INFECTION OF CENTRAL NEURON CULTURES BY HERPES SIMPLEX VIRUS

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SUMMARY

Central neurons in culture represent a limitless substratum for research in neurobiology and experimental neurology; primary cultures of Balb/c mouse neurons were used to study if the special affinity of Herpes Simplex Virus (HSV) for the limbic cortices, was related to a selective infection of certain types of neurons. Neuron cultures were screened for cell subpopulations by using immunological markers: antibodies anti glial fibrillary acidic protein (GFAP) for astrocytes and anti neuron-specific enolase (NSE) for neuron cells. The latter ones were also analyzed by reaction with antibodies anti: dopamine β hydroxilase (DBH),γ aminobutyric acid (GABA), serotonin and choline acetyl transferase (CAT). Mouse neuron cultures showed that neuronal clumps were positives for the neuron marker (NSE), and most of them reacted with anti GABA and DBH (58 and 44%) and, a less proportion with anti serotonin and CAT (23 and 10%). These cultures were infected with HSV-1 or HSV-2, and viral kinetics of growth showed a 40 hours eclipse phase and a titer 100-fold lower for HSV-2 than HSV-1. HSV-1 infected cultures analyzed 18 hours after infection by double immunofluorescence with antibodies anti-HSV and anti-NSE, showed mainly the viral antigen in almost all neuronal clumps (80%), without any predilection for a specific neuron target. Also, HSV-1 was found in astrocytes, detected by their reaction with anti GFAP. When pure astrocytes cultures were used for HSV-1 infection, they showed a similar kinetic to the one found in mouse cultures and HSV-1 antigen at 18 hours after infection was found in all the astrocytes (100%).

INTRODUCTION

Herpes Simplex Virus (HSV) infections range from mild and even asymptomatic recurrent fever blisters, to life threatening disseminated infection and encephalitis. HSV encephalitis fortunately rare, is one of the most devastating of all herpes simplex virus infections (Rawls, 1979; Rand, 1982; Macías *et al.*, 1986; Whitley, 1990; Pertslund, 1991; SkÖldenberg, 1991; Stanberry, 1992). In adults, the virus causes an acute necrosis within limbic structures which may result in localized neurologic abnormalities (Olson *et al.*, Rawls, 1979; Esiri, 1982; Straus *et al.*, 1985; Macias *et al.*, 1986; Griffin, 1990; Nasch & Löhr 1992).

The peculiar affinity of herpesvirus for specific brain areas and the necrotizing encephalitis are not characteristics for other viruses (Olson, 1967). This selective destruction, probably is due to the proximity of those structures to the entry point of the virus (Davis and Johnson, 1979). Damasio and Van Hoesen (1985) have proposed that regardless of the route of entry to the CNS, encephalitis is due to a special affinity of the HSV-1 for the neuroanatomical, neurochemical and neuroimmunological properties of the limbic cortices, furthermore all along the neural chain the affinity of HSV-1 for certain types of neurons would be the determinant factor. In this paper we attempted to study if neuron cells in culture show differences in their permissiveness property for HSV, for that purpose, we used primary cultures of mouse fetal central neurons in which about 80% of the cells are fully differentiated neurons; producing interconnecting axons and dendrite processes (Sotelo *et al.*, 1980; Fukatso *et al.*, 1983; Sotelo *et al.*, 1984;Sotelo and Guevara, 1987), and pure astrocytes cultures; viral infection was detected by double immunofluorescence with antibodies against HSV antigens and markers for neuron cells or astrocytes.

MATERIALS AND METHODS

Viruses and cells

HSV-1 (McIntyre strain) and HSV-2 (G strain) were grown in PC cells (lung guinea pig cells) in medium 199 with Earle's salts, 4% bovine serum, 0.17% $NaHCO_3$, 100 IU penicillin/ml and 100g streptomycin/ml. The titer of viruses were determined by TCID 50 and stored at -70$c until use (Rovozzo, 1973).

