Search for viral nucleic acid sequences in the post mortem brains of patients with schizophrenia and individuals who have committed suicide

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SUMMARY Molecular hybridisation has been used to screen several areas of post mortem brain from 20 patients with schizophrenia, 23 individuals who were suspected of having committed suicide and 21 control cases, for viral nucleic acids. Cloned probes were able to detect picogram levels of viral DNA and to quantify herpes simplex type 1 DNA from encephalitic brain, but no sequences hybridising to cytomegalovirus, varicella zoster virus, herpes simplex type 1 or JC or BK papovaviruses were detected in any of the experimental samples. These findings are discussed with reference to the viral hypothesis of the aetiology of psychiatric disease.

Viral hypotheses of the aetiology of schizophrenia have been based on evidence from several sources, including reports of elevated antibodies to viruses in the serum and CSF from schizophrenic patients,¹⁻³ and the association of schizophrenia-like symptoms with encephalitis lethargica. A "virus-like agent (VLA)" has been described in the CSF of a significant proportion of schizophrenic patients,^{4,5} and immunocytochemical localisation of viral antigens in schizophrenic post mortem brain has been reported.⁶ The neurotropic DNA viruses herpes simplex type 1 (HSV 1) and cytomegalovirus (CMV) are of particular interest. HSV 1 is able to establish latent infections in sensory ganglia,^{7,8} and studies on the distribution of HSV 1 (the commonest cause of encephalitis) in encephalitic brain⁹ have demonstrated an affinity for the temporal cortex and limbic system, where neuropathological abnormalities have been reported in schizophrenia.¹⁰ In addition an increased incidence of herpes virus antibodies has been reported in psychopathic disorder,¹¹ and psychotic depression.¹²

The technique of molecular hybridisation has been reported to detect nucleic acid sequences homologous to HSV 1 in three of four brain smears from chronic psychiatric patients¹³ and in a separate study in six of 11 brain DNA extracts from patients with no clinical signs of herpesvirus infection.¹⁴ Hybridisation studies performed to date have failed to detect CMV

related nucleic acid in the hippocampus of the brains of six schizophrenics¹⁵ and in the temporal cortex of 25 schizophrenics.¹⁶ We present here an extension of these studies to screen DNA preparations from several areas of post mortem brain from 20 schizophrenics, 23 suicide cases and 21 non-neuropsychiatric controls for the nucleic acid sequences of the herpes viruses HSV 1, CMV and Varicella-zoster virus (VZV) and of the human polyoma virus JC (JCV), the aetiological agent of progressive multifocal leukoencephalopathy (PML), as well as a closely related papovavirus BK virus (BKV). JCV and BKV are ubiquitous human agents and as DNA viruses, should be well preserved post mortem. Hybridisation techniques for their detection have been described.¹⁷⁻¹⁹

Materials and methods

Plasmid pBR322 was obtained from Sigma. Plasmid pSG124, a pBR325 derivative containing a 21 kbp insert of HSV 1 DNA was supplied by Dr RM Sandri-Goldin of the University of Michigan Medical School, USA. Plasmid pHIE2, consisting of pAT153 with a CMV Hind III F fragment (21 kbp) insert was supplied by Dr PJ Greenaway of the Central Public Health Laboratories, Porton Down, England. Dr AJ Davison of the Institute of Virology Glasgow, Scotland, supplied plasmid pVb, consisting of the b Kpn I VZV fragment (18 kbp)²⁰ cloned into pAT153, and Dr A Akrigg of the Central Public Health Laboratories supplied total genomic clones of JCV and BKV in plasmid pBR322.

Transformation of *E. coli* HB101 by the above plasmids with viral inserts was by the method of Lederberg and Cohen.²¹ Transformants were isolated by growth on LB

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agar (1.5%) supplemented with appropriate antibiotics. Single colonies were grown up in 10 ml then in 150 ml broth cultures and amplified with chloramphenicol (170 µg/ml). Plasmid DNA was purified by the method of Birnboim.²²

Post mortem brain material

All three series of brains were from the collection at the Division of Psychiatry, Clinical Research Centre, Harrow (see tables 1-4).

