Herpes simplex virus and Alzheimer's disease: a search for virus DNA by spot hybridisation

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SUMMARY A sensitive hybridisation screening technique (spot hybridisation) was used to search post-mortem brain DNA extracts for herpes simplex I virus sequences in non-neurological controls, Alzheimer's disease cases and Herpes simplex virus infected mice. A reconstruction experiment showed that 0.1 herpes genome equivalents per cell could be detected in 100 μ g tissue extracts. Although herpes sequences were readily detected in infected mice, none of the human brains examined could be shown to contain such sequences.

It is well known that type I herpes simplex virus (HSV 1) is present in the trigeminal ganglia of man¹ and experimentally infected animals,² but there are conflicting reports concerning the presence of HSV 1 in the human brain itself. Using solution hybridisation techniques Wetmur *et al*³ and Aulakh *et al*⁴ both reported no detectable HSV 1 sequences in human post-mortem brain material, each claiming a sensitivity of approximately 1 genome equivalent per 10 cells. However Fraser *et al*,⁵ using a filter hybridisation with a sensitivity claimed to be of 1 genome equivalent per 200 cells, reported the detection of HSV 1 DNA in the brains of several normal individuals and patients with neurological disease. In addition Sequeira *et al*⁶ reported the detection in *in situ* hybridisation of HSV 1 sequences in three of four elderly chronic psychiatric cases, but not in brain smears from 2 patients who had acute psychotic episodes. The occasional detection of HSV 1 immunoreactivity in human brain samples, including cases of Alzheimer's disease has also been reported.⁷⁻⁹

It has been speculated¹⁰ that Alzheimer-type dementia may result from chronic HSV 1 infection. The purpose of the present study was to investigate the distribution of HSV DNA in the CNS of Alzheimer's disease patients and controls.

Materials and methods

Plasmids pBR322 and pHSV 106, a pBR322 deriva-

tive containing a 3.4 Kb insert of HSV DNA including the thymidine kinase gene (BRL Ltd) were used to transform *E coli* HB 101.¹¹ Ampicillin resistant colonies were grown in 100 ml of LB broth and purified as described by Birnboim.¹²

Brain DNA was extracted from 100 mg of tissue by homogenisation in 0.4 ml of extraction buffer (150 mM NaCl; 100 mM Tris; 10 mM EDTA, 1% SDS; 50 μ g/ml proteinase K (pH 8). Samples were shaken at room temperature overnight, then extracted twice with phenol: chloroform: iso amyl alcohol (50:50:1). The aqueous phase was precipitated with 2 volumes of ethanol, the precipitate washed in 70% ethanol and then dissolved in 0.5 ml STE buffer (100 mM NaCl; 10 mM Tris; 1 mM EDTA; pH 8). 20 μ l of this solution was examined by agarose gel electrophoresis (6V/cm for 6 hours in 0.8% agarose with Tris acetate buffer, pH 8.3). RNA was removed by boiling in 0.1 M NaOH for 30 minutes followed by neutralisation with HCl and precipitation with 2 volumes of ethanol. The precipitate was redissolved in 1.5 M NaCl, 0.15 M sodium citrate (10 $\times$ SSC, pH 7.2) and DNA concentration estimated using the equation [DNA] mg/ml = OD₂₆₀ nm/22.

The DNA solution was held in a boiling water bath for 5 minutes prior to filtration in duplicate on to prewetted nitrocellulose (Schleicher and Schuell Ltd) or nylon 66 (Biodyne A, Pall Ltd) membrane using a 96 well filtration unit (BRL Ltd). Filters were air dried and baked for 2 hours at 80°C.

Molecular hybridisation was as described by Jeffreys¹³ using a prehybridisation solution of 5 $\times$ Denhardt's solution (0.2% polyvinyl pyrrolidone; 0.2% ficoll; 0.2% bovine serum albumin in 1 $\times$ SSC), 0.1% SDS, 9% dextran sulphate and 100

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$\mu\text{g/ml}$ denatured salmon DNA at 65°C .

DNA probes were labelled by nick translation¹⁴ using ^{32}P dCTP with a specific activity of 400 Ci/mole (Amersham). The specific activity of the plasmid probes was $2\text{--}3 \times 10^6$ cpm/ μg . Human DNA was labelled to a lower specific activity ($5 \times 10^5\text{--}1 \times 10^6$ cpm/ μg).

After denaturation by boiling the probe was added to fresh hybridisation solution to give a final concentration of 10 ng/ml. Filters were incubated in the hybridisation solution at 65°C for 16 hours, followed by 3×30 minute washes in hybridisation solution without probe and without dextran sulphate, then 3×30 minute washes in a solution containing $0.1 \times \text{SSC}$, 50 $\mu\text{g/ml}$ solution DNA and 0.1% SDS. The filters were blotted, dried and autoradiographed using Kodak XS films and Ilford Rapide intensifying screens at -70°C for 36 hours.

