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Impact of cerebrospinal fluid PCR on the management of HIV-infected patients with varicella-zoster virus infection of the central nervous system

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Over a 2 year period, we identified five HIV-infected patients who presented with central nervous system infection caused by varicella-zoster virus, three with myelitits, and two with meningoencephalitis. All five patients were profoundly immunocompromised. Clinical presentation of these patients overlapped to a significant extent with diseases caused by other viruses, e.g. CMV. Indeed, in one case, a dual infection with CMV was diagnosed, but the respective role of each virus was ascertained by *in situ* hybridisation. At the time of CNS involvement, only one patient had active VZV cutaneous lesions, which were instrumental in diagnosing her condition. In contrast, PCR for VZV DNA in the CSF was helpful in making a diagnosis in the four other cases, one of which was confirmed by a post mortem. Of these five patients, two patients developed VZV disease while receiving oral acyclovir and had foscarnet treatment initiated when MRI demonstrated widespread lesions. They did not respond to antiviral therapy. The three other patients had intravenous acyclovir initiated at a time when no or limited parenchymal lesions were observed by MRI. Two of these three patients had VZV infection diagnosed solely on the basis of PCR: all three responded to treatment. Our data show that reactivation of VZV involving the central nervous system occurs frequently in the absence of cutaneous lesions. PCR of cerebrospinal fluid may help in making an early diagnosis which is probably a prerequisite for successful treatment of VZV infection of the CNS.

Keywords: encephalitis; myelitis; varicella-zoster virus; acyclovir; foscarnet

Introduction

Varicella-zoster virus (VZV) is highly contagious and causes varicella in a majority of the population so that by age 15, more than 90% of the population has been infected in the developed world (Arvin, 1996). Typical of the herpesviruses, VZV establishes latency after primary infection wanes, and reactivates later on to cause herpes zoster (Arvin, 1996). In HIV-infected patients, progressive immune dysfunction affects the natural history of VZV infection. Firstly, the incidence of zoster increases as much as 15-fold in HIV seropositive individuals compared to uninfected (Buchbinder *et al*, 1992). As a consequence, in populations with a high prevalence of HIV infections, zoster is a clinical predictor of HIV infection (Colebunders *et al*, 1988). Secondly, the severity of VZV reactivation increases with decreasing $CD4^+$ T cell counts with a higher risk of zoster involving cranial dermatomes, multiple dermatomes, and of recurrent zoster (Buchbinder *et al*, 1992; Colebunders *et al*, 1988; Glesby *et al*, 1995; Whitley and Gnann, Jr. 1995; Veenstra *et al*, 1996). Late in the course of HIV infection, patients may even suffer from visceral involvement, particularly of the central nervous system. Indeed, cases of encephalitis and meningoencephalitis (Poscher, 1994; Gray *et al*, 1992; Gilden *et al*, 1988; Scully *et al*, 1996), ventriculitis (Chrétien *et al*, 1993; Kenyon *et al*, 1996), meningoradiculoneuritis (Snoeck *et al*, 1993; Chrétien *et al*, 1993; Snoeck *et al*, 1994), brain stem encephalitis (Moulignier *et al*, 1995), myelitis (Meylan *et al*, 1995; Manian *et al*, 1995; Gilden *et al*, 1994; Lionnet *et al*, 1996; Grant