The control series of brains were collected from patients dying from a variety of causes in a District General Hospital without a known history of psychiatric or neurological disease. Patients with schizophrenia had died in one or other of two large mental hospitals. The diagnosis was established by application either of the St Louis criteria, or the Syndrome Checklist of the WHO Present State Examination, and in the majority of cases both. The brains of patients in the suicide series were collected with the cooperation of H.M. Coroner for Inner North London (Dr. D. R. Chambers). This series included three individuals who met DSM III criteria for major depression and in seven further cases evidence strongly suggested a past history of depression. Four individ-

uals were thought to have suffered from schizophrenia, two from personality disorder and in the remaining cases no diagnosis was possible.

Brains were dissected according to the standard procedures established at the Clinical Research Centre.

Table 1 Control series brains $n = 21$, sex ratio 9F:12M, mean age = 53 ± 15 (SD) years.

Number	Age (years)	Sex	Cause of death	p.m. delay (hours)
1	26	F	Electrocution	21
2	59	M	Coronary artery occlusion/atheroma	42
3	51	M	Coronary artery occlusion/atheroma	17
4	36	M	Coronary artery thrombosis	66
5	57	M	Not known	72
6	61	M	Coronary artery occlusion/atheroma	26
7	65	M	Coronary artery occlusion	47
8	51	M	Coronary artery occlusion/atheroma	27
9	54	M	Myocardial infarction	72
10	80	F	Carbon monoxide poisoning	78
11	30	F	Diabetic coma	56
12	36	F	Coronary artery occlusion	36
13	68	F	Coronary artery occlusion/atheroma	33
14	59	F	Peritonitis/perforated ulcer/infarction/atheroma	72
15	61	M	Pulmonary embolism	24
16	24	M	Accidental overdose	24
17	61	F	Acute peritonitis/perforated ulcer	62
18	59	F	Coronary occlusion	38
19	51	M	Myocardial infarction	24
20	59	M	Coronary artery occlusion/atheroma	27
21	67	F	Bronchial asthma	36

Table 2 Schizophrenic series brains. $n = 20$, sex ratio 7F:13M, mean age = 65 ± 17 (SD) years.

Number	Age (years)	Sex	Cause of death	p.m. delay (hours)
22	61	M	Myocardial failure	72
23	36	M	Haemorrhage from acute gastric ulcers	72
24	70	M	Bronchial pneumonia	76
25	74	F	Bronchial pneumonia + ischaemic heart disease	58
26	76	M	Bronchial pneumonia	15
27	76	M	Bronchial pneumonia	120
28	83	F	Uraemia, peritonitis	118
29	74	F	Haemorrhage	47
30	71	M	Coronary occlusion/atheroma	48
31	79	M	Myocardial infarction	52
32	59	M	Renal failure	42
33	40	M	Obstruction	69
34	79	F	Pulmonary oedema	120
35	84	F	Bronchial pneumonia	23
36	50	F	Uraemia, renal failure	24
37	69	M	Severe ischaemic heart disease	24
38	69	M	Intestinal obstruction	20
39	46	F	Bronchial pneumonia	45
40	79	M	Cerebro-vascular accident	24
41	22	M	Road traffic accident	72

Fig (a) Detection of HSV1 DNA in DNA prepared from temporal cortex of a case of herpes encephalitis. DNA was prepared as described in Materials and Methods. A duplicate set of serial 10 fold dilutions starting at 50 µg DNA per spot was filtered. Cross hybridisation between HSV-1 sequences and the other probes used in this study was minimal apart from the HSV1 Eco R1A clone, (b) Typical results of hybridisation of brain DNA extracts with serial probes. Hybridisation signals were not significantly above background as defined by signals from hybridisation with pBR322, (c) Hybridisation of brain DNA extracts with labelled human DNA, confirming the retention of denatured DNA by the filters. The amount retained was quantified by densitometry as described previously.²³

Extraction and filtration of DNA

Extraction of DNA from 100 ± 10 mg brain tissue was as previously described,²³ except that RNA was removed from total nucleic acid extracts by adding DNase inactivated RNase to a concentration of 1 mg/ml (total nucleic acids in 0.4 ml STE buffer i.e. 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) and incubating at 30 °C for 30 minutes with shaking. Size of extracted DNA was examined by agarose gel electrophoresis and DNA yields were determined spectrophotometrically, using the equation [DNA] mg/ml = OD260 nm/22.