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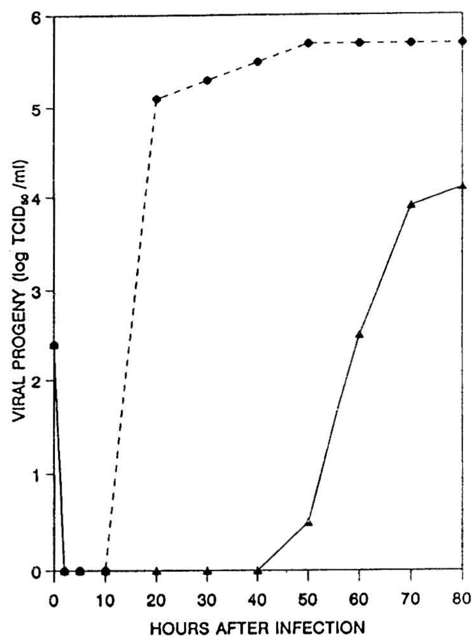


Fig. 1 Kinetics of growth of HSV-1 and HSV-2 in mouse primary central neurons cultures. HSV-1 and HSV-2 virus titer was determined in samples from neurons cultures at various times after infection with 10 $^{2.4}$ TCID^{50}. Values represent the mean of 3 experiments (for each point there was a difference less than log 0.2, p0.05) = HSV-1 = HSV-2.

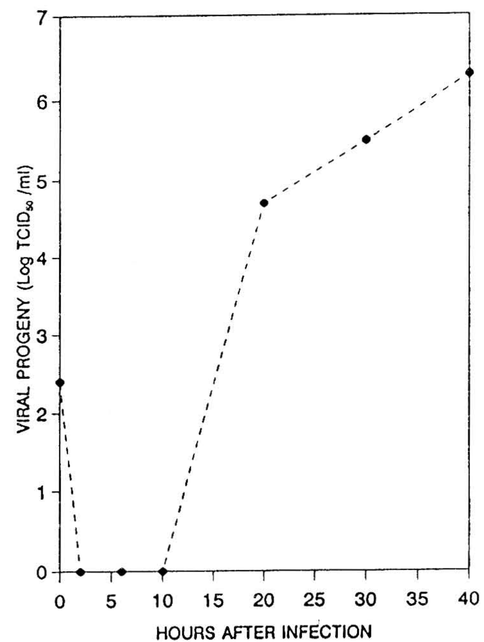


Fig. 2 Kinetic of growth of HSV-1 in mouse astrocytes cultures. Values represent the mean of 3 experiments (for each point there was a difference less than log 0.2, p0.05).

Cultures of central neurons and astrocytes

Primary cultures from fetal Balb/c mice neurons were prepared as described previously (Sotelo *et al.*,1980). The culture were seeded into six well multidishes, in Dulbecco's modified medium supplemented with 10% fetal calf serum, 10% horse serum, 1% dextrose, 0.15% $NaHCO_3$ 2mM L-glutamine, 0.08 UI insulin/ml, 100 UI penicillin/ml and 100g streptomycin/ml. Seven days later, medium was changed to Dulbecco's medium as above but without fetal calf serum and with the horse serum to 20% and 0.001% cytosine arabinoside.

Pure astrocytes cultures from Balb/c mice were prepared according to Mc Carthy and de Vellis (1980). Cells were grow in Eagle basal medium with 15% fetal calf serum and 5% brain mice extract.

Detection of cell subpopulations

Neuron or astrocytes cultures grown and differenciate on coverslips were fixed with cold methanol-acetone and then reacted overnight with rabbit antibodies (IgG) for detection of different cells; anti glial fibrillary acidic protein (GFAP) for astrocytes (Luevano *et al.*, 1986) anti neuron-specific enolase (NSE) for neuron cells, and for different types of neurons, antidopamine βhydroxilase (DBH), antiγaminobutyric acid (GABA), anti serotoin and anti choline acetyl transferase (CAT). As secondary antibody goat IgG anti-rabbit IgG conjugated with rhodamine was used. Primary and secondary antibodies were obtained from Incstar Corporation (Cuello, 1983). For calculation of different cell populations, total neuronal clusters or cells were counted in 20 fields (16x) with white light and a percentage of them stained with rhodamine was determined.

Infection of mouse neurons or astrocytes cultures grown on coverslips were infected with $10^{2.4}$ TCID^{50} in 0.1 ml either HSV-2, and incubated at 37$c with 5% CO_2. At a different hours after infection, medium was harvested and stored at-70$c. Titer of viruses were determined by TCID $_{50}$ in PC cells. Pure astrocytes cultures were infected with HSV-1 as above.

