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Amplification and Characterization of Herpesvirus DNA in Cerebrospinal Fluid from Patients with Acute Encephalitis

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A single pair of oligonucleotide primers selected within a highly conserved region of the DNA polymerase gene of the herpesviruses was designed to amplify related viral genomes, i.e., herpes simplex virus type 1, herpes simplex virus type 2, Epstein-Barr virus, and cytomegalovirus, by the polymerase chain reaction. A simple restriction enzyme analysis of these amplified products allowed accurate characterization of the herpesvirus type. Ninety-nine cerebrospinal fluid samples from 36 patients (including newborns, children, and adults) with acute encephalitis were tested for the presence and identification of herpesvirus DNA by this approach. High levels of viral DNA, which were readily visualized by simple ethidium bromide staining, were found in all these patients from the first days of the disease and, in some cases, until the third week following the onset of acute encephalitis. The herpesvirus type was rapidly identified by enzymatic digestion in 33 patients' samples and by hybridization and direct sequencing in the last 3 patients' samples. Our results show that the polymerase chain reaction provides a highly sensitive and specific technique for the identification of herpesviruses DNA in cerebrospinal fluid that should be of value for early and rapid diagnosis, therapeutic decisions, prognostic evaluation, and epidemiological studies.

Viruses that cause infections of the central nervous system are often difficult to identify. Among them, Epstein-Barr virus (EBV) and cytomegalovirus (CMV) are more common in immunosuppressed patients, while herpes simplex virus (HSV) is the most common cause of sporadic, acute, focal encephalitis (22). The prognosis of severe herpes simplex encephalitis (HSE) has recently been improved by the use of antiviral drugs, provided that they are administered early. However, positive diagnosis cannot be based either on clinical criteria alone, which are nonspecific, or on conventional biological assays, which are rarely of use at the early stage of infection (6, 24). Confirmation requires the isolation of the virus from cerebrospinal fluid (CSF), which is uncommon, or the identification of viral antigens in CSF by the use of specific antibodies, which suffers from a lack of specificity (10). As a last resort, brain biopsy remains the only way to date to establish an early definitive diagnosis of HSE. There is a need, therefore, for the direct detection of the virus, independently of circulating antibodies, in infected individuals. The polymerase chain reaction (PCR) (19) has proved useful for the detection of virus-specific nucleic acids in CSF (1, 7, 16–18, 20). We selected appropriate primers corresponding to conserved sequences of the genome of known members of the herpesvirus family and established conditions for detecting and characterizing, in a single assay, related viral genomes (HSV type 1 [HSV-1], HSV-2, EBV, and CMV). We tested this diagnostic procedure in the investigation of CSF samples from patients with acute encephalitis.

MATERIALS AND METHODS

Clinical specimens. A retrospective search of our CSF bank located 78 samples from 28 individual patients (group A) (Table 1) for whom a diagnosis of HSE had previously been ascertained by the presence of intrathecal synthesis of specific antibodies to HSV and/or isolation of HSV from cutaneous vesicles in newborns, from CSF, or from brain biopsy specimens. In 27 of 28 cases, the level of alpha interferon (IFN-α) was elevated in CSF (12, 13). In one case, a diagnosis of HSE was based on seroconversion associated with elevated IFN-α levels in CSF (patient 9). In addition, 19 CSF samples from seven patients with plausible HSE as well as 2 CSF samples from one child with acute encephalitis associated with EBV infection (group B) (Table 2) were analyzed prospectively. Among all patients, none had an underlying disease except patient 29 (a human immunodeficiency virus-infected newborn) and patient 35 (an immunocompromised child with EBV infection). For most of the patients studied, at least two CSF samples were available. We included in the study 37 other CSF samples from control patients with other central nervous system disorders: eight with meningitis associated with elevated IFN-α CSF levels, six with acute febrile meningoencephalitis of unknown origin, one with varicella-zoster virus encephalitis, six with multiple sclerosis, four with subacute sclerosing panencephalitis, four with Guillain-Barré syndrome, three with meningoradiculitis, three with congenital encephalopathies, one with Rasmussen encephalitis, and one with meningitis after vaccination for mumps-measles-rubella.

Virus strains. We used virus-infected cell cultures as positive controls in PCR assays. HSV-2 and CMV strains were isolated in our laboratory and were identified with specific monoclonal antibodies (Syva-Merieux and Clonatec, respectively), HSV-1 (Shealey strain) and HSV-2 were cultivated in a Vero cell line, CMV was cultivated in MRC5 diploid cells, and EBV was obtained from the B95-8 cell line.