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et al, 1993; de Silva *et al*, 1996; Edelstein, 1994), and acute retinal necrosis (Hellinger *et al*, 1993; Galindez *et al*, 1996; Batisse *et al*, 1996) have been described. This variety of anatomical location of CNS disease caused by VZV in HIV-infected patients has been confirmed in pathological studies (Gray *et al*, 1994; Kleinschmidt-DeMasters *et al*, 1996; Gray *et al*, 1991). Given this variety and the fact that a typical cutaneous involvement may be lacking (Meylan *et al*, 1995; Manian *et al*, 1995; Moulignier *et al*, 1995; Poscher, 1994; Gomez-Tortosa *et al*, 1994; Bergstrom, 1996), the diagnosis of CNS disease caused by VZV is particularly difficult. While VZV is only rarely cultured from the cerebrospinal fluid of such patients (Heller *et al*, 1990; Gomez-Tortosa *et al*, 1994), the detection of VZV DNA in the CSF appears a promising tool in the diagnosis of CNS disease caused by VZV (Puchhammer-Stockl *et al*, 1991; Shoji *et al*, 1992; Gilden *et al*, 1994; Lionnet *et al*, 1996; Grant *et al*, 1993; Bergstrom, 1996). Two recent studies have documented the frequency and significance of finding VZV DNA in the CSF among HIV+ patients presenting with neurologic symptoms (Cinque *et al*, 1997; Burke *et al*, 1997). In the present paper, we report a series of five cases of HIV-infected patients with definite or probable VZV infection of the CNS, stressing the role of PCR in the management of four of them.

Results and Discussion

In the present study, we report five cases of central nervous system infection due to the varicella zoster virus. As shown by Table 1, all had advanced HIV-induced immunosuppression, a history of herpesvirus infections, and had received antiherpetic drugs before or until the current episode.

Clinical presentation included three cases of myelitis and two instances of meningoencephalitis. In patient 2, the diagnosis was definite, based on the detection of VZV and CMV DNA by *in situ* hybridisation in samples from the CNS obtained post mortem. In the other cases, the diagnosis was presumptive, based once on a PCR signal, MRI myelitis lesions (Figure 1a) and a histopathology at post mortem consistent with a herpesvirus infection (patient 1), once on a history of relapsing zoster, spinal cord lesions (Figure 1b), and improvement with acyclovir treatment (patient 3), and twice on PCR data and improvement with acyclovir treatment (patients 4 and 5). In patient 2, a dual infection by VZV and CMV was evidenced by PCR of the CSF, which was confirmed by the observation of positive CMV and VZV signal in encephalitis lesions by *in situ* hybridisation (Figure 3). Other instances of dual infection of the CNS by VZV and CMV have been described (Cinque *et al*, 1997; Burke *et al*, 1997; Meylan *et al*, 1995).

Table 1 Anamnestic characteristics of the patients[a].

Patient number Age/sex	*Stage, CD4$^+$ T cell/μl*	*Previous OI*	*Antiretroviral treatment at presentation*	*History of herpes virus infection*	*Previous exposure to anti-herpetic drugs*	*CNS localisation (symptoms)*	*Final diagnosis*
1, 36/M	C3, 3/μl	diss. tuberculosis, KS	Didanosine	Facial zoster	Acyclovir, 5 × 800 mg/d up to presentation time	Myelitis (thoracic hyperpathic pain, paraparesia, sphincter disturbances)	VZV myelitis, probable
2, 38/F	C3, 3/μl	T. gondii encephalitis, CMV oesophagitis, Cryptosporidiosis	None	Relapsing anal Herpes simplex, CMV stomatitis and oesophagitis	Acyclovir, 2 × 400 mg/d up to presentation time ganciclovir	Meningo-encephalitis (fever, meningeal irritation, left side ataxia, confusion)	VZV and CMV ventriculo-encephalitis, definite
3, 30/F	B3, 3/μl	Oral candidiasis	None	Relapsing thoracic and sacral zoster	Acyclovir	Myelitis (thoracic hyperpathic pain, T8 sensory level, paraparesia, sphincter disturbances)	VZV myelitis, probable
4, 49/M	C3, 3/μl	Candida oesophagitis, Giardiasis, Molluscum contagiosum	Zalcitabine	Relapsing anal Herpes simplex	Acyclovir	Myelitis (fever, spastic paraparesia, T10 sensory level)	VZV myelitis, probable
5, 31/F	C3, 5/μl	Oral candidiasis, PcP, CMV colitis, disseminated M. avium	None	Genital Herpes simplex CMV colitis	Acyclovir, ganciclovir, foscarnet (90 mg/kg bid up to presentation time)	Meningo-encephalitis (confusion)	VZV encephalitis, probable

[a]Abbreviations in the table: OI, opportunistic infections, KS: Kaposi sarcoma, PcP: *Pneumocystis carinii* pneumonia.