Double immunofluorescence staining

Neurons cultures seeded over coverslips and infected with HSV-1, were fixed with cold methanol-acetone (1:1) at 18 hours after infection. Then, cultures were stained by double fluorochrome immunofluorescence: with anti-NSE as marker for neurons. The second chromogen was anti-HSV conjugated with fluorescein for HSV antigen detection. Mock infected controls were included. Along the studies, neuronal clusters stained with rhodamine were determined in 20 fields (16x), in cudruplicate; and in the same fields the clumps stained with fluorescein were counted. The percentage of HSV-1 infected neuronal clumps was calculated.

Pure astrocytes cultures 18 hours after infection with HSV-1 were stained as above, using anti-GFAP (Luevano *et al.*, 1986) for astrocytes detection instead of NSE antibodies.

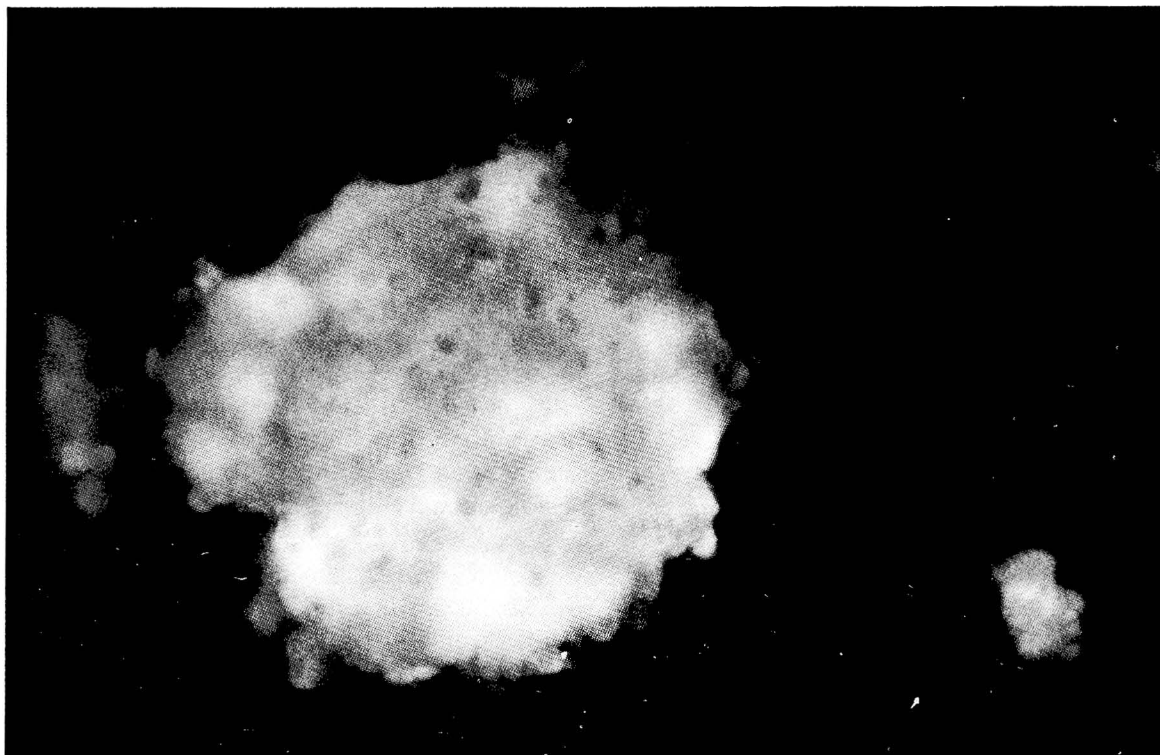
Statistical analysis

Student's t-test was used

RESULTS

Identification of cell subpopulations in neuron cultures

Balb/c neuron cultures showed that cell clusters producing interconnecting axons and dendrite process were neurons which reacted with anti-NSE antibody. In this cultures the neurons were the major