DNA preparation. Approximately 200 μl of CSF was obtained from each specimen and was incubated with 200 μg of proteinase K per ml at 56°C for 1 h. Samples were extracted with phenol-chloroform (vol/vol); this was followed by ethanol precipitation. The pellet was resuspended

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TABLE 1. Results of PCR for HSV DNA in serial samples from patients with proven HSE[a]

Patient no. in group A	Sex/age[b]	Diagnostic test	PCR results in serial CSF samples on:			HSV type
			Days 1–7	Days 8–14	Post day 15	
1	M/11 days	ITS	++D			2
2	M/11 days	Culture CSF	++	−	− − − − −	1
3	M/15 days	ITS	+	+	D	2
4	M/19 days	Culture vesicle and IFN	+	NA	NA	2
5	F/17 days	ITS	+	−	D	2
6	F/10 days	Culture CSF	++	D		1
7	M/21 days	ITS	+	+	+	2
8	F/30 days	ITS	−	+	−	1
9	F/4 mo	Serology and IFN	+	+	− −D	2
10	M/6 mo	ITS	+	+	NA	1
11	F/7 mo	ITS	+	+−	NA	1
12	M/8 mo	ITS	+	+	NA	2
13	M/9 mo	ITS	++	−	++	1
14	M/10 mo	ITS	+	−	− − − − −	1
15	M/11 mo	ITS	+	−	D	1
16	F/14 mo	ITS	+	−	NA	1
17	M/24 mo	ITS	+	−	NA	1
18	F/3 yr	ITS	NA	NA	+++D	2
19	M/7 yr	ITS	+	−	NA	1
20	F/11 yr	ITS	++	−	NA	1
21	F/12 yr	ITS	+	−	−	1
22	F/13 yr	ITS	+	NA	NA	1
23	M/84 yr	ITS	+	+	−	1
24	F/67 yr	ITS	+	+	D	1
25	M/67 yr	ITS	+	−	NA	1
26	F/41 yr	ITS	+	−	−	1
27	M/54 yr	ITS	+	+	NA	1
28	M/50 yr	Culture autopsy	+	+	D	1

[a] Each + − sign (+, positive; −, negative) refers to the PCR result in a single sample. ITS, intrathecal synthesis of specific antibodies directed to HSV; IFN, intrathecal synthesis of IFN-α; culture CSF, autopsy, and vesicle refer to isolation of virus by culture from CSF, at autopsy, and from cutaneous vesicle, respectively; serology, seroconversion; NA, not available; D, deceased.
[b] M, male; F, female

in 25 μl of distilled water, and the entire suspension was used directly for PCR.

Design of oligonucleotide primers. Two 20-base oligonucleotides (P_1 and P_2) deduced from the published sequence of the DNA polymerase gene from HSV (11, 21) were used in PCR assays (see Fig. 1A). This set of primers, which was chosen within a highly conserved region of the DNA polymerase gene from the herpesvirus group, allows amplification of a selected fragment of the HSV-1, HSV-2, EBV, and CMV DNA polymerase genes (9).

TABLE 2. Results of PCR testing for herpesvirus genomes in serial CSF samples from patients with acute focal encephalitis[a]

Patient no. in group B	Sex/age[b]	PCR results in serial CSF samples on:			Virus type	Diagnostic confirmation
		Days 1–7	Days 8–14	Post day 15		
29	F/30 days	NA	++	NA	HSV-2	IFN
30	M/6 mo	+	+	−	HSV-1	Ag CSF
31	M/11 yr	+	+	NA	HSV-1	ITS
32	F/17 mo	+	NA	− +D	HSV-1	ITS
33	M/44 yr	+	+	−	HSV-1	ITS
34	F/57 yr	++	+	−	HSV-1	ITS
35	F/64 yr	+	−	NA	HSV-1	ITS
36	M/5 yr	+	+	NA	EBV	Serology

[a] Ag CSF, detection of HSV antigen in CSF; for other abbreviations, see footnote *a* of Table 1.
[b] M, male; F, female.

PCR amplification. Reaction mixtures contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 5% dimethylsulfoxide, 200 μM (each) deoxynucleoside triphosphates, 10 pmol of each oligonucleotide primer, and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). The reactions were performed in an automated thermal cycler (Perkin-Elmer Cetus); the cycle, which consisted of 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 1 min of elongation at 72°C, was repeated 40 times.

