Patients with normal anti-GMl antibody titres also respond to immune modulating therapy.

There are three possible reasons for the normal anti-GMl antibody titres observed in our patients: firstly, the polyneuropathies of unknown origin investigated might not be immune mediated[11]; secondly, these polyneuropathies might be immune mediated but induced by antibodies directed against epitopes other than those of gangliosides; and thirdly, the assay applied was of low sensitivity and specificity and may not have recognised disease specific anti-GMl antibodies. Other authors, however, have detected raised anti-GMl antibody titres using the EL-GMl assay and, hopefully, the specificity of anti-GMl antibody testing will be increased by analysing GMl target epitopes other than Gal(β1-3)GalNAc.[9]

In conclusion, our results suggest that quantification of anti-GMl antibody titres is of little help in the diagnosis of polyneuropathies of unknown origin.

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Quantification of HIV DNA in the brain by PCR: differences between fresh frozen and formalin fixed tissue

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Abstract

HIV-1 DNA extracted from frozen and formalin fixed brain tissue can be detected using PCR. This work has been extended by amplifying, using semiquantitative PCR, HIV DNA extracted from frontal lobe tissue of 16 patients with AIDS (eight positive and eight negative for p24 antigen). DNA was amplified using HIV-1 *pol* gene digoxigenin labelled primers and detected by chemiluminescence and densitometry. Cloned standards were amplified in parallel for quantification. HIV DNA levels detected in frozen tissue showed a correlation with p24 positivity and the severity of the histological diagnosis. This correlation was less clear in the formalin fixed material.

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Keywords: HIV, PCR, frozen tissue, formalin fixed tissue.

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About 30% of adult patients and 50% of paediatric patients with AIDS are likely to develop symptoms encompassed by the term HIV associated dementia complex (HIV-ADC) and it is probable that these percentages would be greater if patients did not succumb to earlier complications. The HIV-ADC includes a wide range of neurological problems in which the pathogenetic processes involved are not fully understood. There are two likely pathogenetic mechanisms, both of which may be contributing to disease. Firstly, the direct cytopathic effects of viral infection may be responsible for the cell destruction and loss of neurons which are normally observed in HIV-ADC. This type of pathogenesis may be important during the terminal stages of AIDS.[1] However, neurons in vivo show no evidence of HIV infection while microglia and macrophages are typically infected and the formation of multinucleated giant cells (MGC) is a diagnostic feature of HIV encephalitis (HIVE) and HIV leucoencephalopathy (HIV lep).[1] Secondly, damage is the indirect consequence of HIV infection and is probably macrophage mediated. Neuropathological changes are the result of secretion of neurotoxic factors including arachidonic acid, cytokines and toxic oxygen metabolites.[2] Several reports have im-

Table 1 Pathology, HIV p24 antigen status and copies of HIV DNA per μg total DNA extracted from frozen and formalin fixed tissue

Patient number	Tissue		p24 antigen status	Pathology
	Frozen	Formalin fixed		
1	1020	340	Positive	HIVE
2	700	200	Positive	HIVE
3	150	<10	Positive	HIVE
4	90	110	Positive	HIVE
5	130	<10	Positive	HIVE, PML
6	100	<10	Positive	HIVE
7	30	130	Negative	HIVE, CMV ventricilitis
8	<10	<10	Negative	HIVE
9	25	20	Positive	HIV leucoencephalopathy
10	180	180	Negative	Diffuse gliosis, vasculitis
11	125	30	Negative	Diffuse non-specific changes
12	100	<10	Negative	Non-specific changes
13	30	160	Negative	Moderate gliosis
14	<10	50	Positive	HIV, necrosis, encephalitis
15	<10	40	Positive	PML
16	<10	<10	Negative	Cryptococcosis

PML = progressive multifocal leucoencephalopathy; CMV = cytomegalovirus; HSV = herpes simplex virus.

plicated viral proteins in the pathogenetic process—for example, the viral tat protein and gp 120, the latter being expressed in transgenic mice which showed some HIV-ADC-like symptoms.[3] Recently, it was suggested that neuronal loss was due to apoptosis occurring after the local release of neurotoxic factors during HIV infection.[4] All of these possibilities may well be involved in the pathogenesis. An important consideration in attempting to understand the pathogenesis of HIV-ADC in the light of these possibilities is whether or not there is a link between viral load and the extent of neuropathology. This has been noted in previous reports where there was correlation between increasing pathology and the amount of virus present in the tissue.[5] It was these observations which were of interest for the present study, particularly the link between viral load and the more severe diseases, such as HIVE and HIV lep. We have chosen to estimate the amount of HIV in the brain in patients with AIDS by measuring levels of HIV DNA. This DNA represents a total of integrated provirus and extrachromosomal viral DNA, which is often present in relatively high amounts in the brain.[5] Viral particles (containing RNA only) are normally found in the brain tissues at low levels so the HIV DNA represents the consequence of latent rather than active infection.

We also wished to assess the effects of formalin fixation on the tissue being examined by PCR quantification. Postmortem brain material is typically fixed in 4% formaldehyde solution (formalin) for long periods of time (that is, weeks or months). This procedure has the advantage of rendering the tissue non-infectious, preventing deterioration and preserving the morphology. However, formalin crosslinks proteins and nucleic acids, which can make their analysis more difficult. Determination of the molecular weight for DNA extracted from frozen tissue shows that it is normally intact, whereas the molecular weight of DNA from formalin fixed material is typically less than 500 base pairs (bp)[6] (our unpublished observations). This may cause problems for some DNA studies. However, in a previous study comparing amplification in frozen and formalin fixed brain material, we showed that the fixation process only slightly compromised the ability to detect HIV DNA by PCR.[7]

Methods

Frozen and formalin fixed frontal lobe tissue from postmortem brain material of 16 patients with AIDS was studied. The samples selected for quantification had previously been shown to be HIV DNA positive by PCR[7] and the quantification studies were done without prior knowledge of the histological details of each sample. Negative controls, consisting of PCR reagents only, were included with all batches of samples being tested to ensure no contamination was present. Histological examination of the brain tissue from each patient was done on a routine basis to determine the type of HIV encephalopathy.

