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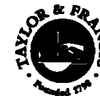

Detection of infectious agents in brain of patients with acute hemorrhagic leukoencephalitis

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Acute hemorrhagic leukoencephalitis (AHL) is a rare and usually fatal disorder characterized clinically by an acute onset of neurologic abnormalities. It may occur in association with a viral illness or vaccination. Radiology and brain biopsy are essential for the diagnosis. The etiology of AHL is unclear. We postulated that viral/bacterial infection might be responsible, directly or through an immune-mediated mechanism, for this acute inflammatory myelinopathy. Fifteen cases of AHL were studied. Infectious agents, including varicella zoster virus (VZV), herpes simplex virus (HSV), human herpes virus-6 (HHV-6), cytomegalovirus, Epstein-Barr virus, and *Mycoplasma*, were investigated in brain specimens using the polymerase chain reaction (PCR), reverse transcriptase (RT)-PCR, and immunohistochemistry. Using PCR, HSV DNA was found in four cases, VZV DNA in two, and HHV-6 DNA in one. Among the control cases, two were HSV DNA positive. Further investigation to detect HSV RNA and antigens in HSV DNA–positive cases revealed that two cases with AHL were both HSV RNA and antigen positive. AHL is a hyperacute disease, which is considered the most acute form of acute disseminated encephalomyelitis (ADEM). Our findings suggests that a viral infection may be implicated in its pathogenesis, most likely through an indirect mechanism; however, as only a few cases of this rare disease were examined, statistical significance was not achieved. As a number of patients with disorders of the ADEM group may progress to develop multiple sclerosis (MS), we argue that an organism that has produced the former may remain in the brain tissue and be subsequently involved in the production of a self-sustained disorder such as MS. *Journal of NeuroVirology* (2002) **8,** 439–446.

Keywords: acute hemorrhagic leukoencephalitis; herpes simplex virus; immunohistochemistry; reverse transcriptase–polymerase chain reaction

Acute hemorrhagic leukoencephalitis (AHL), first described by Hurst in 1941, is a rare hyperacute process whose clinical presentation includes a preceding flu-like illness, variable motor, sensory, visual, and cognitive symptoms, changes on neuroimaging, and the presence of red cells and neutrophil pleocytosis in the cerebral spinal fluid (CSF). Despite broad-spectrum antibiotics and antiviral therapy, the disease may take a rapidly progressive course with seizures and coma, leading to death. Radiology and brain biopsy are essential for diagnosis (Markus *et al*, 1997). Pathological changes are localized to the white matter and consist of perivenous small cell infiltrates, edema with demyelination, and prominent petechial and ringlike hemorrhages.

Following the early paper by Russell (1955), AHL is presently seen by a number of authors as the most acute and severe variant of acute disseminated encephalomyelitis (ADEM) (Hartung and Grossman, 2001), a disorder often preceded by an infectious illness or a vaccination. Both are monophasic disorders

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involving the white matter and showing a prominent inflammatory perivascular infiltrate. However, clinical and pathological differences should also be noted. AHL is a hyperacute disorder, usually developing after a respiratory tract infection and leads to death in 1 to 2 weeks. CSF is under increased pressure and contains polymorphs. Neuroradiology reveals diffuse high T1 and low T2 signal. Conversely, ADEM follows measles, rubella, and mumps and runs a more protracted and less severe course over weeks to months. CSF cytology reveals lymphocytes. Radiological findings include presence of multifocal, symmetrical, and often confluent signals. AHL/ADEM can be defined as postinfectious, postimmunization, and post–organ transplantation leukoencephalomyelitides.

The more protracted forms of AHL/ADEM share features with multiple sclerosis (MS) as well as obvious differences: the former may have a multiphasic presentation (Khan *et al*, 1995) and CSF may contain oligoclonal bands, albeit they may subsequently disappear (Nasr *et al*, 2000). Importantly, both are demyelinating disorders (Kesselring *et al*, 1990). Furthermore, it is known that a number of patients with ADEM progress to develop MS (Hartung and Grossman, 2001).