Analysis of PCR products. Ten percent of each amplified product (10 μl) was loaded onto a 1.5% agarose gel containing ethidium bromide and was subjected to electrophoresis before and after digestion with the restriction enzymes *Sma*I and *Bam*HI (Bethesda Research Laboratories), which were used under the conditions recommended by the supplier.

The PCR products which remained undigested, despite the use of these two enzymes, were hybridized with an internal ^{32}P-end-labeled oligonucleotide probe specific for HSV.

Single-stranded DNA suitable for sequencing was generated in PCR assays by using unequal amounts of the two primers (P_1/P_2 = 50 pmol/1 pmol), as described previously (4). The amplification reaction mixture (100 μl) was mixed with 2 ml of distilled water, applied to a Centricon 30 microconcentrator (Amicon), and spun at 3,000 × *g* to remove excess deoxynucleoside triphosphates and buffer components. Approximately 7 μl of the final retentate was used for sequencing by the dideoxynucleotide chain-termi-

nation method (DNA sequencing kit; United States Biochemical).

Precautions against contamination. The high level of sensitivity of the PCR procedure may easily lead to false-positive results because of minute contaminations. Precautions were taken, therefore, to ensure the validity of the results that were obtained. Precautions to avoid carryover of the PCR products included physical separation of pre-PCR and post-PCR mixtures, aliquoting of reagents, the use of positive displacement pipettes, and the avoidance of aerosols. Negative controls, including other DNA sources such as human genomic DNA, control CSF samples, varicella-zoster virus, as well as a reaction mixture without any DNA, were run in each experiment. DNA extractions were repeated when the volume of CSF available was sufficient.

RESULTS

Analysis of sensitivity and specificity of the PCR assay on control samples. A fragment of the DNA polymerase gene from different viruses belonging to the *Herpesviridae* family (i.e., HSV, CMV, and EBV) was successfully amplified from infected cell cultures by using the same set of primers (P_1 and P_2) located within a highly conserved region of this gene. The presence on an ethidium bromide-stained gel of PCR products of the expected size (518, 524, and 589 bp corresponding to HSV-1 and HSV-2, EBV, and CMV, respectively) attested to the specificity of the PCR assay (Fig. 1A and B). While the CMV amplified product was accurately identified by its molecular weight, HSV and EBV, which led to PCR products of similar sizes, needed further characterization. The specific *Sma*I and *Bam*HI patterns of these amplified PCR products clearly distinguished HSV from EBV. In addition, this restriction analysis permitted an unambiguous discrimination between HSV-1 and HSV-2 genomes. The 518-bp HSV-1 amplified product was cleaved by *Sma*I into 476- and 42-bp fragments and remained undigested by *Bam*HI; conversely, the 518-bp HSV-2 amplified product was cleaved by *Bam*HI into 225- and 293-bp fragments and remained undigested by *Sma*I. The 524-bp EBV amplified product was cleaved by *Sma*I and *Bam*HI into two fragments (100 and 424 bp, and 277 and 247 bp, respectively) (Fig. 1B).

We were also able to discriminate the two PCR products generated from HSV and EBV genomes (518 and 524 bp, respectively) using a specific internal ^{32}P-end-labeled oligonucleotide probe. Hybridization with this HSV probe yielded a highly specific signal without cross-hybridization with EBV (Fig. 2) or CMV amplified products (data not shown).

The HSV genome has a high GC content which adversely affects amplification. We found that the addition of 5% dimethyl sulfoxide enhanced amplification of the chosen sequences efficiently, leading to a visible band on an ethidium bromide-stained gel after 40 cycles of PCR. We estimate the threshold of detection to be 3 PFU by amplification of serial dilutions of HSV-1 viral stock (Fig. 3).

Detection of PCR-amplified herpesviruses genomes in CSF from patients with acute encephalitis. We first analyzed CSF samples from group A patients. In all these cases, a 518-bp fragment was amplified from at least one of the serial CSF samples and was readily visible on an ethidium bromide-stained gel. In view of this 100% agreement (for 28 cases) between the diagnosis of HSE and PCR-positive results, we included in the course of this study 19 CSF samples from 7 patients (group B) with plausible HSE. In all these cases, the PCR was positive for the presence of the HSV genome in CSF. Diagnosis was confirmed by intrathecal synthesis of specific antibodies or detection of HSV antigen in CSF from six of seven patients. We also analyzed two CSF samples