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p24 HIV antigen screening was done routinely on all sections as described previously.[7]

EXTRACTION OF DNA

Formalin fixed tissue samples were dewaxed with three 15 minute xylene washes followed by five minutes in ethanol. Small blocks of brain tissue from each source were finely diced before being placed in DNA extraction buffer. DNA was prepared by two methods. (1) Standard DNA extraction procedures using proteinase K digestion (0·25 mg/ml 56°C, overnight) followed by chloroform/phenol purification and ethanol precipitation.[7] For the formalin fixed tissue it was necessary to increase the proteinase K concentration to 5 mg/ml to extract sufficient DNA. (2) A technique using guanidium isothiocyanate to dissolve tissue was found to produce slightly better quality DNA, especially from formalin fixed brain so this latter technique was eventually used on a routine basis.[8] The purified DNA was finally quantified by spectrophotometry.

PCR AND DNA QUANTIFICATION

A semiquantitative PCR technique was used to estimate the number of HIV DNA copies in each sample.[9] The primers used amplified a 143 bp fragment from the HIV *pol* gene; the upstream primer was 5′ labelled with digoxigenin. The amplified DNA incorporated the digoxigenin, which enhanced detection and enabled the amount of reaction product to be quantified. The amplification reaction included identical amounts of brain DNA (normally 1 μg) and reagents as specified by the manufacturer (Bioline, UK) under the following conditions: denaturation, five minutes at 94°C; annealing/extension, two minutes at 62°C; for 30–32 cycles followed by five minutes extension at 72°C. This protocol maintained the reaction in the exponential phase which is essential for quantification, as during this phase there is a direct correlation between the quantity of target DNA and the amount of amplified product

containing digoxigenin. Serial dilutions of cloned HIV DNA were run in parallel with brain DNA samples to permit quantification within a range of 20–2000 copies. After amplification, aliquots of reaction sample were separated by electrophoresis in 2% agarose and transferred to nylon membranes (Boehringer). The membrane bound digoxigenin was then reacted with a chemiluminescent substrate (Boehringer), detected by autoradiography and quantified by densitometry (Molecular Dynamics). The amplified standards were used to plot the HIV copy number against densitometry values to produce a standard curve. From this curve the number of HIV copies in each brain sample was extrapolated. The number of HIV copies was then expressed relative to the amount of total brain DNA in each reaction.

Results

The patients' diagnoses and the results are given in table 1. Patients 1–9 were ranked together because their diagnoses reflected a uniform type of pathology. These were HIVE and HIV lep which are associated with the presence of MGC and p24 antigen positivity. Immunostaining of the tissues for p24 antigen was usually observed with HIVE, but two non-HIVE cases (patients 14 and 15) were also positive. There was a perifocal pattern of staining in these two patients. This was due to local boosting of HIV levels around the region of pathology which is often seen when there is coinfection with another virus, such as DNA viruses, and did not represent typical distributions of p24 antigen seen in HIVE. It was possible to detect HIV DNA in all patient samples from both frozen and formalin fixed tissues. However, in many cases the levels of HIV DNA were barely detectable and therefore were not quantified and were designated as <10 copies. Increasing the number of PCR cycles confirmed that these cases were positive (data not shown). The 1 μg of total DNA used in each case represents an analysis of approximately 150 000 cells. Plots of cloned standards of HIV DNA against densitometer values produced straight line graphs in the range of 10–2000 copies from which test samples values were extrapolated (data not shown).

Discussion

The results from frozen tissue suggested that there was a slight, direct correlation between viral load and a more severe pathology—that is, HIVE and HIV lep. In particular, patients with considerably higher HIV DNA levels—for example, patients 1 and 2, would be expected to have HIVE. However, a converse conclusion that patients with HIVE will always have a greater HIV DNA load was not indicated by our data. The precise significance of brain HIV DNA load in relation to pathogenesis is not clear, although it may represent a latent infection with intermittent expression of viral antigens at low levels with consequent immune mediated cytopathology.[5 10] It is not known whether or not these antigens and HIV nucleic acid are being assembled into intact virions which may reinfect local cells. The cellular localisation of HIV is restricted mainly to macrophages and MGC, both of which are thought to be reservoirs of infection in the brain. In situ DNA studies will help to define more precisely this localisation and determine whether or not other cell types are involved.

The quantification of HIV DNA in formalin fixed material resulted in lower values than in frozen material in most cases, with limited agreement between the two, especially in patients 1 and 2 where HIV DNA levels were high. In those samples where there was more HIV DNA in the formalin fixed tissue this was possibly due to local fluctuations within the brain tissue under analysis. The ability to detect and quantify DNA from formalin fixed tissue will be largely determined by the size of the fragment to be amplified. In this study the amplified *pol* gene fragment was 143 bp, which was significantly smaller than the average size of DNA fragments extracted from formalin fixed tissue of approximately 500 bp.[6] However, as the reduction in molecular weight of extracted chromosomal DNA is likely to be a random process it is inevitable that some of the target sequences will no longer be present. The discrepancy between the results of frozen and formalin tissue may also be due to differences in the fixation times of the samples and to vagaries in the fixation process. Although quantification of HIV DNA in formalin fixed tissue using PCR was feasible, the results must be interpreted with caution and are likely to be lower than those from fresh material.

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