These five cases, as well as a patient presenting with VZV myelitis who has already been reported (Meylan *et al*, 1995) were observed over a 4 year period (1992–1995). Five of them were enrolled in a prospective cohort study including the majority of the patient population attending our HIV outpatient clinic. Patient 1 was referred from another hospital. Throughout this period, approximately 500 patients were receiving care at our outpatient clinic. Of these, around 70 were deeply immunocompromised (< 50 $CD4^+$ T cells/mm^3) and probably at risk of developing CNS opportunistic infections. Thus, we observed grossly a 1% incidence of VZV infection of the CNS in severely immunocompromised patients over this period, greater than that reported in studies of consecutive cases of CNS diseases in HIV-infected patients. For instance, Levy *et al* (1985) reported only one case of VZV encephalitis among 128 HIV-infected patients presenting with CNS diseases at University of California, San Francisco, while Gray *et al* (1991) found

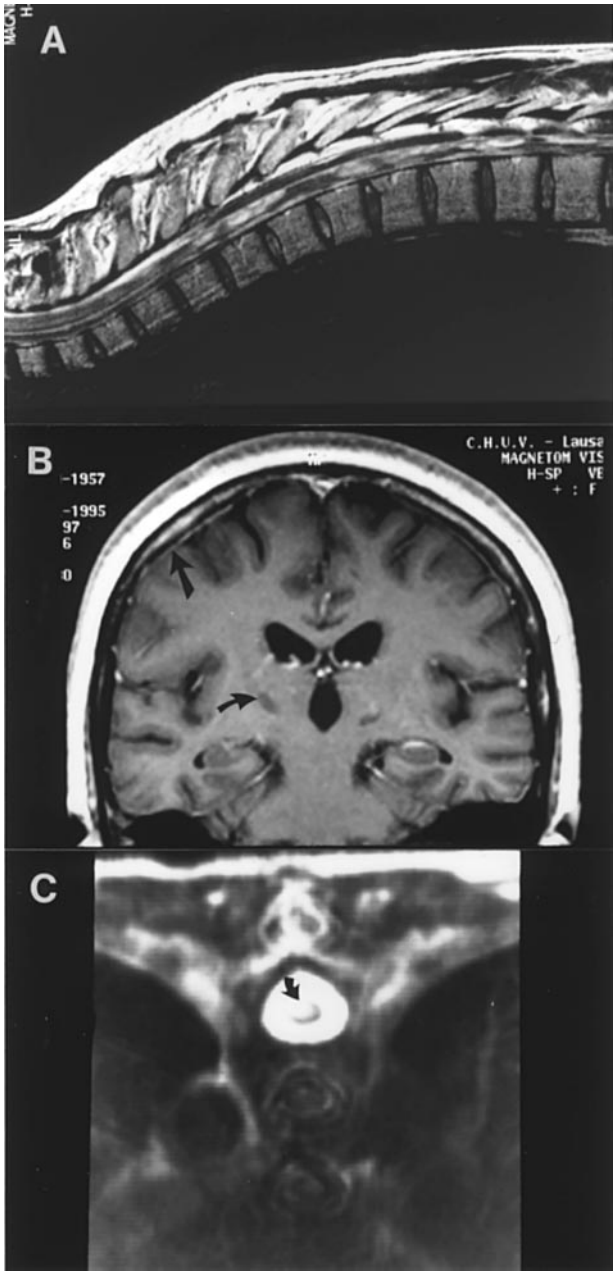

Figure 1 Nuclear magnetic imaging of central nervous lesions. (**a**) patient 1 T2-weighted sagittal cut of the spinal cord, showing disseminated hyperintense lesions in the cord. (**b**) Patient 2 T1-weighted coronal section, showing hypointense residual lesions of Toxoplasma encephalitis in the basal ganglia (e.g. curved arrow) as well as positive meningeal contrast enhancement (straight arrow). (**c**) Patient 3 T2-weighted transversal image of the cord at bone level T9, showing a hyperintense intramedullary lesion (arrow).