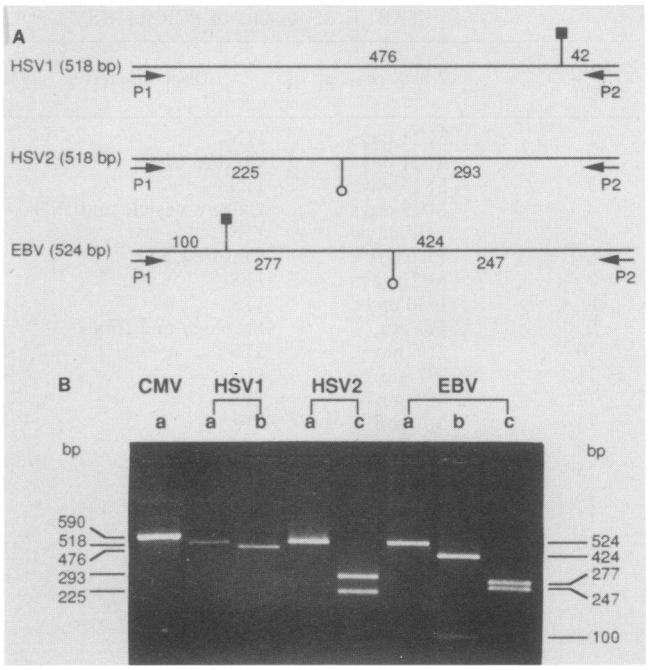

FIG. 1. Characterization of PCR products generated from different herpesviruses by using one set of primers. (A) Fragments of the DNA polymerase genes of HSV-1, HSV-2, EBV, and CMV were amplified by PCR by using primers P_1 (5′-CGACTTTGCC AGCCTGTACC-3′) and P_2 (5′-AGTCCGTGTCCCCGTAGATG-3′), which are represented by horizontal arrows. The PCR products were characterized with reference to their specific molecular masses (numbers in parentheses) and restriction profile. The symbols (■, *Sma*I; ○, *Bam*HI) mark the locations of the restriction enzyme sites. The resulting restriction fragment sizes are expressed in base pairs above and under each line. (B) Ethidium bromide-stained agarose gel analysis of undigested (lanes a) and digested (lanes b, digestion with restriction enzyme *Sma*I; lanes c, digestion with restriction enzyme *Bam*HI) products.

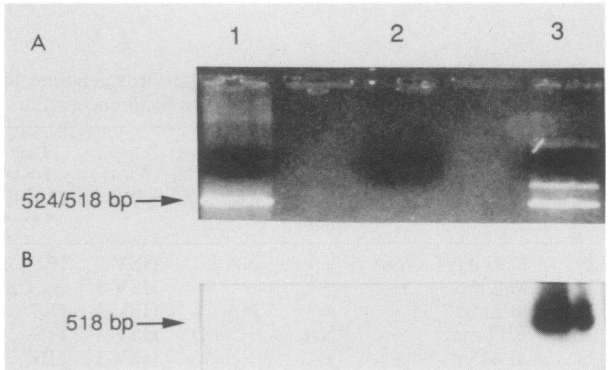

FIG. 2. Specificity analysis of hybridization of amplified DNA generated with primers P_1 and P_2. Lane 1, EBV DNA; lane 2, negative control DNA; lane 3, HSV DNA. (A) Ethidium bromide-stained gel. (B) Corresponding autoradiograph after hybridization with the HSV-specific probe (5′-GATTCCCCAGAGCAGCCCCG-3′).

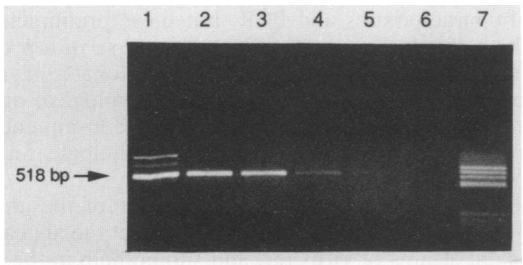

FIG. 3. Sensitivity analysis of PCR assay. DNA amplification of fivefold dilutions of HSV-1 stock virus titrated at 10^6 PFU/ml visualized on an agarose-stained gel. Lanes 1 to 5, 2×10^3, 4×10^2 80, 16, and 3 PFU, respectively; lane 6, negative control DNA; lane 7, size marker generated by *Hae*III digestion of pBR322.