The etiology of both AHL/ADEM and MS is unknown. Among the various possibilities, infectious agents have been suggested in both of them, which, in the former group, might operate through an autoimmune mechanism. This hypothesis is supported by clinical and pathological similarities between AHL/ADEM on the one hand, and experimental hyperacute allergic encephalomyelitis and allergic encephalomyelitis on the other. There is some evidence that an autoimmune mechanism could be the case also for MS (Noseworthy, 1999). Unfortunately, data from literature to understand the role of an infection in all these disorders are scanty, as only case reports or small series are available.

The aim of this study was to detect the presence of organisms in brains of patients with the acute form of AHL/ADEM. The rationale behind our decision to study the most acute type within the group (AHL) is that if these disorders are triggered by an infectious agent, the chances of finding it in the tissue are higher the closer to the acute event it is sought.

Results

Patients

Among the 15 patients with AHL, clinical signs of recent or relapsing flulike or infectious illness (respiratory and urinary tract), skin burns, or dental extraction were reported in 7 (in particular patient 9, an 11-year-old boy, who died shortly after sustaining burns affecting 55% of his skin), nonspecific changes in CSF in 4, computed tomography/magnetic resonance imaging (CT/MRI) abnormalities in 6. All died within 4 weeks from the onset of neurological symptoms. Detailed clinical history was available in 10 patients. In these, it appeared that, with one exception, all died within 1 week from the onset of the symptoms; unfortunately no tests for detection of organisms had been carried out during their hospital admission. Moreover, there was no record of recent vaccination.

Histological examination

All cases shared similar morphological features, consisting of a severe and diffuse hemorrhagic process localized to the white matter. On histological examination, the hemorrhages appeared to surround small veins and, with few exceptions, were not confluent. Blood cells were intermingled with an inflammatory process, represented by lymphocytes with a minor component of polymorphs. There was also a diffuse microglial hyperplasia and discrete presence of macrophages. Meninges showed discrete lymphocytic infiltration, whereas the grey matter was mostly spared. Myelin stains revealed a demyelinating process, which was particularly obvious around the blood vessels, with relative preservation of the axons.

Polymerase chain reaction (PCR)

PCR revealed herpes simplex virus (HSV) DNA in four cases, varicella zoster virus (VZV) in two, and human herpes virus-6 (HHV-6) in one. No more than one type of virus was found in any single case and cytomegalovirus (CMV), Epstein-Barr virus (EBV), and *Mycoplasma* could not be detected. Among the 10 controls, HSV DNA (but no other viral DNA) was detected in 2. Figure 1**a** shows the PCR results in agarose gel electrophoresis. Data are listed in Table 1.

Reverse transcriptase (RT)–PCR and Southern blot hybridization

RT-PCR was applied to detect presence of HSV RNA in the four AHL- and two HSV DNA–positive control cases. Among them, two (patients 8 and 9) with AHL revealed HSV DNA polymerase RNA (Rogers *et al*, 1991; Cui and Carr, 2000). RT-PCR results were detected by gel electrophoresis and confirmed by Southern blot hybridization using internal probe (Figure 1**b**). Data are also included in Table 1.

Immunohistochemistry (IHC)

Two cases with AHL, both of which were also PCR/RT-PCR–positive for HSV, were HSV antigen positive. The pattern of immune reactivity with HSV antibody in these cases differed from that seen in brains with acute HSV encephalitis (used as positive control), in which antigen was revealed both in neurons (Figure 2**A**) and glia in tissue sections at a dilution of 1/2000. By contrast, in each of the two AHL cases, immunoreactivity appeared largely restricted to cells (Figure 2**B**) within and near inflammatory lesions that were selectively demyelinated, as well as to vessel wall and endothelial cells; moreover, these