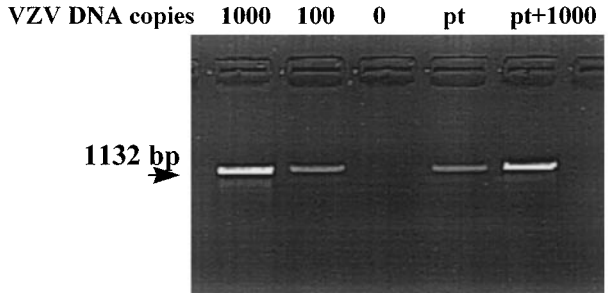

Figure 2 Example of PCR analysis of the CSF of patient 1. CSF was extracted and amplified as described in Materials and methods. In each assay, positive controls containing known amounts of VZV DNA were performed (lanes 1000 and 100). A sample of buffer containing no VZV DNA served as a negative control (lane 0). A plain aliquot of the patient sample (pt) was amplified along with an aliquot spiked with 1000 copies VZV DNA (pt+1000) aimed at detecting the presence of inhibitors in the patient extract. A test was ruled positive only when the patient sample was positive and the negative control negative, and a negative result only when the spiked control amplified efficiently. In the sample of patient 1, the amount of VZV DNA in the sample corresponding to 20 μl of CSF had a signal intensity similar to that of the 100 copies control suggesting about 5000 copies per ml of native CSF. Similar positive PCR tests were observed with CSF samples from patients 2, 4 and 5.

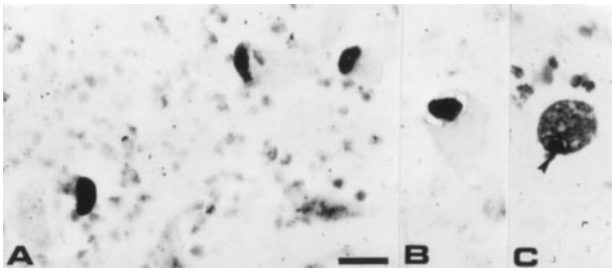

Figure 3 *In situ* hybridisation detection of CMV and VZV DNA in the CNS of patient 2. Photomicrographs (**a**) and (**b**) show the presence of CMV DNA in the subependymal necrotic areas adjacent to the lateral ventricle. Photomicrograph (**c**); in the same necrotic area, VZV DNA was also detected. The arrowhead points to a viral inclusion in the nucleus of a phagocytic cell. The bar in (**a**) corresponds to 50 μm, and enlargement is the same for (**b**) and (**c**).

only two cases of VZV encephalitis (one without cutaneous involvement) among 135 patients with a neuropathological study. More recently, Cinque *et al* (1997) observed a positive PCR signal for VZV in only 13 of 514 HIV-infected patients with neurological disease. In four of these patients, VZV CNS disease was diagnosed, while in nine others, the neurological symptoms were likely caused by other HIV-related complications.

Burke *et al* (1997) reported a series of 84 consecutive HIV-infected patients investigated for new neurological symptoms by PCR for VZV DNA in the CSF. Six were found positive, correlating with various CNS lesions and in two cases with progressive outer retina necrosis.

