DETECTION OF HERPES SIMPLEX VIRUS DNA BY POLYMERASE CHAIN REACTION IN THE CEREBROSPINAL FLUID OF PATIENTS WITH VIRAL MENINGOENCEPHALITIS USING PRIMERS FOR THE GLYCOPROTEIN D GENE

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Summary. – A novel set of primers for polymerase chain reaction (PCR) which amplified the portion of US6 sequence coding for the main type-common neutralizing epitope of glycoprotein D (gD) was used for detection of herpes simplex virus (HSV) DNA in 44 cerebrospinal fluid (CSF) samples from 29 patients with clinical symptoms of viral meningitis or meningoencephalitis. The primers in question amplified the DNA of 9 out of 10 low-passage HSV-1 isolates and of 5 out of 10 HSV-2 low-passage isolates as well as the DNA of all laboratory strains examined when tested in the supernatant fluid of infected cells cultures. The PCR was positive in 5 CSF samples (taken on days 2, 4, 8, 10 and 56 after the onset of symptoms, but not later than day 8 after starting acyclovir (ACV) therapy) obtained from 4 patients with intrathecal antibody response. The PCR was repeatedly negative in CSF of 15 patients who had antibodies to HSV in serum and CSF, but did not show intrathecal antibody production. It was also negative in 10 patients who had no HSV antibodies in CSF. Our results confirmed that positive PCR for HSV DNA in the CSF is an indication for starting and/or continuing ACV therapy even in the absence of classical symptoms of HSV encephalitis.

Key word: herpes simplex virus; polymerase chain reaction; cerebrospinal fluid; meningitis; encephalitis

Introduction

Early diagnosis of HSV encephalitis has been difficult till now (Nahmias, 1982), because virus isolation from CSF is consistently negative (Johnson, 1982) and the appearance of detectable amounts of virus-specific glycoproteins is rare (Chen *et al.*, 1978). The virus can be isolated from brain biopsy specimens only (Johnson *et al.*, 1968). Supporters of cerebral biopsy argued that temporal lobe decompression may also be regarded as a method of treatment (Hanley *et al.*, 1987), but this is true only for advanced stages of HSV encephalitis (Barza and Parke, 1980), which would not develop under conditions of successful acyclovir treatment. Non-invasive diagnosis of HSV encephalitis, however, has been mainly indirect and suffered from imperfections. EEG changes such as low theta and delta waves, temporarily localized flat irregular rhythms interrupted by periodic sharp peaks (Upton and Grumpert, 1970) are only suggestive of the disease. Elevation of HSV antibodies in CSF occurs at late stages of encephalitis (McCallum *et al.*, 1974). The estimation of leakage of serum antibodies through impaired blood-CSF barrier by calculating the antibody index (HSV-specific antibody ratio in CSF and serum) makes the serological diagnosis cumbersome (Klapper *et al.*, 1981; Koskiniemi and Vaheri, 1982; Bos *et al.*, 1987). Alternative indices can be calculated by relating the CSF to serum HSV-specific and measles-specific antibody titres.

The introduction of PCR for amplification of HSV DNA in CSF has provided a powerful tool for early non-invasive diagnosis of HSV encephalitis (Puchhammer-Stockl *et al.*, 1990; Rowley *et al.*, 1990; Klapper *et al.*, 1990; Rogers *et al.*, 1991; Aurelius *et al.*, 1991; Pohl-Koppe *et al.*, 1992).

Different conditions for PCR have been reported, with variations in the annealing temperature, the number of cycles, the way of preparation of CSF DNA samples, and the type of primers. Concerning the pretreatment of CSF samples, many authors stress the importance of deproteinization and precipitation (Dennett *et al.*, 1991) or ultrafiltration (Rogers *et al.*, 1991). Also the choice of primers varies. Klapper *et al.* (1990) used primers flanking a portion of the thymidine kinase gene, Rozenberg and Lebon (1991) employed primers amplifying a conserved portion of the DNA polymerase gene, Puchhammer-Stockl *et al.* (1990) had primers flanking a part of the UL42 ORF, Pohl-Koppe *et al.* (1992) described primers amplifying a portion of gB gene, while Aurelius *et al.* (1991) used primers from the gD gene. Here we describe a new primer-set flanking a 421 bp sequence coding for the virus-specific type-common neutralization epitope of gD. The sensitivity of detection of viral DNA is less than 1 pg. We report its application in detection of HSV DNA in CSF of patients showing symptoms of viral meningitis and meningoencephalitis.

Patients, Materials, and Methods

Cells and viruses. Human embryonic lung (LEP) cells, Vero cells or primary quail embryo (PQE) cells were used for isolation and growth of different HSV type 1 and type 2 strains. DNA extracts of type 1 laboratory strains KOS, 17, HFEM and HSZP (Szántó, 1960) and of type 2 laboratory strain G were employed in addition to 10 clinical isolates (routinely typed by monoclonal antibodies Syva Microtrak from Syva Co., San José, CA, USA).