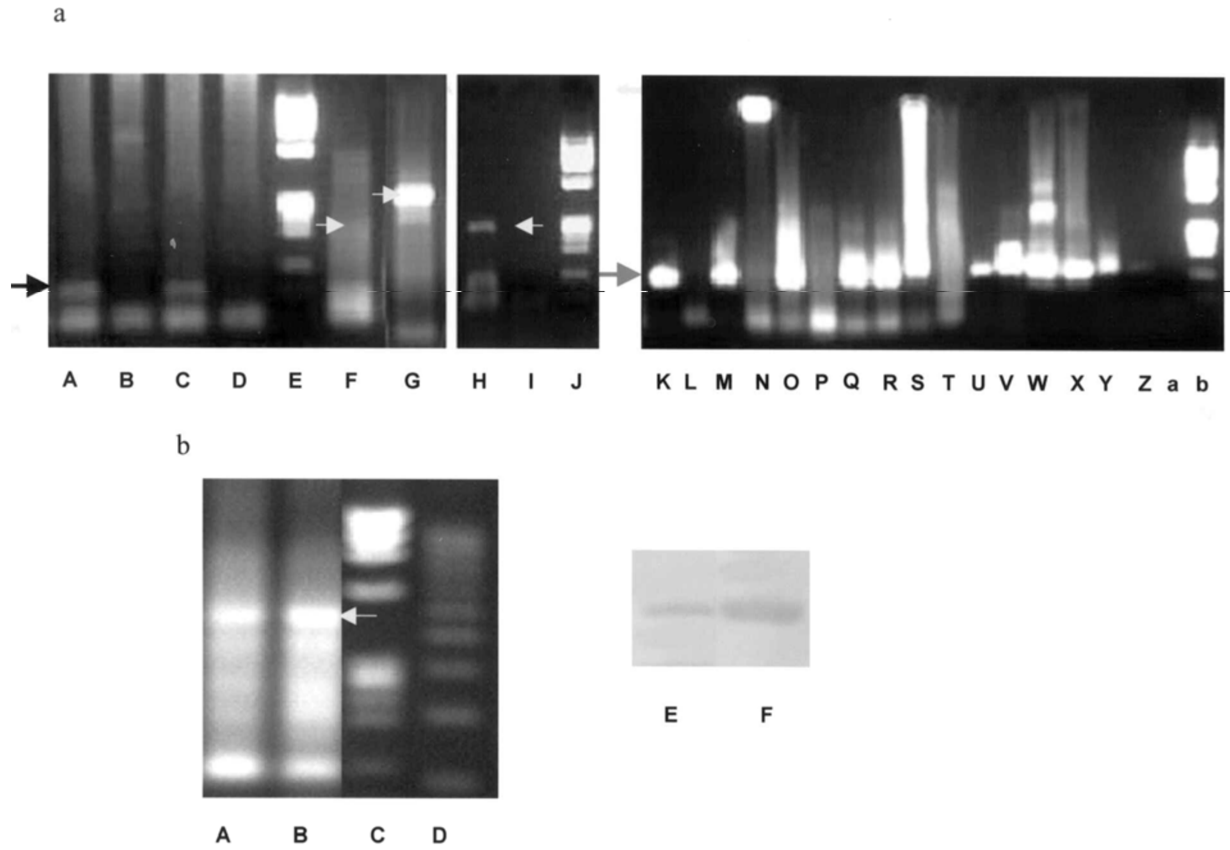

Figure 1 Electrophoresis shows some PCR/RT-PCR–positive cases. (**a**) Lanes A–D: HHV-6 (A: case no. 3; B and D: negative cases; C: positive control); E: $\phi\times174$/Hae III molecular weight marker; F: VZV positive control; G: CMV positive control; H and I: *Mycoplasma* positive and negative controls; J: $\phi\times174$/Hae III marker; K–Z, a, b: HSV (K, M, O, Q, R, U, V, W, X, Y, Z: HSV-positive cases; S: positive control; L, N, P, T, a: negative cases); b: $\phi\times174$/Hae III marker. (**b**) Lanes A, B: HSV RNA (arrow); C: $\phi\times174$/Hae IV molecular weight marker; D: 100 bp ladder; E, F: Southern blot hybridization.

cases required a much higher HSV antibody concentration (1:50) to detect the antigen, which, in double-immunofluorescent preparations with CD68 or GFAP antibodies, showed the virus only in macrophages, both within (Figure 2**C**) and outside (Figure 2**D**) the demyelinated areas.