Owing to the very high rate of VZV latent infection throughout the temperate climates, it is unlikely that the frequency of VZV reactivation will vary among different patient cohorts, controlling for the level of immunodeficiency. We suspect therefore that our observation of six cases of VZV CNS infection either reflected a clustering of cases due to coincidence or the fact that the numbers of profoundly immunocompromised patients were maximum during those years. Indeed, we have not observed any VZV infection of the CNS in 1996–7, a time when the use of highly efficacious antiretroviral treatment increased with a concomitant decrease in the risk of progression to AIDS and mortality in Switzerland (Egger *et al*, 1997). In addition, our increased interest in this problem and the development of a PCR assay for VZV DNA may have introduced an observation bias. In any case, the retrospective nature of the present study, and the fact that submitting CSF samples for PCR detection of VZV DNA was left to the discretion of the clinicians does not allow an estimate of the true incidence of VZV CNS disease in the present patient population.

Our small series of cases adds however to the knowledge about the clinical and pathological presentation of CNS VZV infection in HIV-infected patients. Myelitis (patients 1, 3 and 4) presenting with paresia and a sensory level, and intraspinal lesions as demonstrated by MRI (e.g. Figure 1a and c) may appear as a relatively characteristic presentation of CNS infection caused by this virus (Meylan *et al*, 1995; Manian *et al*, 1995; Gilden *et al*, 1994; Lionnet *et al*, 1996; Grant *et al*, 1993; de Silva *et al*, 1996; Edelstein, 1994). Presentations of meningoencephalitis were observed in patients 2 and 5, with ventriculitis demonstrated post mortem in patient 2 (Figure 3a) who was dually infected with VZV and CMV. In these two patients, MRI revealed a meningeal contrast enhancement (Figure 1b).

In this case series, only one patient had cutaneous evidence of zoster at the time of CNS involvement pointing to the diagnosis. This adds to a growing number of case reports demonstrating that visceral dissemination of VZV, in particular to the CNS, can occur in the absence of simultaneous cutaneous involvement (Meylan *et al*, 1995; Manian *et al*, 1995; Moulignier *et al*, 1995; Poscher, 1994; Gomez-Tortosa *et al*, 1994; Débat Zoguéreh *et al*, 1996; Scully *et al*, 1996; Chrétien *et al*, 1993), and even suggests that it may be so in a majority of cases (Gray *et al*, 1994; Burke *et al*, 1997).

Thus, our observations support the evidence that the diagnosis of VZV involvement of the central nervous system cannot rest on the clinical observation alone and stress the need for the development of non invasive diagnostic tools, such as PCR on CSF samples. The PCR test described here amplifies the whole thymidine kinase open reading frame, a choice made to allow the development of rapid tests aiming at detecting acyclovir resistance mutations (Boivin *et al*, 1994; Talarico *et al*, 1993). The primers were chosen so as not to amplify sequences from other herpesviruses. Its analytical sensitivity was determined as less than 100 copies of VZV DNA, and its specificity verified by testing samples of nucleic acids purified from fibroblasts infected with CMV, HSV-1 and HSV-2. No amplification signal was observed. This assay was indeed instrumental in the diagnosis in cases 1, 2, 4 and 5. A positive signal for VZV DNA in patient 1 CSF sample correlated with pathological findings at post mortem consistent with an infection with a virus of the herpes group. However, immunohistochemistry for HSV, HIV and CMV was negative as was *in situ* hybridisation for VZV and CMV. The reason for this discrepancy is unclear, but based on imaging and PCR data, a diagnosis of VZV myelitis remains probable, and the negative *in situ* for VZV might reflect the effect of treatment reducing the viral load below threshold of detection or a sampling problem. In patient 2, the detection of amplification signal for VZV and CMV in a CSF sample obtained *intra vitam* correlated with *in situ* hybridisation data in tissue samples obtained post mortem 2 months later, demonstrating that the PCR data were due to a *bona fide* dual infection caused by CMV and VZV. In patients 4 and 5, the favourable short term outcome after initiation of intravenous acyclovir precluded a correlation of PCR results with pathological data. Others have also reported on the successful use of PCR in the diagnosis of CNS VZV infections either in the context of varicella, i.e. primary VZV infection or zoster (Puchhammer-Stockl *et al*, 1991; Shoji *et al*, 1992) or in the context of VZV reactivation in the immunocompromised host (Lionnet *et al*, 1996; Débat Zoguéreh *et al*, 1996; Grant *et al*, 1993; Gilden *et al*, 1994; Bergstrom, 1996).