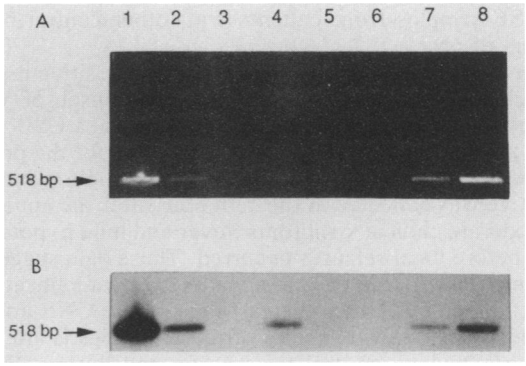

FIG. 4. Follow-up of PCR signal in serial CSF samples. Lanes 1 to 5, samples from patient 13 at days 1, 7, 20, 22, and 28, respectively, after the onset of clinical symptoms; the PCR was positive for the presence of HSV DNA in the first two samples; a clinical improvement under antiviral therapy was found to be associated with a negative PCR in the third sample collected on day 20; the last two CSF samples analyzed were positive again 2 and 6 days later, respectively, at the time corresponding to a significant worsening of the neurological status of the patient; lane 6, negative DNA control; lanes 7 and 8, samples from patient 1 at days 2 and 4, respectively, after the onset of the disease. (A) Ethidium bromide-stained gel. (B) Corresponding autoradiograph (3 h of exposure) after hybridization with the HSV-specific probe.

from one immunocompromised child with acute febrile encephalitis and recent EBV infection. In this case, a 524-bp amplified product was clearly visible after ethidium bromide staining.

None of the 37 control CSF samples was positive. The risk of false-positive PCR results was minimized, since extensive control experiments (described in above) were repeatedly negative.

Characterization of the type of herpesvirus detected in CSF from patients with acute encephalitis. To characterize which herpesvirus was associated with encephalitis, amplified DNAs from all patients (36 subjects) were subjected to restriction enzyme analysis with *Sma*I and *Bam*HI endonucleases. In 33 patients, this analysis allowed accurate characterization of the herpesvirus type (23 HSV-1, 9 HSV-2, 1 EBV). In three patients from group A, the PCR products remained undigested, but a strong positive signal was obtained after hybridization under stringent conditions with an internal HSV-specific oligonucleotide probe. Direct sequencing of the PCR products established the diagnosis of HSV-1 infection in all three patients.

Among the nine infants with neonatal encephalitis, six (67%) were positive for HSV-2, whereas three (33%) were positive for HSV-1. HSV-1 had already been isolated and identified in two of these three newborns (patients 2 and 6) by viral culture of CSF. HSV-1 infection was much more frequent than HSV-2 infection in the children and adults. In view of the results of restriction enzyme analysis and direct sequencing, the frequency of HSV-1 infection in 17 children and 9 adults was found to account for 82 and 100% of cases, respectively (Tables 1 and 2).

Sequence analysis of the three products which remained undigested with *Sma*I and *Bam*HI (patients 14, 21, and 28) allowed us to identify a single base mutation that was responsible for the lack of recognition of a *Sma*I restriction site (data not shown). This cytosine-to-thymidine transition in the codon for amino acid 875 of the DNA polymerase resulted in a proline-to-serine substitution.

Follow-up of PCR signal in serial CSF samples. A follow-up of PCR testing was performed for CSF samples from most of the patients studied (during the first and second weeks and after the third week following the onset of acute encephalitis). The results, which are summarized in Tables 1 and 2, show a significant decrease of the number of CSF-positive samples after day 15. From days 1 to 7, 38 of 39 CSF specimens gave a positive PCR response, while 17 of 31 CSF specimens were positive between days 8 and 14, and only 7 of 27 were positive after day 15.

It is noteworthy that in three cases in which PCR was positive after day 15 (patients 13, 18, and 32), CSF was collected at the time corresponding to a clinical relapse. In patient 13 (Fig. 4), five serial CSF samples were available for DNA analysis. In another case (patient 32), we analyzed three CSF samples obtained over 3 months; the first sample analyzed at day 6 was positive; the second one, obtained 1 month after therapy, was negative; 2 months after the onset of HSE, the patient's neurological status worsened. A third CSF sample obtained at that time was again positive for HSV-1 DNA, with a strong signal.

DISCUSSION

Results of this study demonstrate, by means of a highly sensitive and specific procedure (PCR), that herpesvirus genomes can be detected and characterized in CSF from patients with acute encephalitis.