Statistics analysis
Therate of detection of viral DNA in cases with AHL was higher than in controls, butstatistical analysis did not show significant difference between the two groups ($P = .1417$). With regards to HSV RNA and antigen, 2 out of 4 cases of AHL resulted positive,

Table 1 Results of viral/bacterial DNA and antigens detection in cases with ADEM by PCR and immunohistochemistry

Case	*Age/sex*	*VZV, IHC/PCR*	*HHV-6B, IHC/PCR*	*HSV, IHC/PCR*	*CMV, IHC/PCR*	*EBV, PCR*	*Mycoplasma, PCR*
1	29/F	−/+	−/−	−/−	−/−	−	−
2	60/M	−/−	−/−	−/+/−*	−/−	−	−
3	47/M	−/−	−/+	−/−	−/−	−	−
4	68/F	−/−	−/−	−/+/−*	−/−	−	−
5	n.a./F	−/+	−/−	−/−	−/−	−	−
6	48/M	−/−	−/−	−/−	−/−	−	−
7	41/F	−/−	−/−	−/−	−/−	−	−
8	n.a.	−/−	−/−	+/+/+*	−/−	−	−
9	11/M	−/−	−/−	+/+/+*	−/−	−	−
10	34/M	−/−	−/−	−/−	−/−	−	−
11	27/M	−/−	−/−	−/−	−/−	−	−
12	21/n.a.	−/−	−/−	−/−	−/−	−	−
13	n.a./M	−/−	−/−	−/−	−/−	−	−
14	n.a.	−/−	−/−	−/−	−/−	−	−
15	30/n.a.	−/−	−/−	−/−	−/−	−	−

M = male; F = female; + = positive; − = negative; * = RTPCR result; n.a. = not available.

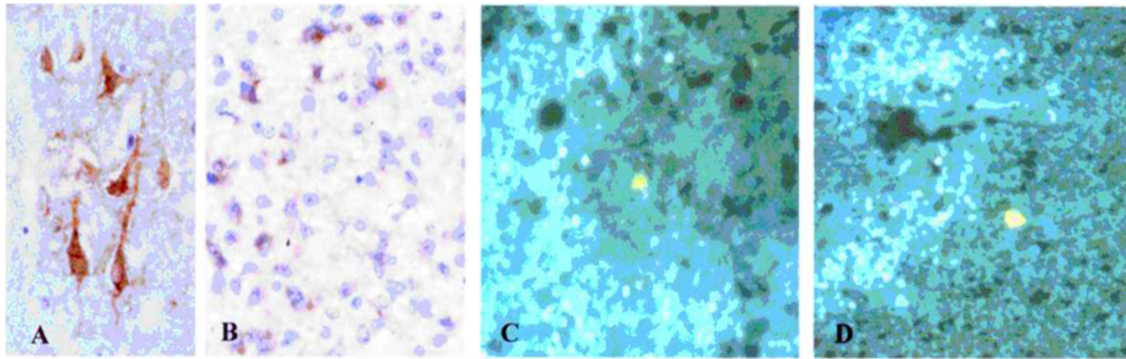

Figure 2 HSV antigen immunohistochemical staining in cases with typical HSV encephalitis (**A**) as well as AHL (**B**, **C**, **D**). (**A**) Signals are located in both grey and white matters in HSV encephalitis. Positive cells are morphological identified as neurons and glial cells. By contrast, in cases with AHL (**B**), immunostaining are located in white matter and glial and endothelial cells are selectively stained. **A** and **B**, DAB and hematoxylin; (**A**) ×444, (**B**) ×283. Double-fluorescent staining show HSV-positive macrophages locate within (**C**) and outside (**D**) lesion area. **C** and **D**, fluorescein and Texas Red; (**C**) ×575, (**D**) ×500.

whereas no single control case was positive for HSV RNA and antigen.