Two recent studies have ascertained the significance of finding VZV DNA in the CSF of patients infected with HIV, supporting the view that its detection by PCR correlates in many patients with

Table 2 Patient characteristics (MRI, CSF and treatment data).

Patient number	*MRI (contrast enhancement, CE)*	*CSF WBC (% PMN)*	*CSF proteins*	*CSF PCR*	*Time from first symptoms to start of therapy*	*Treatment*	*Outcome*	*Autopsy*
1	T2-weighted hyperintense lesions lower thoracic cord CE negative	7/μl (0.5%)	975 mg/l	VZV+ CMV−	8 months	Foscarnet, then ganciclovir added	Worsened, death 1 month after admission	Necrotizing radiculomyelitis with histo-pathology consistent with herpesvirus infection. No signal detected by IHC or ISH
2	T1-weighted hypointense lesions in basal ganglia, consistent with previous TE, positive meningeal CE	24/μl (13%)	1650 mg/l	VZV+ CMV+	Several days	Foscarnet	Worsened, died of pneumonia 2 months later	Multiple cystic lesions consistent with past TE. Acute necrotic subependymal meningoencephalitis. *In situ* hybridisation+for VZV and CMV. A greater number of cells were positive for CMV than for VZV
3	T2-weighted hyper-intense extramed lesion compressing cord levels T1-T7 (cystic arachnoiditis) T2-weighted hyperintense T8-T9 intramed lesion	Declined spinal tap	ND	ND	1 year	Acyclovir (iv, then p.o.)	Improved (resumed walking), and stable for 6 months, then worsening general condition and neurological status. Died 8 months after admission in a chronic care facility	No post mortem performed
4	No detectable abnormality of the spinal cord	0.1/μl (ND)	325 mg/l	VZV+ CMV−	38 days	Acyclovir (iv, then p.o)	Improved (resumed walking), and stable for 4 months. Then worsening condition due to severe diarrhea. Died 6 months after admission in a chronic care facility	No post-mortem performed
5	Cortical atrophy, T2-weighted hyper-intense cerebellar and basal ganglia lesions, positive meningeal CE	0/μl	ND	VZV+ CMV−	5 days	Acyclovir (iv, then p.o.) added to foscarnet	Improved, and stable for 6 months. Antivirals stopped because of renal failure. 4 weeks later, worsening condition and neurological status. Died 8 months after presentation in a chronic care facility	No post-mortem performed

neurological or ophthalmological disease caused by VZV, but may also reflect subclinical reactivation (Cinque *et al*, 1997; Burke *et al*, 1997).

Importantly, the information provided by the PCR assay was instrumental in initiating early specific antiviral treatment and perhaps affecting the clinical outcome in our patients (Table 2). Three years ago, we reported a patient who presented before the PCR assay was available and died 1 month after the onset of myelitis (Meylan *et al*, 1995). A diagnosis of VZV encephalomyelitis was made only at the post mortem. This diagnosis was not even entertained before death and no specific treatment was initiated, for lack of cutaneous lesions. Patient 1 had the diagnosis of VZV myelitis made at a time when widespread lesions could be observed by MRI. This disease occurred despite chronic acyclovir treatment suggesting that the VZV strain might have been resistant to acyclovir, as already described (Snoeck *et al*, 1994). The initiation of foscarnet treatment did not appear to affect the outcome, which might have been due to cross resistance to acyclovir and foscarnet as is observed in the case of polymerase resistance mutations (Reusser, 1997), or to the fact that the lesions had so progressed that curtailing viral replication could no longer affect the course of the disease, a hypothesis that would be consistent with the negative *in situ* hybridisation tests at post mortem. Patient 2 developed encephalitis due to both CMV and VZV while receiving suppressive oral acyclovir and failed to respond to the addition of foscarnet, again perhaps due to cross resistance between acyclovir and foscarnet, or to established, irreversible lesions. In contrast, patient 4 had a VZV myelitis diagnosed by PCR before any lesions could be observed by MRI. In this case, the initiation of acyclovir treatment had a striking favourable effect. A similar positive outcome was observed in patients 3 and 5, who, at the time of starting treatment, had respectively limited lesions in the spinal cord, or evidence of meningeal involvement without brain parenchymal lesions. In patient 3, cutaneous zoster lesions at the time of myelitis presentation suggested the diagnosis of probable VZV myelitis. In contrast, in patients 4 and 5, only the PCR results made us aware of a VZV infection and prompted the initiation of specific antiviral treatment.