DNA samples were then denatured in a boiling water bath for 10 minutes, an equal volume of $20 \times$ SSC buffer (3M NaCl, 0.3 M sodium citrate, pH 7.0) was added, and then 200 µl aliquots (5 µg) were filtered in duplicate through pre-

wetted ($3 \times$ SSC) nitrocellulose membranes (Schleicher and Schuell) using a 96 well filtration manifold (BRL Ltd). After air drying and baking filters were stored at -20 °C in sealed bags until use.

Molecular hybridisation

Hybridisation and washing of filters was as previously described.²³ Plasmids were labelled with deoxycytidine 5'-[α -³²P] triphosphate (Amersham) by the nick translation method of Rigby *et al*²⁴ to specific activities of the order of $3 \times 10^8 - 2 \times 10^9$ cpm/µg DNA. Human DNA was labelled to a specific activity of $1-2 \times 10^6$ cpm/µg. Probes were used at a concentration of 10 ng/ml, and the volume of hybridisation buffer: area of nitrocellulose ratio was maintained at > 0.1 ml/cm². Hybridisation was at 65 °C for 16 hours. After washing, filters were rinsed in $3 \times$ SSC at room temperature, dried and exposed to Kodak X-Omat AR film, using Ilford Rapide intensifying screens at -70 °C for 1 to five days. Intensity of hybridisation was measured by densitometric scanning.

Filters were hybridised in sequence with labelled pBR322 (a control for non-specific hybridisation), pHIE2 (CMV), pSG124 (HSV), pVb (VZV), pJCV then pBKV. To determine assay sensitivity and ensure assay specificity control filters were included in each hybridisation. Controls for the pSG124 hybridisation were of serial dilutions of pure HSV 1 DNA and of DNA extracted from the temporal cortex of a confirmed case of HSV 1 encephalitis and pHIE2 controls were serial dilutions of purified CMV DNA. For the pVb, pJCV and pBKV hybridisations fragments of viral nucleic acid were excised by restriction digestion, run on agarose gels, blotted onto nitrocellulose membranes by the method of Southern²⁵ and hybridised to the appropriate ³²P-labelled plasmid. Data presented in table 5 show assay sensitivity.

Results

DNA yields from frozen post mortem brain tissue were of the order of 0.45 mg/g wet wt tissue. Agarose gels (1%) of the extracts stained with ethidium bromide and viewed under UV illumination showed preservation of fragment sizes greater than 20 kb, suitable for dot blot hybridisation. RNA contamination was minimal.

Hybridisation signals between all the post mortem DNA preparations and labelled pBR322 were minimal. We did not observe any hybridisation between sample DNA and any of the viral probes which was above background but we were able to demonstrate

Table 3 Suicide series brains. n = 23, sex ratio 12F:11M, mean age = 46 ± 19 (SD) years.

Number	Age (years)	Sex	Cause of death	p.m. delay (hours)
42	21	F	Drug overdose	69
43	68	F	Fall	24
44	37	M	Cardiac arrest/burns	48
45	74	M	Alcohol/drug overdose	17
46	61	M	Fall	48
47	24	M	Fall	19
48	36	M	Hanging	37
49	33	M	Alcohol/drug overdose	24
50	26	F	Struck by train	64
51	41	F	Drug overdose	21
52	27	F	Poisoning/drug overdose	48
53	71	M	Drug overdose	48
54	24	M	Drug overdose	25
55	73	M	Drug overdose	41
56	48	M	Drug overdose	36
57	63	F	Drug overdose	48
58	22	M	Hanging	43
59	70	F	Drowning	24
60	50	F	Drug overdose	36
61	65	F	Drug overdose	24
62	21	F	Alcohol/drug overdose	48
63	51	F	Drug overdose	96
64	57	F	Drug overdose	72

Table 4 Areas of control, schizophrenic and suicide post mortem brains screened by molecular hybridisation.