Discussion

AHL and ADEM are monophasic illnesses characterized by progressive, widespread demyelination and share many features. They may be part of a spectrum of diseases rather than distinct entities (Russell, 1955), AHL representing the hyperacute form (Hartung and Grossman, 2001). Both are believed to be immune-mediated and inflammatory processes, inducing a combination of motor, sensory, visual, and cognitive disturbances, which reflect the diffuse nature of the lesions. A number of viruses have been named as possible trigger of these disorders: VZV (Westenend and Hoppenbrouwers, 1998), EBV (Shoji *et al*, 1992), measles (Pearl *et al*, 1990) in addition to bacteria (Kumada *et al*, 1997), TB (Adams *et al*, 1986) among them, and fungi (Hall *et al*, 1998). AHL is also a known complication of vaccination (Huber *et al*, 1999) and organ transplantation (Horowitz *et al*, 1995).

We addressed the possible infectious etiology of AHL in 15 patients, in 10 of whom we were able to retrieve the clinical history. Duration of illness was between 2 days to 4 weeks. In seven of them, an acute monophasic or relapsing flu-like/infectious illness preceded the neurological deterioration, in keeping with the view that the majority of cases with AHL/ADEM follow an infection (Stüve and Zamvil, 1999). Although the finding of viral DNA in the brain of seven patients in the whole group (47%) does not confirm a pathogenetic role of a viral infection, we note that each of the seven positive brains contained only one type of viral DNA.

At present, the role of viral DNA in this acute disease is only speculative and, possibly, not a direct one. However, an indirect action is possible, through a still unknown mechanism, as suggested by other infections. In acquired immunodeficiency syndrome (AIDS), a disorder in which a condition of immune activation develops (Gendelman *et al*, 1994), various groups have given evidence supporting an indirect mechanism of brain damage (Schnittman and Fauci, 1994; Buttini *et al*, 1998). We studied brains of a number of presymptomatic individuals, in which we detected the presence of human immunodeficiency virus (HIV) DNA (An *et al*, 1996b), together with expression of cytokines (An *et al*, 1996a), discrete neuronal loss (An *et al*, 1996c), and axonal damage (An *et al*, 1997). Consequently, we suggested that an indirect mechanism might operate also at this stage of HIV infection.

Notwithstanding obvious differences between the changes observed in the presymptomatic stages of HIV infection and those seen in AHL, it is possible for other infectious organism to use a similarly indirect mechanism to produce different lesions from those resulting from the action of the mature replicating form.

The demonstration of HSV antigen, HSV DNA, and HSV RNA in the brain of 2 of 15 cases of AHL is unusual and at variance with the negative results of most studies done on brain, CSF or serum (Markus *et al*, 1997; Yamashita *et al*, 1999; Hirano, 1997). These findings suggest that HSV was replicating at the time the patient's death, although the absence of data on circulating immunoglobulins makes it impossible to ascertain whether the replicating HSV resulted from a primary infection or reactivation of a virus latent in the brain. The latter event ('Esiri's theory,' Esiri, 1982a, 1982b) has been observed in experimentally infected mice (Cook and Stevens, 1976; Cabrera *et al*, 1980; Kastrukoff *et al*, 1981). A previous report by Lach and Atack (1988), describing a patient with AHL complicating brain stem HSV encephalitis, was thought to be relevant to our study. However, this patient clearly presented two types of lesion: one

in the brain stem resulting from a direct action of HSV; the second, demyelinating, was localized to the cerebral hemispheres. In both, the replicating virus could be detected. With regard to the pathogenesis, although not dismissing a direct cytotoxic effect of HSV, the authors consider one immunologically mediated to be more likely.