Patients. Paired CSF and serum samples were obtained from 29 neurological patients with suspected viral meningitis, or meningoencephalitis who attended the Clinic of Infectious Diseases in Bratislava during a 1.5 year period from December 1990 to May 1992 (Table 1). All samples were examined immediately and stored at -20 °C. At enrollment, there were 18 patients with signs of viral meningoencephalitis and 11 with aseptic meningitis. Only in 10 of 29 patients serial paired CSF/serum samples were available. In 4 cases the diagnosis of HSV encephalitis was suggested according to CT, EEG examinations and intrathecal HSV antibody production. In the rest of patients who had high serum HSV antibody levels and positive HSV antibody in CSF, intrathecal antibody production could not be demonstrated. From 11 subjects in whom the early diagnosis at enrollment was aseptic meningitis, 1 developed later the signs of HSV encephalitis.

Serological tests. Enzyme immunoassay (EIA) was performed using a domestic HSV-1 antigen whose preparation has been described elsewhere (Murányiová *et al.*, 1991*a*,*b*). Briefly, KOS-infected LEP cell extract was enriched in HSV-1 specific proteins by affinity chromatography on CNBr-activated Sepharose-4B (Pharmacia) with bound anti-LEP IgG raised in pigs immunized with the LEP cell extract. After titration of the specific HSV-1 antigen content in a modified EIA with anti-HSV-1 IgG bound to solid phase, the antigen was diluted to contain 2 – 5 μg protein per ml and used for coating of microplate wells (20 antigenic units per well). For antibody titrations, EIA was carried out in two modifications, namely using anti-human IgG/Px or anti-human IgM/Px (both peroxidase labelled conjugates were from Sevac, Prague, Czech Republic, and were used diluted 1.3×10^{-3}). For each serum (or CSF) dilution, uninfected LEP cell extract (not subjected to affinity chromatography) was used as control antigen. Results were calculated by dividing A_{492} value of infected cell extract by that of the control antigen; value above 2.1 was taken to estimate the end-point titer.

Table 1. Sum of patients whose CSF samples were amplified to detect HSV DNA by PCR

Diagnosis at enrollment	Number of patients	Number of CSF PCR-negative	PCR-positive
Encephalitis	3		4/6[a]
	15	22	
Subtotal	18		
Meningitis	1		1/2
	10	14	
Subtotal	11		
Positive together	4		5/8
Negative together	25	36	
Total	29	44	

[a]PCR-positive out of total CSF samples examined (2 samples per patient).

To assess the leakage of HSV-specific immunoglobulins through the blood-brain barrier due to inflammation, albumin levels in serum and CSF were measured, related to the HSV IgG titers in CSF and serum using the formula of antibody index AI of Klapper *et al.* (1981).

$$AI = \frac{\text{CSF HSV antibody}}{\text{Serum HSV antibody}} : \frac{\text{CSF albumin}}{\text{Serum albumin}}$$

The index allows to distinguish between intrathecal antibody production and serum-derived antibody leakage. In cases of unpaired CSF and serum specimens we calculated the index from HSV-specific IgG to albumin ratio in CSF only (I. Orolín, personal communication).

Polymerase chain reaction. The downstream primer (5'-A ACT ACC CCG ATC ATC AGT TAT CCT-3') and the upstream reversed primer (5'-GAT GGT CAG GTT GTA GGG TTG TTT C-3') flank a 421 bp sequence of the gD gene (nt 186- 606) (Watson *et al.*, 1982) which corresponds to the section nt 5761-6181 of US6 (Fig. 1) in the short unique region of HSV-1 genome (McGeoch *et al.*, 1985). This region codes for the type-common domain of gD which induces neutralizing antibodies (Cohen *et al.*, 1984) and represents a sequence significantly distinct from mammalian and microbial genes as determined by GCG computer analysis (University of Wisconsin, WI, USA) using data available in GenEMBL.

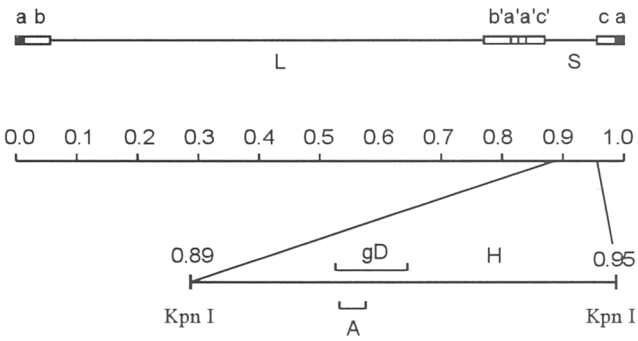

Fig. 1
Assignment of the strain 17 *Kpn*I H DNA fragment and the position of gD amplimer (A) in US6 (nt 5761-6781) corresponding to the gD gene

In the middle of the amplicon in question is a *Pvu*II site separating the 421 bp fragment into two nearly identical (210 and 211 bp) subfragments (Fig. 2). The primers were synthesized in PCR-MATE EP DNA synthesizer from Applied Biosystems following the instructions of the manufacturer.

PCR was performed as described by Saiki *et al.* (1988). