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HERPES SIMPLEX VIRUS DNA IN THE BRAIN OF PSYCHOTIC PATIENTS

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Summary.— Herpes simplex virus (HSV) DNA was found by spot blot hybridization in the right *nc. amygdalae* of 3 out 10 patients who underwent curative stereotactic surgery for severe mental retardation with aggressive behaviour and/or paranoic schizophrenia. Of these, 6 were also tested for the presence of CMV DNA sequences with negative results. Biopsy specimens from *nc. amygdalae* of another 7 psychotic patients were cultured *in vitro* but no virus was isolated.

Key words: mental disorders; herpes simplex virus; DNA; latency; hybridization

Introduction

Latent herpes simplex virus (HSV) is known to reside for lifelong in the human sensory as well as vegetative ganglia, which yield virus when cultured in vitro (Bastian *et al.*, 1972; Baringer and Swoveland, 1973). The reactivated virus causes skin or mucosal lesions by reversed axonal transport. It is less clear, however, whether centripetal virus transport occurs upon reactivation of the latent virus in trigeminal ganglion and whether encephalitis develops due to the reactivated endogenous virus. It has been suggested that nonproductive latency can be established in the olphactory bulb and entorhinal cortex with a virulent strain introduced by the nasal route. Encephalitis would develop upon reactivation of the latent virus in the entorhinal and temporal cortex (Stroop, 1986) rather than from reactivation of less virulent strains harboured in trigeminal ganglion, brain stem, thalamus and in the area of parietal cortical projections of sensory neurons.

The cardinal hallmark of HSV latency is the persistence of viral DNA in the absence of any transcription or under conditions of limited transcription of immediate early mRNA (Cantin *et al.*, 1984; Fraser *et al.*, 1986). HSV-1 DNA was found by *in situ* hybridization in 3 out 4 brain samples taken at autopsy from patients with chronic psychiatric illness but in none out 2 patients with acute psychoses (Sequiera *et al.*, 1976). Fraser *et al.* (1981)

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found HSV-1 DNA in 6 out 11 brain samples examined. Of these, 3 were coming from the frontal lobe gray matter, or undefined brain areas in multiple sclerosis, where another 3 positive samples were from the frontal or parietal lobe cortex or from brain stem of normal CNS. Gannicliffe *et al.* (1985) described positive HSV DNA hybridization with the extracts of temporal lobe of 5 out 6 epileptic patients, who underwent lobectomy and in 5 out 11 necroptic specimens from 4 nonepileptic cases (parkinsonism, 2 normal subjects).

We have focused our interest on the search for latent HSV in *nc. amygdalae* of patients with paranoic schizophrenia or mental retardation with aggressive behaviour in which curative stereotactic surgery had been performed (Pogády and Nádvorník, 1982). The findings are of interest in the light of enhanced serum antibody levels to HSV-1 in schizophrenic patients (Líbiková, 1983) and the presence of antibodies to this virus in the cerebrospinal fluid of such patients (Bártová *et al.*, 1987).

Materials and Methods

Brain specimens (18) were coming from patients (17) on which curative stereotactic surgery was performed (Pogády and Nádvorník, 1982). The patients were hospitalized in the Regional Psychiatry Hospital at Pezinok (near Bratislava). Specimens were taken from the right *nc. amygdalae*, in two patients from both *nc. amygdalae* (Table 1).