The obvious question that follows is whether the results obtained from the study of AHL/ADEM may be relevant to MS. Although the former is a predominantly monophasic disorder, cases of ADEM with a multiphasic pattern are known (Khan *et al*, 1995; Ünal *et al*, 2000), with some of them progressing to MS (Dale *et al*, 2000). Thirty-five percent of 40 patients with ADEM recently reported by Schwarz *et al* (2001) developed clinically definite MS over a mean observation period of 38 months. As for MS, it has been suggested that the various types of inflammation, de- and remyelination, and tissue damage may reflect different pathogenetic mechanisms (Ozawa *et al*, 1994). With regard to its etiology, it is regarded as multifactorial (Willer and Ebers, 2000), genetic (Compston, 2000) and exogenous, including viral (Noseworthy, 1999), factors playing a role.

Results of studies aiming at identifying the presence of virus in patients with MS vary considerably. HSV 2 was reported by Martin *et al* (1988) in three cases, but only using high concentrations of antibody. Nicoll *et al* (1992) found PCR evidence of HSV 1 DNA only in 1 of 77 MS plaques, whereas Ferrante *et al* (2000) detected it in acute MS plaques. In addition, Ferrante *et al* (2000) found no significant difference in EBV and HHV 6 between MS and control patients, and detected CMV only in MS cases. Results by Ablashi *et al* (1998) support the role of HHV 6 as a cofactor in the pathogenesis of some cases of MS. Blumberg *et al* (2000) suggest a role for the activation of HHV 6 genome in this disease and Friedman *et al* (1999) revealed presence of both provirus and replicating virus in a significant number of cases of MS. A more sceptical view on the role of this virus is that taken by Liedtke *et al* (1995) and Sanders *et al* (1996), although the results of the latter lack statistical significance.

Our results have established the presence of a number of viruses in the brain tissue in cases of AHL, an encephalitis regarded as the hyperacute form within the group of ADEM. As in most of our cases, only viral DNA could be found, and the clinical and pathological patterns shown by this disorder are rather stereotyped and quite different from those produced by the mature form of each of the viruses detected in this study; an indirect common mechanism is more likely to be in action. Furthermore, the hyperacute presentation and the inflammatory and necrotizing vasculitis often observed in AHL plead for an autoimmune process. As patients with disorders in the ADEM group may progress to full blown MS, we argue that it is possible that, at least in some patients, once the organism has entered the central nervous system (CNS) and established the lesion, it may remain in the tissue and could be reactivated and be involved in the production of a self-sustaining and, eventually, permanent lesion.

Materials and methods

Subjects and tissues

Brains of 15 cases of AHL were obtained at postmortem. They included six from the series (1964–1994) of the Department of Neuropathology, Institute of Neurology, University College London, and nine from other institutions in the United Kingdom. Normal control tissue consisted of brain of 10 patients who had died from road traffic accident.

Neuropathology and detection of infectious pathogens

Neuropathological examination used routine and immunohistochemical methods. HSV, CMV, VZV, and HHV-6B antigens were studied by immunohistochemistry. Infectious agents, including VZV, HSV, HHV-6, CMV, EBV, and *Mycoplasma*, were also investigated in paraffin-embedded brain specimens using PCR. Procedures for PCR and immunohistochemistry have been described previously (An *et al*, 1996a). For RT-PCR, RNA was extracted from formalin-fixed samples as described by Masuda *et al* (1999, 2000) and Godfrey *et al* (2000), with minor modification. Details of primers used for PCR/RT-PCR and antibodies applied for immunostaining are listed in Table 2. In addition, β-globin DNA and hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA were used as positive control for PCR and RT-PCR respectively. Commercially available viral and bacterial DNAs (HSV, CMV, and *Mycoplasma* DNA) and viral DNAs extracted from known cases (VZV, EBV, and HHV-6) were used as positive controls for PCR. For immunohistochemical identification of the various viruses, the following tissues were used: HSV limbic encephalitis (two brains), VZV myelitis (one spinal cord), CMV encephalitis (one brain), HHV6 (one salivary gland). Tissue sections from known infectious cases (HHV-6, HSV, CMV, and VZV) were used as positive controls for immunostaining. Two HSV immunopositive AHL cases were further studied using two different dilutions of the antibody (1:2000 as routinely used and 1:50) to confirm the earlier results.

