. Lane 4 shows that NC membranes treated with trypsin eliminated the 65 kDa band, while the treatment with neuraminidase (lane 5) or endoglycosidase H (lane 6) did not affect the interaction between the virus and the 65 kDa protein.

Discussion

The presence of viral receptors on the cellular plasma membrane is a fundamental step for early interaction of virus and cell infection (Tyler, 1990). The difference in neurotropic behavior associated with flavivirus infection could be explained by the presence of specific receptors expressed in a subset of neurons (Monath, 1989). The molecular basis for the vulnerability of neural cells to flaviviruses remains largely unknown and a possible interaction of virus with neurotransmitter receptors has been suggested (Monath, 1989).

Our results strongly suggest that a 65 kDa membrane cell protein may be involved in the binding of dengue virus to the mouse (N1E-115) and human neuroblastoma (SK-N-SH) cells. Treatment of N1E-115 cells with trypsin diminished the number of infected cells (Table 1) suggesting that a protein is the binding molecule for dengue virus as pointed out by Daughaday *et al* (1981) who showed that the infection of adherent human monocytes to dengue virus is mediated by a trypsin-sensitive receptor and by a trypsin-resistant immune complex receptor. To obtain more information about the nature of the putative receptor for dengue virus, we used the Virus Overlay Protein Binding Assay (VOPBA) as described by Williams *et al* (1991) with slight modifications. The use of partially purified cellular membrane proteins from mouse (N1E-115) and human (SK-N-SH) neuroblastoma cells revealed a 65 kDa protein which binds dengue virus;

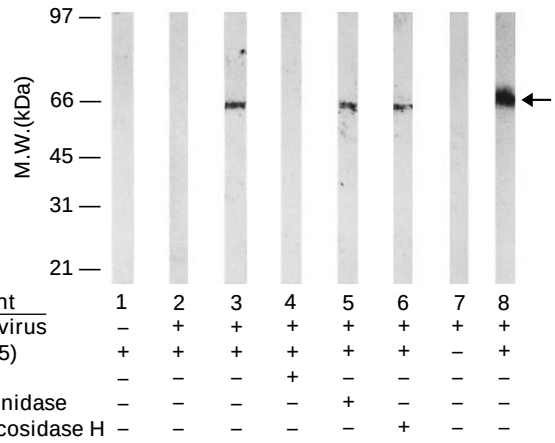

Figure 3 Virus Overlay Protein Blot Assay (VOPBA). Membrane proteins from the neuroblastoma N1E-115 cell line (Lanes 1, 3–7), rat glial C6 cells (lane 2) and from SK-N-SH human neuroblastoma (lane 8) were transferred to NC membrane (see Material and methods) and reacted as indicated in the bottom of Figure. The arrow indicates the position of 65 kDa membrane cell protein. The figure is a representative experiment of three independent assays.

Table 1 Effect of enzymatic treatment on the infection of N1E-115 cells with dengue virus

	Days of infection			
	5		*7*	
Enzyme	*% of fluorescent cells*[1] ± s.d.	*% of viable cells*[2] ± s.d.	*% of fluorescent cells*[1] ± s.d.	*% of viable cells*[2] ± s.d.
Trypsin	37 ± 14.8	82 ± 5.2	51 ± 21.2	73 ± 9.7
Neuraminidase	95 ± 2.4	87 ± 3.1	97 ± 0.2	72 ± 3.6
Endoglycosidase H	98 ± 1.2	85 ± 1.5	93 ± 1.0	76 ± 2.9

[1]% of fluorescent cells was calculated as follows: 100 × (number of treated fluorescent cells/number of control fluorescent cells) ± standard deviation (s.d.) of three independent experiments.
[2]% of viable cells was determined by trypan blue exclusion ± s.d.