The present case reports exemplify the complex problems posed by patients with advanced immunosuppression and atypical presentations of opportunistic infections. Our data, though anecdotal, support previous observations suggesting that early treatment of VZV infection of the CNS in immunocompromised hosts, based on PCR diagnosis, can positively affect the outcome of this infection in a subset of patients (Poscher, 1994; Lionnet *et al*, 1996; de Silva *et al*, 1996; Cinque *et al*, 1997; Burke *et al*, 1997).

Material and methods

Patients, specimens and clinical data

In 1995–96, CSF samples from 38 HIV-infected patients presenting with neurologic symptoms were submitted to the virology laboratory of the Centre Hospitalier Universitaire Vaudois (Lausanne) for the detection by PCR of VZV, CMV, EBV and JC Virus DNA. Four of them tested positive. Clinical and laboratory data were retrieved from their clinical chart and from the pathology chart. An additional patient whose probable VZV myelitis was diagnosed based on cutaneous lesions was also included in this study.

Definitions

VZV disease of the central nervous system was considered as definite when histopathological evidence of infection was found on routine staining of tissue samples and the presence of VZV confirmed using either immunohistochemistry or *in situ* hybridisation.

VZV disease was defined as probable, when patients presented with : (i) CNS symptoms and/or lesions consistent with VZV etiology on neuroimages, and no other CNS disease that could account for the neurologic findings, (ii) PCR evidence of VZV DNA in the CSF, or (iii) concurrent relapsing zoster and a response to acyclovir treatment.

DNA extraction from cerebrospinal fluid (CSF)

DNA was extracted from CSF samples by adding 200 μl of TE, 1% (v/v) Nonidet NP-40 (Sigma), 1% (v/v) Tween-20 (Sigma) and 30% (w/v) Chelex 100 (BioRad) to 200 μl of CSF (Walsh *et al*, 1991). This mixture was kept at 95°C for 30 min with constant vortexing in a Thermomixer shaker (Eppendorf), and centrifuged at maximum speed for 5 min. The supernatant was harvested and the DNA precipitated by adding 40 μl of sodium acetate 3 M, pH 5.5, and 800 μl ethanol. The DNA was pelleted by centrifugation at maximum speed for 30 min, washed and dissolved in 50 ml of 10 mM Tris, 0.1 mM EDTA, pH 7.4.

PCR amplification of VZV DNA

Five μl of the extract (corresponding to 20 μl native CSF) was added to 95 μl of 1 × PCR Amplitaq buffer (Perkin Elmer) supplemented with 2.5 mM $MgCl_2$ and 20 pmol of sense (5′-AACGAGTGTGG-CAACGTTGTC-3′) and antisense (5′-TTAG-GAAGTGTTGTCCTG-3′) primer and 2.5 U of Taq polymerase (Perkin Elmer). These sequences span position −106 to +1026 of the thymidine kinase open reading frame, producing a 1132 bp amplicon. The cycling parameters were 95°C, 4 min once, followed by 40 cycles of 95°C, 1 min, 52°C, 1 min, 72°C, 1 min 30 s and a final 10 min extension at 72°C. For analysis, 10 μl of the reaction was loaded on a 0.8% ethidium bromide-stained agarose gel,

and observed with a UV transilluminator. Every specimen was amplified together with a negative control, standards containing respectively 1000 and 100 copies of VZV DNA purified from fibroblasts infected with a clinical isolate of VZV. To control for the presence of inhibitors in the test sample, an aliquot was spiked with 1000 copies of VZV DNA and amplified (see Figure 2). PCR for CMV DNA was performed as recently described (Chatellard *et al*, 1998).