HSV-1 DNA was isolated from nucleocapsids sedimented by differential centrifugation of the infected cell extract. Strain HSZP -infected (Szántó, 1960) quail embryo cells (MOI 0.5 PFU per cell) were harvested 18—20 hr post-infection (p.i.) into a hypotonic buffer (10 mmol/l Tris-HCl, pH 7.5, 10 mmol/l KCl, 15 mmol/l $MgCl_2$), and lysed by addition of an equal volume of the same buffer containing 1 % Nonidet P-40, and 2 mmol/l dithiothreitol for 20 min at 4 °C. After low speed centrifugation (3000 rev/min) the supernatant was centrifuged through 30 % sucrose cushion at 105 000 g for 3 hr at 4 °C. The pellet was treated with 1 mg/ml pronase (Calbiochem) in 0.1 mol/l Tris-HCl buffer pH 7.9 containing 1 % SDS and 0.1 mol/l NaCl for 2 hr at 37 °C. The DNA was extracted with distilled phenol saturated in TEN buffer (0.1 mol/l Tris-HCl pH 8, 0.1 mol/l NaCl and 1 mmol/l EDTA). The water phase was further extracted with chlorophorm: isoamylalcohol (24 : 1) and then precipitated with a 3-fold vol of ethylalcohol at —20 °C overnight. The precipitate was treated with pancreatic RNase (Sigma) (20 μg per ml in 10 mmol/l Tris-HCl pH 7.8 for 1 hr at 37 °C), DNA was extracted in chlorophorm-isoamylalcohol as above and then precipitated with 3-fold vol of ethylalcohol in the presence of 0.1 mol/l NaCl (—20 °C, overnight). The DNA was purified by equilibrium centrifugation in 1× SSC containing CsCl at a density of 1.72 g/cm^3 for 44 hr at 36 500 rev/min, in Beckmann L8 centrifuge rotor SW 50.1.

Cytomegalovirus (CMV) DNA fragments Eco RI E, HindIII T and HindIII R were kindly provided by prof. B. Fleckenstein (Institute for Clinical and Molecular Virology, Erlangen, F.R.G.).

Nick translation of the HSV-1 DNA and of the CMV DNA fragments was made according to Rigby *et al.* (1977) in the presence of α ^{32}P-dCTP and α ^{32}P-dGTP (specific activity 110 TBq per mmol, IZINTA, Budapest, Hungary). The activity of the labelled HSV-1 DNA was $3-4\times 10^7$ cpm/ μg, that of the labelled CMV DNA fragments 2.6×10^7 cpm/μg.

The DNA from brain specimens was extracted using a lytic buffer (0.2 mol/l Tris-HCl pH 7.9 containing 0.5 mol/l EDTA and 0.5 % SDS), with proteinase K (100 μg/ml for 2 hr at 65 °C) and then further extracted with phenol-saturated buffer (0.01 mol/l Tris-HCl pH 7.5, 0.15 mol/l NaCl and 1 mmol/l EDTA). The water phase was treated with RNase and chlorophorm-isoamylalcohol as described above and precipitated with alcohol. The pellet of infected and uninfected human embryo lung (HEL) cells was treated in the same manner.

Spot blot hybridization was performed as described by Fraser *et al.* (1981). The DNA extracted from the brain specimens of from positive and negative control (HEL) cells was spotted either

on nitrocellulose or on nylon (Gene Screen NEF-972) filters. The nitrocellulose filters were baken at 80 °C for 2 hr under vacuum, the DNA on nylon filters was cross-linked by UV-irradiation (30W germicide lamp, 12 cm for 2 min). Nitrocellulose filters were incubated in prehybridization mixture (20 % formamide, 4× SSC, 5× Denhardt's solution, 0.1 % SDS, 1 mmol/l EDTA and 25 μl/ml heat denaturated salmon sperm DNA) overnight at 48 °C and then hybridized with the hybridization mixture (the same as prehybridization mixture with exception of 50 % formamide) in the presence of denatured radioactive probe. Then filters were washed twice in buffer 5× SSC with 0.1 % SDS 10 min at 48 °C, once 20 min at 50 °C in hybridization mixture without radioactive probe, then in buffer 1× SSC with 0.1 % SDS 20 min at 37 °C and 20 min in the same buffer at 64 °C; the last washing buffer contained 0.25× SSC with 0.1 % SDS (15 min at 64 °C). Nylon filters were hybridized according Gene Screen's protocols under high stringency conditions. The dried filters were autoradiographed with XR film (Medix- -Rapid) for 2—3 days at —70 °C.

The brain samples were cultured in medium CMRL-1415 (Connaught, Med. Labs, Toronto) supplemented with 5 % foetal calf serum and 5 % newborn calf serum as described (Rajčáni *et al.*, 1975). By day 7 in culture, the fragments from the same brain sample were collected, homogenized in about 200 μl of medium and inoculated intracerebrally into newborn mice (10 μl per mouse, 10 sucklings each). The medium of the cultured fragments was assayed in Vero cells.