this band was absent when cell membrane proteins from the non-susceptible rat glioblastoma (C6) cells were used or when NC membranes containing proteins from N1E-115 and SK-N-SH cells were treated with trypsin. The VOBPA procedure has been used to identify receptors for virus such as lymphocytic choriomeningitis virus (Borrow and Oldstone, 1992) and Visna virus (Dalziel *et al*, 1991). Using a similar experimental approach, Rothwell *et al* (1996) suggested that binding of dengue virus to human bone marrow could be mediated by a membrane protein of approximately 100 kDa. The difference in the observed molecular weight binding protein of dengue virus could reflect the diversity of cellular receptors for dengue virus and their abundance and/or binding activity could explain the preference of the virus for the lymphoid or neural cells. Although both mouse and human neuroblastomas are susceptible to dengue virus infection, the number of infected human neuroblastomas under similar conditions was less than for mouse neuroblastoma. SK-N-SH cells have high levels of dopamine-beta-hydroxylase (Biedler *et al*, 1973), however, we do not know if this difference influences their susceptibility to dengue virus infection. Additionally, a rat glial (C6) cell line was included as a negative control since, under similar experimental conditions these cells were not susceptible to dengue virus infection. These data agree with results published by Imbert *et al* (1994) who demonstrated that dengue virus infects cultured mouse neurons but not astrocytes. It is known that C6 cells are susceptible to vesicular stomatitis virus, herpes simplex and vaccinia viruses, but not susceptible to polyovirus type 3 (ATCC catalogue 1988).

Recently, Chen *et al* (1997) demonstrated that dengue 2 virus and its recombinant envelope protein bind to a highly-sulfated heparan sulfate molecule present on the membrane of Vero and CHO-K1 cell lines. Despite the fact that these cells are not the natural target for dengue virus infection, this works opens an important discussion on the tropism determinants of dengue virus, since as Chen *et al* suggested, differences in the degree of sulfatation of glycosaminoglycans could be the main determinant of dengue virus tropism. However, as remarked by Putnak *et al* (1997), this scenario could be more complex and they suggest that a second interaction with a high affinity receptor on the cell membrane could be an important factor for virus infection. In this context, our results provide experimental support for this issue.

In epidemics of dengue fever and dengue hemorrhagic fever alterations of the CNS have been reported and dengue virus has been recovered from cerebrospinal fluid (Lum *et al*, 1996). Characterization of the dengue virus infection in neural cells could be a valuable tool to understand the molecular basis of the pathophysiologic mechanisms involved in the infection of the CNS in experimental models and humans. The knowledge of virus receptors in susceptible cells will be important to develop effective strategies to control dengue virus infections. In addition, the characterization of dengue virus receptors on neuronal cells will be important to understand the neurologic alterations in patients with dengue virus.

Materials and methods

Virus

The prototype dengue virus serotype 2 NGC (Den-2) strain kindly donated by Dr Duane J Gubler (CDC, Fort Collins, CO) was used in all experiments. The virus was amplified by intracerebral inoculation in newborn mice and virus stock was produced in mosquito cell cultures (TRA-284 cell line), and titered by the lytic plaque assay in LLC-MK_2 cells (CDC, 1981). In some experiments the virus was concentrated using the Ultrafree LGC system (Millipore, Bedford, MA).

Cells

The mouse neuroblastoma N1E-115 cell line was kindly provided by Dr M Niremberg (NIH, Bethesda, MD), the rat glial C6 cell line was purchased from the American Type Culture Collection (Rockville, MD) and the human neuroblastoma SK-N-SH cell line was kindly provided by Dr Jesus Calderon (CINVESTAV, México City). All cells were cultured in DMEM high glucose (GIBCO, Gaithersburg, MD) supplemented with HEPES 20 mM, 10% fetal bovine serum, and penicillin-streptomycin, and for N1E-115 cells the culture medium was additionally supplemented with 10% of inactivated horse serum.

Infection of cell cultures

Cell monolayers were infected with different multiplicity of infection (moi) of Den-2 diluted in Hank's Balanced Saline Solution (HBSS; GIBCO, Gaithersburg, MD) supplemented with 1% (w/v) Bovine Serum Albumin (BSA, Cohn fraction V, Sigma Chemical). The infected cell cultures were incubated for 1 h at 37°C in humidified atmosphere with 5% CO_2, washed twice with HBSS and complete fresh medium was added. The infection was detected by indirect immunofluorescence using the mouse monoclonal antibody 3H5 against Den-2 (Gubler *et al*, 1984).