Area	Controls	Schizophrenics	Suicides
Caudate	20	17	22
Hippocampus	17	14	18
Putamen	17	17	18
Temporal cortex	21	16	19
Frontal cortex	17	15	18

Table 5 Sensitivity of molecular hybridisation using viral probes, derived from dot blots (*) and from Southern blots of excised viral fragments after agarose gel electrophoresis (†).

Probe	Virus	Size of genome (kbp)	Fragment size (kbp)	Amount of homologous DNA detectable (pg)	Amount of viral DNA detectable (pg)
pHIE2	CMV	225 (1.5×10^6 daltons)	21	—	10.7*
pVb	VZV	120 (0.8×10^8 daltons)	18	0.6†	4.0
pSG124	HSV	150 (1.0×10^8 daltons)	21	—	10.0*
pJCV	JCV	4.9 (3.3×10^6 daltons)	4.9	0.8†	0.8†
pBKV	BKV	5.2 (3.5×10^6 daltons)	5.2	0.6†	0.6†

HSV1 DNA at a copy number of 1.38×10^3 copies/cell in a post mortem cortical sample of HSV1 temporal lobe encephalitis. Cross-reaction between viral probes and pBR322 was low and hybridisation to Southern blots of restriction fragments of CMV, VZV, JCV and BKV showed sensitivities of between 0.6 pg and 10 pg viral DNA.

Discussion

Molecular hybridisation utilising cloned viral probes is able to detect viral DNA sequences at picogram levels. The sensitive and specific assays used in this study were unable to detect viral sequences in the post mortem brains of control, schizophrenic or suicide cases, and hence our results are not in agreement with reports of the detection of HSV DNA in the brains of three of four chronic psychiatric patients,¹³ five of six epileptic patients,²⁶ and in six of 11 control patients with no clinical signs of HSV infection.¹⁵ By incorporating cloned fragments of nucleic acid unique to viral genomes in our study we have minimised the possibility of cross hybridisation sometimes seen in hybridisations using whole genomic probes resulting from regions of homology between mammalian cell DNA and the genomes of herpes viruses,²⁷ and by contamination of the probes with cellular sequences.

Our detection limits for HSV, CMV and VZV were 1.7×10^3 , 1.2×10^3 and 0.8×10^3 genome equivalents respectively. The amount of material filtered per sample onto nitrocellulose was $5 \mu\text{g}$ (10^6 cells) and hence the optimal detection limits in terms of genome equivalents/cell were 1 genome/588 cells for HSV, 1 genome/833 cells for CMV and 1 genome/1250 cells for VZV, comparable with or better than those previously described with similar techniques.^{14 15 26 28-30} Assay sensitivity of the above limits would have allowed detection of viral genomes at the levels previously described in latent viral infections. Puga *et al*⁸ have reported levels of 0.11–0.30 HSV DNA copies/cell in the trigeminal ganglia of mice chronically infected with HSV, and Cabrera *et al*²⁸ have shown levels of HSV DNA of 0.01–0.05 copies/cell in the hemispheres and 0.22–1.78 copies/cell in the brain stem of mice with latent HSV. We were able to detect HSV sequences in the post mortem brain (temporal cortex) of a confirmed case of HSV encephalitis and demonstrate virus DNA at approximately 1×10^3 copies/cell demonstrating an active infection (Gilden *et al*³¹ reported VZV at a level of 700 copies/cell in infected cells).

In this study we report limits of detection of JCV and BKV of 0.8 pg and 0.6 pg respectively. This represents 1 genome/300 cells for JCV and 1 genome/350 cells for BKV. Our negative hybridisation results for these papovaviruses are in accordance with those

reported by Chesters *et al*³² who were unable to detect BKV or JCV in control or disease brain tissue (other than PML).

We conclude that our assay sensitivities should have been able to detect active infection (and probably viral latency) in the brain areas described, and although we cannot exclude a localised latent infection, or an infection in an area not examined in this study, our findings and those of Pogo and Elizan³³ suggest that intracranial infection with these viruses is rare. Our findings suggest that these viruses are not systematically associated with either schizophrenia or types of affective illness which lead to suicide. The list of viruses surveyed is by no means exhaustive however, and serological evidence indicates that searches for other viruses should be undertaken.³⁴

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