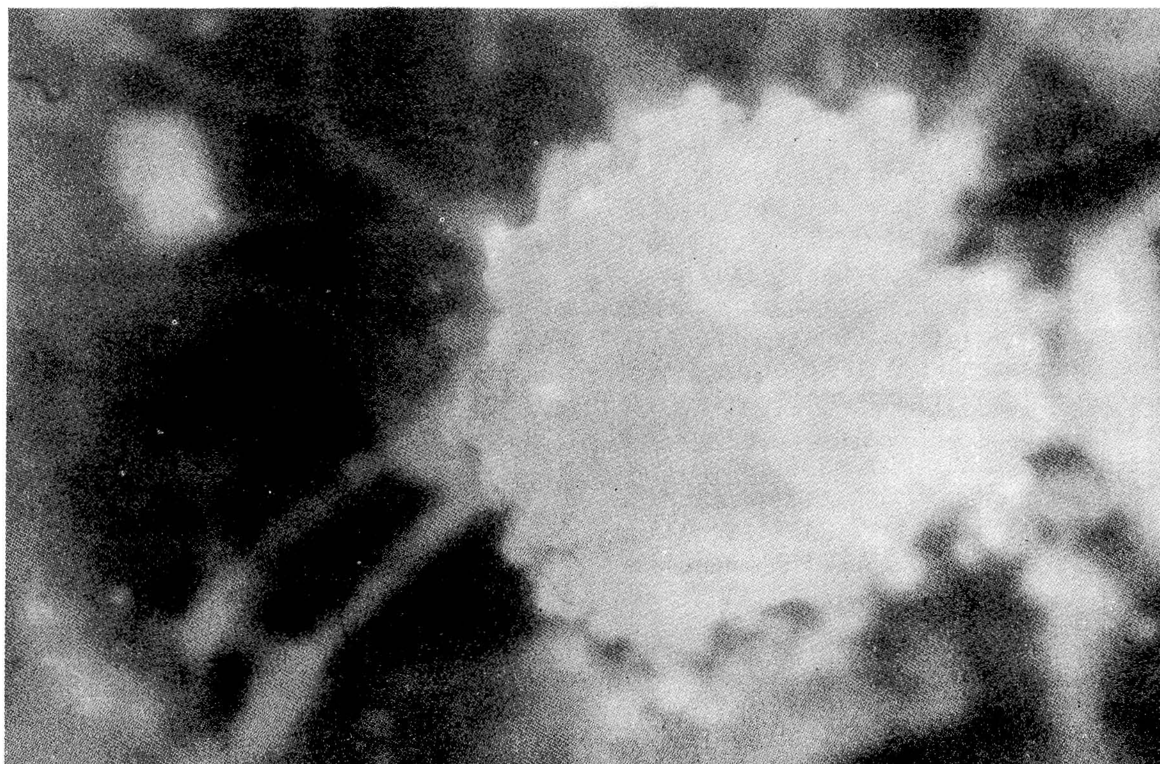


Fig. 3 A neuron cluster from a Balb/c central neurons culture 18 hours after infection with HSV-1 and stained by double fluorochrome: A) anti-HSV with fluorescein and B) anti-NSE with rhodamine (400x).

observed cells (80% of the total cells), and astrocytes identified by their reaction with anti-GFAP were approximately 10% of the total observed cells.

By using antibodies to detect several types of neuron cells, 58% of neuron clusters reacted with anti-GABA, 44% with anti-DBH, 23% with anti serotonin and 10% with anti-CAT (table 1)

Identification of cell populations in astrocytes cultures

In these cultures 100% of the cells were identified as astrocytes by their reaction with the antibody anti-GFAP.

Growth of HSV in central neurons

HSV could be propagated in primary neuronal cells from Balb/c mice. Kinetic of growth of HSV-1 was faster than HSV-2, with about a 100-fold higher titer; HSV-2 had an eclipse phase of 40 hours (fig. 1), compared with 12 hours found with HSV-1. As a consequence all next experiments were carried with HSV-1 showed a similar kinetic of growth to the one found in neuron cultures (fig.2) but with a higher viral yield.