Results

Positive hybridization of the HSV-1 DNA was found in 3 out of 10 specimens coming from the *nc. amygdalae*. As seen in Table 1, the patients whose brain biopsy specimens contained HSV DNA were operated on for imbecility associated with aggressive behaviour. In two cases (patients Š. Z. and K. F.) samples were taken from both *nc. amygdalae*, but only one (left side) specimen showed positive hybridization. This seems to support the specificity of the results. The control spot, which contained DNA from 10^6 uninfected HEL cells was repeatedly negative under the given high stringency conditions. This along with negative hybridization of the remaining 8 biopsy samples also seems to confirm the specificity of the positive hybridization. As seen on Figs. 1—2, the intensity of the positive spot corresponded approximatelly to the positive control spot containing the DNA extract from 10^4—10^5 virus-infected HEL cells. Assuming that the amount of DNA copies in acute infection was in the range of 10^2—10^3 HSV DNA molecules per cell and provided that each HEL cell had been infected at given high multiplicity, we can estimate the number of DNA copies present in the positive brain sample to be equal to 10^6—10^8. As the bioptic sample contained 10^6—10^7 cells, we may conclude that we were able to detected about 0.1—1 viral DNA copy per cell. It is not clear, however, whether neurons only or both neurons and glial cells contained the HSV genome.

Further proof for the specificity of our hybridization results comes from the negative hybridization of brain samples of the patients No. 12—17 (Table 1) with the CMV DNA. The latter extracts were spotted on nylon filters and hybridized with the labelled CMV DNA fragments. No positive hybridization was found. After washing off the radioactive probe, the same filters were rehybridized with ^{32}P-HSV-1 DNA. One sample showed positive hybridization confirming a specific annealing of the HSV DNA probe.

Tab.e 1. Clinical diagnosis, spot blot hybridization and explantation results in patients which underwent curative stereotactic surgery

Patient	Sex	Age (years)	Diagnosis	Localization	Explantation	Hybridization HSV DNA	Hybridization CMV DNA	Note
1 H. D.	F	23	Schizophrenia, paranoides	Nc. amygd., l.dx.	neg	n.d.	n.d.	
2 P. L.	M	21	Debility, encephalopathy	Nc. amygd., l.dx.	neg	n.d.	n.d.	
3 D. P.	F	25	Imbecility	Nc. amygd., l.dx.	neg	n.d.	n.d.	
4 G. V.	F	28	Schizophrenia, paranoides	Nc. amygd., l.dx.	neg	n.d.	n.d.	
5 V. J.	M	34	Idiocy, aggressivity	Nc. amygd., l.dx.	neg	n.d.	n.d.	
6 K. B.	M	30	Idiocy, aggressivity	Nc. amygd., l.dx.	neg	n.d.	n.d.	
7 K. F.	M	49	Schizophrenia, paranoides	Nc. amygd., l.dx.	neg	n.d.	n.d.	
				Nc. amygd., l.sin.	neg	n.d.	n.d.	
8 D. T.	M	22	Imbecility	Nc. amygd., l.dx.	n.d.	pos	n.d.	Fig. 1
9 A. E.	F	17	Mental retardation, aggressivity	Nc. amygd., l.dx.	n.d.	neg	n.d.	
10 B. M.	F	40	Depression, obsendanty sy.	Nc. amygd., l.dx.	n.d.	neg	n.d.	
11 Sz. M.	F	14	Imbecility, aggressivity	Nc. amygd., l.dx.	n.d.	neg	n.d.	
12 L. L.	F	32	Imbecility, aggressivity	Nc. amygd., l.dx.	n.d.	pos	neg	Fig. 2
13 Š. Z.	M	18	Imbecility, aggressivity	Nc. amygd., l.dx.	n.d.	neg	neg	
				Nc. amygd., l.sin.	n.d.	pos	neg	
14 D. Š.	M	14	Idiocy, encephalopathy	Nc. amygd., l.dx.	n.d.	neg	neg	
15 V. M.	M	33	Imbecility, aggressivity	Nc. amygd., l.dx.	n.d.	neg	neg	
16 L. J.	M	15	Idiocy, aggressivity	Nc. amygd., l.dx.	n.d.	neg	neg	
17 B. P.	M	20	Imbecility, aggressivity	Nc. amygd., l.dx.	n.d.	neg	neg	
			Total (patients)		0/7	3/10	0/6	