Recent retrospective studies mentioned the use of PCR for the diagnosis of HSE (1, 7, 16, 18). Aurelius et al. (1) used nested PCR, which could be a source of contamination. Indeed, this technique, which is based on two rounds of PCR, requires the addition of the nested primers between the two rounds. At this step, contaminations with the PCR products generated from the first round could occur, especially when the PCR is used for routine diagnosis. Under our experimental conditions, a single round of PCR was sufficient to detect HSV DNA. Although PCR is known to be only a semiquantitative method of evaluation of the amount of DNA in a sample, we showed that it is possible to detect as little as 3 PFU of HSV.

We presented here the results of a retrospective analysis of CSF samples from patients with confirmed HSE and a prospective study of patients with acute focal encephalitis. In 36 (100%) of the 36 patients studied, the herpesvirus genome was amplified successfully, as revealed by the presence of PCR products of the expected size. The PCR results were in agreement with the diagnosis made by serological methods. There was no positive result among the

37 CSF samples from patients with other central nervous system disorders.

The use of PCR gave a rapid and early positive result for the diagnosis of HSE, relative to the onset of clinical symptoms. In all cases except one, the first CSF sample analyzed by PCR (days 1 to 7) was positive for the presence of the HSV genome. The first CSF sample which was negative was collected at the 12th hour after the appearance of moderate clinical symptoms (fever and mild hypotonia), 2 days before focal seizures occurred. These data suggest that the amounts of HSV DNA compatible with amplification are detectable in CSF from the first days of the CNS infection. The high degree of reliability of these findings is attested to by the extensive control tests performed during DNA extractions and PCR assays.

Moreover, in our study, the reality of detection of HSV DNA in CSF was strongly reinforced by the characterization of the type of HSV genome. In previous reports, the primers used by the investigators permitted detection of only HSV-1 (1, 16) or could not differentiate the two types of HSVs (7, 18). Such identification is of crucial importance for a better understanding of the epidemiology and evaluation of the prognosis associated with the type of HSV infection in newborns and infants (2, 23).

Our results show that it is possible to distinguish, on the basis of PCR, which viral genome is present in CSF. We designed one pair of primers to amplify four members of the *Herpesviridae* family. Such use of a single pair of primers is of particular importance when small amounts of biological fluid are available. A simple digestion of the amplified products by two enzymes, *Sma*I and *Bam*HI, allowed us to identify accurately and rapidly the type of HSV responsible for HSE in 32 (91%) of 35 patients studied. Moreover, in one case of acute encephalitis associated with EBV infection, we could demonstrate the presence of EBV DNA in the CSF using the same strategy. In the last three patients (group A), HSV infection could be ascertained by using an internal HSV oligonucleotide probe, and the viral type was established by direct sequencing.

The results of the relative frequencies of the HSV-1 and HSV-2 infections by age (newborns, children, and adults) are in agreement with those of previously published studies (2, 23): three of nine newborns were found to have HSV-1 infection (33%). In the children and adults, we found a high frequency of HSV-1 infection (82 and 100%, respectively), as expected.

In three patients, only specific hybridization and direct sequencing of PCR products permitted us to identify HSV-1. Interestingly, the same single-base mutation that altered the *Sma*I recognition site and that generated a serine in place of a proline at position 875 of the DNA polymerase was present. This nucleotide substitution further illustrates the variations in restriction endonuclease profiles of HSV genomes (14, 25). Its location within a DNA region not known to be of crucial importance (3) suggests that it could be a simple polymorphism. However, only an in vitro assay could test the functional importance of this variant which contains a polar amino acid (serine) in place of a nonpolar one (proline), which is conserved among HSV-1 and HSV-2.

In addition, results of our study illustrate the usefulness of the PCR in the follow-up of HSE. Its high sensitivity permitted us to recover HSV DNA as long as 15 days after the onset of clinical symptoms. Moreover, this approach also allowed us to show in two patients that the relapse of encephalitis was associated with the reappearance of the HSV genome. We cannot yet establish a correlation between clinical characteristics and PCR, but these preliminary observations should be completed in the course of a prospective study. Indeed, on the basis of conventional assays, it is most often impossible to attribute the neurological deterioration observed in such patients to the consequences of primary infection involving possible immunological mechanisms (8) and/or to active viral replication.

PCR analysis should allow the efficiency of the antiviral therapy to be followed, should help to detect the appearance of resistant strains of virus (5), and should help in the direct analysis of sequences associated with drug resistance (15).

Lastly, the design of this single pair of primers selected in a highly conserved region of herpesvirus DNA polymerase genes permits the study of other encephalopathies, such as those associated with EBV infection and, probably, with CMV infection as well.

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