Three sets of hybridisation experiments were performed using the same samples. First a negative control using pBR322, next after washing the filters pHSV106 was used to detect HSV 1 DNA, and finally human DNA retention was checked by probing with labelled human DNA.

Results

The preservation of DNA in the necropsy samples was confirmed by electrophoresis (fig 1a). A typical set of extracts had a substantial proportion of the

DNA present as large fragments (>23 kilobase pairs) with some smaller components including some RNA. The mean yield of DNA was 0.5 μg per mg of tissue.

Plasmids prepared by the rapid alkaline extraction method were found to be predominantly supercoiled molecules (fig 1b), which could be labelled with high efficiency using *in vitro* nick translation without further purification.

Successful binding and retention of DNA to filters was evaluated by the final hybridisation experiment using labelled human DNA. Comparison with a series of standards enabled an estimate of the quantity of bound DNA to be made (fig 2). Cases where less than microgram quantities of DNA were retained on the filter, or where non-specific binding of pBR322 was observed were not included in the analysis of results. The table summarises the number of samples which were included in the analysis of results. The total of 112 samples include 57 samples from nine non-neurological control patients and 55 from eight cases of dementia of the Alzheimer type.

Nitrocellulose filters gave a slightly better retention of DNA at higher concentrations than nylon filters. Figure 3 shows the result of a filtration experiment in which 100 μl samples from the same stock of denatured human DNA in $10 \times \text{SSC}$ were filtered through two superimposed membranes, either nylon or nitrocellulose. Nearly all of the 5 μg standard was retained by the first (upper) nitrocellulose mem-

Fig 1 Agarose gel electrophoresis of nucleic acids. (a) Total undigested nucleic acid extract from a typical set of necropsy brains used in this study (tracks 1-14). Track 15 is a λ hind III digest, the largest (upper) fragment has a size of approximately 23 kilobase pairs. Electrophoresis was 6 V/cm and pH 8.3 for 4 hours in 1% agarose. (b) Plasmids used in this study: track 1 is a λ hind III digest, track 2 is pHSV 106 and track 3 is pBR322. Electrophoresis was for 16 hours at pH 8.3 in 0.8% agarose.

Fig 2 DNA retention by filters. After hybridisation with pBR322 and pHSV 106 the filters were washed and hybridised with ³²P human DNA (see text for details). The intensity of autoradiograph spots was compared to a series of standards (S1 = 1 µg, S2 = 100 ng, S3 = 10 ng human DNA) to obtain an estimate of the amount of DNA bound. Numbers 1–12 are a typical set of extracts used in this study.

brane, whereas a substantial amount leaked through the first nylon membrane when either 5 µg or 1 µg of DNA was filtered. For this reason we used nitro-

cellulose membranes in the rest of this study. The sensitivity and specificity of hybridisation was examined using a series of controls. Figure 4a shows the result of a hybridisation reaction using pHSV 106 as a probe for homologous sequences in filter bound pBR322. A strong hybridisation signal was seen, and as little as 2 pg of pBR322 could be detected.

Next the ability of pHSV 106 to detect HSV 1 sequences in infected brain tissue was confirmed using control and infected mouse brain DNA extracts. The infected mouse brain homogenate contained 1 × 10² plaque forming units/mg of HSV 1 after freezing and thawing. 10 mg of this homogenate was extracted and the DNA extract was diluted in a series of 10 fold steps. Figure 4b shows the result of hybridisation with labelled pHSV 106. A signal was clearly detectable in the rows 3 and 4 (neat and 10⁻¹) equivalent to 10 and 1 plaque forming unit (pfu) of HSV, and a signal slightly higher than background was visible at 10⁻³. It was not

Table Post-mortem brain areas used in hybridisation experiment

Control	1	2	3	4	5	6	7	8	9	Total
Substantia nigra	+	+	+	+	+	+	+	+	+	8
Putamen	+	+	+	+	+	+	+	+	+	9
Globus pallidus	+	+	+	+	+	+	+	+	+	8
Caudate	+	+	+	+	+	+	+	+	+	7
Olfactory tubercle	+	+	+	+	+	+	+	+	+	7
Temporal cortex	+	+	+	+	+	+	+	+	+	6
Amygdala	+	+	+	+	+	+	+	+	+	8
Hippocampus	+	+	+	+	+	+	+	+	+	4
Dementia	1	2	3	4	5	6	7	8		Total
Substantia nigra	+	+	+	+	+	+	+	+		7
Putamen	+	+	+	+	+	+	+	+		7
Globus pallidus	+	+	+	+	+	+	+	+		8
Caudate	+	+	+	+	+	+	+	+		7
Olfactory tubercle	+	+	+	+	+	+	+	+		8
Temporal cortex	+	+	+	+	+	+	+	+		8
Amygdala	+	+	+	+	+	+	+	+		4
Hippocampus	+	+	+	+	+	+	+	+		6

Controls n = 9, mean age = 75 (±8) yrs, sex ratio 6 M; 3 F. Dementia N = 8, mean age = 71 (±10) yrs, sex ratio 4 M; 4 F.