n.d. = not done; M = man; F = female; pos = positive; neg = negative; Nc. amygd., l.dx. (l.sin) = Nucleus amygdalae, right side (left side)

None of the 8 samples which had been cultured for 7 days and then assayed in suckling mice yielded infectious virus.

Discussion

The presence of HSV DNA sequences in the *nc. amygdalae* biopsy samples from human brain confirms the previous studies indicating that the latent HSV genome might reside in various brain areas in a nonproductive form (Sequiera *et al.*, 1979; Fraser *et al.*, 1981; Gannicliffe *et al.*, 1985). No information is available on the positive rate of HSV DNA in normal human brain, but the findings of Fraser *et al.* (1981) point at the possibility that the latent genome can be detected also in the brain tissue of healthy subjects. Whether such persons are at higher risk of development of any brain pathology such as multiple sclerosis, epilepsy or of mental deterioration needs further elucidation.

The route of involvement of *nc. amygdalae* by HSV is of particular interest. As mentioned in Introduction, two possibilities are well documented: neural spread along the trigeminal sensory pathway and neural spread along the olphactory-limbic system. In series of experiments made for an another purpose, we found positive immunofluorescence of HSV-1 antigen (virulent strain SC 16) in *nc. amygdalae* and temporal cortex of hairless mice infected by intracutaneous route which had developed typical zosteriform herpes and were dying under signs of focal encephalitis (unpublished observation). The haematogenous spread of HSV from skin to the temporal lobe cannot be excluded in this particular situation. It is tempting to speculate—owing to the hypothesis of Stroop (1986)—that especially virulent strains causing severe destruction of neurons in the temporal lobe could emerge by recombination of the endogenous HSV DNA with the DNA of another virus entering the brain by haematogenous route.

The absence of virus reactivation in the cultured fragments of *nc. amygdalae* is not contradictory to the finding of HSV DNA sequences. Under experimental conditions, the presence of HSV DNA sequences was demonstrated in the brain stem of mice with established latent infection by various hybridization techniques (Cabrera *et al.*, 1980; Stroop *et al.*, 1984). Noteworthy, the positive rate of virus reactivation from the brain stem explants in culture is usually much lower than the frequency of positive hybridization results (Cabrera *et al.*, 1980; Cantin *et al.*, 1984; Kúdelová *et al.*, in preparation). This interesting observation could indicate that the nonproductive genome in the brain is probably incomplete (or at least uninducible) in contrast to nonproductive persistence of the complete and inducible genome in a proportion of pseudounipolar neurons of trigeminal or other ganglia. As discussed above, the incomplete (inducible) genome cannot be reactivated by simple culturing of the brain fragments. Superinfection with a ts mutant would be a useful approach for complementation of such genomes.

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Explanation to Figures (Plate LXXVIII):

Fig. 1. Spot blot hybridization of the DNA extracts on a nitrocellulose filter. 1 — patient D. T.; 2 — patient A. E.: 3 — patient B. M.; 4 — patient M. S.; 5 — human embryo lung (HEL) cells, 10^4, HSV-1 infected; 6 — HSV-1 infected HEL cells, 10^6; 7 — HSV-1 infected HEL cells; 10^5; 8 — (in the middle) noninfected 10^6 HEL cells.

Fig. 2. Spot blot hybridization of the DNA extracts on nylon filter. 1 — patient L. L.; 2 — 10^4 HSV-1 infected HEL cells; 3—10^5 HSV-1 infected HEL cells; 4—10^6 HSV-1 infected HEL cells; 5—10^6 noninfected HEL cells.