Detection of HSV antigen in neurons and astrocytes

Cultures of neurons at 18 hours after infection showed morphological alterations such as, swollen and refrigerent neuronal bodies with granulations in axons; 80% (214/265) of neuron clusters reacted with the double fluorochrome; fluorescein for anti-HSV and rhodamine for anti-NSE (maker for neurons). Fluorescence was observed essentially in the clusters of neurons. The astrocytes in these cultures were also labeled by double fluorochrome;fluorescein for HSV antigen and rhodamine for anti GFAP. Figure 3 shows same neuron cluster positive for fluorescein which detected HSV antigen and rhodamine for anti NSE used for neuron cells identification. Pure astrocytes cultures 18 hours after HSV-1 infection showed 100% (332/332) of the cells labeled with double fluorochrome, fluorescein for anti-HSV rhodamine for anti-GFAP.

DISCUSSION

HSV encephalitis fortunately rare is the most disastrous complication in adults (Rand, 1982; Macias *et al.,* 1986; Sköldenberg, 1991). The reasons why a characteristic distribution of lesions particularly in limbic parts of the brain are unknown. An explication for it, could be the virus spread along the olfactory pathway (Dinn, 1979; Dinn, 1980). Other hypothesis is that virus travels through tentorial branches of the trigeminal nerve in the direction of the dura mater then, virus travels to the meninges subjacents cortices (Davis and Johnson, 1979; Stroop *et al.,* 1984), but others have proposed a specific affinity of HSV for limbic cortices, and the determinant factor would be, the affinity of the virus for certain types of neurons (Damasio and Van Hoesen, 1985). Kennedy *et al.,* 1983,. using human fetal neural cultures found that neuron cells were markedly non-permisive for HSV-1.

We used an *in vitro* model, primary cultures of central neurons from Balb/c mice, to study if there was a selective affinity of HSV for a particular type of neurons. These neuron cultures usually from a confluent cell culture in which about 80% of the total cells are fully differentiated neurons, lying upon a layer of a mixed cell population of 10% astrocytes either fibrous or protoplasmic, 2% oligodendrocytes and 8% fobroblast liked cells (Sotelo, *et al.,* 1980). These cultures screened with different antibodies as immunological markers to detect neurons, astrocytes and several types of neurons, showed that the cellular clumps (the most abundant cells) producing interconnecting axons and dendrite process were neurons. These neuronal clumps were mainly positives for GABA (58%) and DBH (44%) and in less proportion for serotonin (23%) and CAT (10%).

In these cultures HSV-1 grew faster than HSV-2, as it is observed in cell lines *in vitro*. HSV started to be released at 12 hours after infection and HSV-2 after 40 hours postinfection (figure 1). These kinetics of growth of both virus were similar to those obtained by Borcholte *et al.,*1990 in murine spinal cord neurons. The studies by double immunofluorescence in cultures infected with HSV-1 did not show the HSV antigen in a specific neuron target, it was detected in almost all the neuron clusters (80%). Kinetic of HSV-1 and double fluorescence results contrast with the findings on human fetal neural cultures in which neuron cells were markedly nonpermissive for herpes simplex virus (Kennedy *et al.,*1983); maybe this finding is related to the low number of neuron cells in these cultures, from 10 up to 50% of the total cells.

In primary mouse neuron cultures, astrocytes were also infected by HSV-1, this result was confirmed using mouse pure astrocytes cultures (McCarthy and de Vellis, 1980) and these cells were totally permisive for HSV-1, as it was observed by immunoperoxidase reaction in autopsies of HSV encephalitis (Esiri,1982).

Apparently in this mouse central neuron culture, there was not a significant difference in neuron permissiveness for HSV which could explain the HSV affinity properties for limbic cortices like Damasio and Van Hoesen(1985) have proposed.

On the other hand, this system seems to be very useful for evaluation of antiviral substances, and also studies of interaction among neurotropic agents and different cells of the central nervous system, since by a simple method one can obtain a large population of central neurons which grow to form a network of processess on top of a population of supporting cells which could be representative of the different cell population from central nervous system (Sotelo *et al.*,1980; Fukatsu *et al.*,1983; Sotelo *et al.*, 1984; Sotelo and Guevara, 1987) in contrast with the use of neuron cell lines, which besides their tumoral origin have shown among them, high variability in cell sensitivity to virus infection and also different response to antiviral effect of some drugs (Hanada *et al.*,1989; Borcholte *et al.*,1990).

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