Fig 3 Comparison of the retention of DNA by nylon and nitrocellulose. Denatured human DNA solutions were filtered through two membranes and after baking the membranes retention of DNA was checked by hybridisation at 5 µg per spot the membranes were both saturated, and above this DNA leaked through to the lower membrane. Under the conditions used (see methods) the performance of nitrocellulose was superior, giving sharper spots and better retention at higher concentrations.

Fig 4 Sensitivity and specificity of hybridisation (a) Detection of pBR322 sequences by pHSV 106 (b) Detection of HSV sequences in infected mouse brain homogenates by pHSV 106. Rows 1 and 2 control mouse brain, rows 3 and 4 HSV infected mouse brain (c) Detection of HSV DNA by pHSV 106. Rows 1 and 2: DNA extract from a human cortex white matter only (5 µg), rows 3 and 4: serial dilutions of HSV DNA, rows 5 and 6: serial dilutions of HSV DNA mixed with 5 µg of human DNA.

possible to interpret these results in terms of HSV DNA since the DNA to infectivity ratio in the infected brain homogenates is not known. A direct quantitative measure was obtained using DNA extracted from glycerol gradient purified HSV 1 (fig 4c). The HSV DNA concentration was measured by ultraviolet absorption and serial dilutions were made and filtered either alone or in the presence of 5 µg of DNA extracted from human cortex. Most cortex samples alone gave low background signals, and HSV DNA could be detected at 100 pg levels with or without additional host material.

When the control and Alzheimer's disease brain samples were hybridised with pBR322, two samples gave a hybridisation signal. The reason for this occasional non-specific hybridisation is not known, but it occurred repeatedly and always with the same samples. Those samples were excluded from the analysis of results and are not shown in the table. Subsequent hybridisation with pHSV 106 did not produce any detectable binding in any of the human samples.

Discussion

Since we were able to detect 100 pg of HSV 1 DNA in the presence of 1–5 µg of human DNA on a filter, we estimate the limits of sensitivity to be between 0.1 and 0.5 HSV genome equivalents per cell. Fraser *et al*⁵ reported the detection of HSV DNA sequences using a probe similar or identical to pHSV 106, containing a 3.4 kilobase Bam H1 insert including the HSV thymidine kinase gene, so in studies using that probe it is likely that our techniques were of comparable sensitivity. The claimed sensitivity of 1 genome per 200 cells presumably applied to studies using either whole HSV sequences or much larger cloned fragments. In this case, as in the study by Sequeira *et al*⁶ there is a risk of false positive findings due to the hybridisation between mammalian DNA and HSV short terminal repeated sequences.¹⁵ On the basis of this study there is no evidence of replicating HSV in the human central nervous system, either in Alzheimer's disease patients or in non-neurological controls. This included areas of the limbic system (amygdala, hippocampus, temporal cortex) where HSV replicates during acute encephalitis¹⁶ and which may be associated with the pathology of Alzheimer's disease.¹⁷

The possibility cannot be excluded that latent HSV could be sporadically reactivated, perhaps from another site, or that we failed to detect a small number of latently infected cells. Small numbers of isolated cells carrying the HSV genome may only be detectable using *in situ* hybridisation, where large numbers of individual cells can be examined. As the brain is a large organ, a systematic survey would be a time consuming endeavour, but there are no alternative methods offering a sensitivity comparable to hybridisation. Although Morawetz *et al*¹⁸ report a high success rate in the cultivation of HSV from biopsies of acute HSV encephalitis, direct infectious assay has been less successful when applied to latently infected CNS tissue. Cabrera *et al*¹⁹ isolated HSV from 5% of infected mouse brains after the acute phase, yet could detect HSV sequences in 30% of the brains. Similarly Rock and Fraser²⁰ could detect HSV sequences in mouse brain stem 2 months post infection, but were not able to grow viruses from brain stem explants.

In conclusion, our results do not support the findings of Fraser *et al*⁵ that HSV DNA is commonly found in the human CNS; in the brains we examined HSV DNA was either absent or present at a very low level. However by analogy with mouse studies²⁰ there are other areas that might be expected to harbour virus sequences after a CNS infection, such as the brain stem. It may be fruitful to look in these

areas and employ techniques such as immunocytochemistry, which has been reported to detect HSV antigenicity in brains, both non-neurological and Alzheimer's disease cases,⁹ in addition to molecular hybridisation.

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