Post mortem examination

The post mortem examination was performed within 24 h of the patient death. From the autopsied, unfixed brain and spinal cord, samples were harvested for microbial cultures. After the macroscopical examination of the formalin-fixed brain and spinal cord, blocks from representative regions of the neocortex, basal ganglia, thalamus, brainstem, cerebellum, but also from several levels of the spinal cord were taken and embedded in paraffin. Sections (7 μm) were cut from each block and were used for routine staining as well as immunohistochemistry and *in situ* hybridisation.

Immunohistochemistry for the antigen detection of HSV, HIV and CMV

Herpes Simplex Virus antigens were detected using rabbit polyclonal antisera, one recognising both HSV-1 and -2 antigens (BioGenex RH0B6093, dil 1 : 20), the other recognising specifically HSV-1(DAKO, B114, dil. 1 : 1000).

HIV-1 antigens were detected using two monoclonal antibodies recognising respectively the p24 capsid protein of HIV-1 (Specialty Diagnostics, Dupont, NEA-9283, dil. 1 : 100) or both the p24 capsid protein and its precursor p55 proteins (DAKO, M 857).

CMV early antigens were detected using a monoclonal antibody (DAKO, M757, dil. 1 : 30).

Immunostaining was carried out with the avidin-biotin-peroxidase technique. Sections were incubated with the primary antibodies overnight at 4°C. Sections stained omitting the primary antibodies served as negative controls.

In situ *hybridisation for the detection of CMV and VZV*

For the detection of CMV and VZV DNA by *in situ* hybridisation, paraffin slides were dewaxed in xylene, hydrated in 99% then in 95% ethyl alcohol, rinsed in running tap water, then immersed in distilled water. Sections were treated with Proteinase K (Boehringer), 10 μg/ml in 50 mM Tris HCL, pH 7.6 for 30 min at 37°C followed by 2 min washes in distilled water at 4°C. The sections were then dehydrated in 95% ethanol and air-dried. For *in situ* hybridisation, the slides were transferred to a Thermal cycler (Hybaid) fitted with an *in situ* hybridisation chamber block.

Hybridisation was performed using a cocktail of fluorescein-conjugated CMV probes (CMV Probe ISH Kit, NCL-CMV-K, Novocastra Laboratories Ltd., Newcastle, UK) or with a digoxigenin labelled PCR product representing a fragment of the CMV UL97 open reading frame, as described previously (Chatellard *et al*, 1998).

For the detection of VZV, a digoxigenin labelled PCR product probe was prepared using the PCR system described above. The hybridisation step was initiated by adding 10 pmol of probe per 100 μl of hybridisation solution and a 10 min denaturation step at 100°C, followed by an overnight incubation at 42°C. The hybridisation solution consisted of 1 ml 0.5 M Tris HCl, pH 7.4, 50 μl 20 × SSC, 1 μL 0.5 M EDTA, 100 μl dextran sulphate, 250 μl formamide and 500 μl water. After hybridisation, the slides were washed three times in TBS buffer containing 0.1% Triton X-100, followed by a blocking step in TBS containing 20% normal rabbit serum, 3% BSA and 0.1% Triton X-100 for 10 min. For the detection of the hybridisation products, anti-fluorescein and anti-digoxigenin alkaline phosphatase or peroxidase conjugates (Boehringer) were used. Alkaline phosphatase substrate solution (Novocastra, Boehringer) or diaminobenzidine (DAB) were used as chromogens for the visualisation of the signal by standard immunohistochemistry techniques.

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