Enzymatic treatment of cell cultures

Monolayer cultures of N1E-115 cells were treated separately with trypsin 0.075% (w/v) during 15 min at 37°C, with 150 mU/ml of neuraminidase type V from *C. perfringens* (Sigma Chemical) during 1 h at 37°C, and with 10 mU/ml of endoglycosidase H

(Boehringer-Mannheim) during 12 h at 37°C. Treated cells were washed five times with cold HBSS and infected with Den-2 (moi=1). After 1 h of incubation in cold, the cells were washed twice with cold HBSS, pre-warmed complete DMEM was added and cell cultures were incubated during 5 or 7 days in an atmosphere humidified with 5% CO_2. Untreated cell cultures were included as controls. A cell culture was included as control to exclude the possible residual effect of the enzymatic treatment on virus infectivity. Briefly, the cell culture was treated as above, the cells were harvested and sonicated with 10 strokes of 10 s at maximum output, the cell lysate containing the virus was clarified by centrifugation at 14 000 r.p.m. and used to infect susceptible mosquito cells (TRA-284). Virus infection was detected by direct immunofluorescence using a polyclonal antibody labeled with FITC and the number of fluorescent cells was recorded.

Extraction of cellular membrane proteins
In the present work, the procedure described by Takács and Staehelin (1981) was used with slight. Briefly, 10^8 cells were washed with cold D-PBS ($CaCl_2$ 0.9 mM, KCl 2.7 mM, KH_2PO_4 1.2 mM, $MgCl_2$ 0.5 mM, Na_2HPO_4 8.1 mM, pH 7.2), pelleted by centrifugation at $1000 \times g$ during 15 min at 4°C and an equal volume of the following solution was added: Tween 20/NP 40 (7 : 3 v/v) 5% in TNM buffer (Tris-HCl 20 mM pH 7.3, NaCl 145 mM, $MgCl_2$ 1.5 mM, Aprotinin 100 KIU/ml and PMSF 1 mM). The mixture was incubated for 15 min, and sucrose (Merck) was added to a final concentration of 3%. The sample was applied to a discontinuous sucrose gradient (10–32% in TNM buffer) and it was centrifuged at $81\,000 \times g$ for 1 h at 4°C. The cell membrane fraction was obtained from the gradient interphase, diluted in TNM buffer and centrifuged at $100\,000 \times g$ for 1 h at 4°C; The pellet was resuspended in the original volume of cell suspension and stored at −70°C until use; the presence of cell membranes was corroborated by phase contrast microscopy.

Virus overlay protein blot assay (VOPBA)
This procedure was carried out as described by Boyle *et al* (1987). Briefly, 10 μg of total cell membrane protein was separated in a discontinuous (4–12.5%) gel of SDS-acrylamide under denaturing conditions. The proteins were transferred to a nitrocellulose membrane (NC) according to the procedure described by Towbin *et al* (1979). The NC membranes were blocked with a solution of 3% of BSA (Sigma Chemical) and 0.2% of Tween 20 in PBS 10 mM, pH 7.4 for 2 h at 37°C, washed three times with 0.2% Tween 20 in PBS (PBS-Tween) and incubated with 10^5 PFU/mm^2 overnight at 4°C. The NC membranes were washed three times with PBS-Tween and incubated at room temperature during 4 h with monoclonal antibody (3H5) against Den-2 diluted 1 : 10 in BSA-Tween-PBS solution; a non-related monoclonal antibody was included as a control. After three washes with Tween-PBS, the NC membranes were incubated with conjugated anti-mouse Ig coupled to biotin diluted 1 : 30 during 1 h at 37°C, followed by three washes in Tween-PBS and incubated for 1 h at room temperature with ^{125}I-Streptavidin, 0.5 μCi/ml (specific activity 20–40 μCi/mg, Amersham UK). The NC membranes were washed five times with Tween-PBS and three times with PBS, dried and exposed to X-Omat film for 48 h at −70°C. Some NC membranes were pre-treated separately with trypsin, neuramidase and endoglycosidase H, as described above.

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