Double-fluorescent immunostaining

Selected sections were double-stained with HSV and one of the following cellular markers: anti-human CD68 for mononuclear cells or anti-human GFAP for astrocytes (both DAKO, Cambridge, UK).

Table 2 Details of primers used for amplification of HSV, CMV, HHV-6, EBV, and *Mycoplasma*, as well as antibodies for detection of HSV, CMV, VZV, and HHV-6

	PCR/RTPCR		*IHC*
Primer	*Sequences*	*Length of fragment*	*Antibodies*
	HSV		
HSV-1	ATC ACG GTA GCC CGG CCG TGT GAC A	1st round, 221 bp;	Anti-HSV polyclonal
HSV-2	CAT ACC GGA ACG CAC CAC ACA A	2nd round, 138 bp	(1:50, 1:2000), Dako
HSV-3	CCA TAC CGA CCA CAC CGA CGA		
HSV-4	GGT AGT TGG TCG TTC GCG CTG AA		
	HSV RNA		
HSVD-1	CAG TAC GGC CCC GAG TTC GTG	476 bp	
HSVD-2	GTA GAT GGT GCG GGT GAT GTT		
Internal probe	TAC TGC ATA CAG GAT TCC C		
	CMV		
CMV-1	CCA AGC GGC CTC TGA TAA CCA AGC C	435 bp	Anti-CMV monoclonal
CMV-2	CAG CAC CAT CCT CCT CTT CCT CTG G		(1:25), Dako
	VZV		
VZV-1	CGT CAC ATA TTA TGC AAA CAT G	224 bp	Anti-VZV monoclonal
VZV-2	CGT TTT TAA TAT TAC AAA TCC CGC		(1:25), Novocastra Lab
	EBV		
EBV-1	TGT CTG ACG AGG GGC CAG GTA CAG GAC	239 bp	—
EBV-2	GCA GCC AAT GCA ACT TGG ACG TTT TTG		
	HHV-6		
HHV-6ex1	GGA GTG ACA GAC AAC GTC	1st round, 380 bp;	Anti–HHV-6B monoclonal
HHV-6ex2	ACG GAA GTA CAA AAC ATG ACC	2nd round, 100 bp	antibody (1:50), Chemicon
HHV-6int3	AAG AAC CCA CAA ATC CTA CCC		International
HHV-6int4	TGG GTT TCG TTT GCG T		
	Mycoplasma		
Myco1	GGG AGC AAA CAG GAT TAG ATA CCC T	Minerva Biolabs Mycoplasma	—
Myco2	TGC ACC ATC TGT CAC TCT GTT AAC CTC	Detection Kit, 280 bp	

After incubation with 2.5% normal horse serum for 10 min, sections were incubated in primary antibody against HSV (see Table 1) at 4°C overnight. After three washes, sections were incubated with biotinylated pan-specific secondary antibody (R.T.U. Vectastain Universal Quick kit, Vector, UK) at RT for 20 min. Between incubations, three 5-min washes with phosphate-buffered saline (PBS) were applied. Avidin D Texas Red (Vector Fluorescent Avidin Kit, 1:50, UK) was used to reveal signals of HSV.

Before application of anti-CD68 and -GFAP antibodies, sections were incubated with 5% normal mouse, goat, rat, and rabbit serum for 30 min. Sections were then incubated in antibodies against cellular markers (see above) for 1 h before being incubated in biotinylated secondary antibody, followed by fluorescein-conjugated Avidin DCS (Vector, 1:50, Peterborough, UK). Slides were mounted with Citifluor, which contains DAPI (1:1000) for counterstaining.

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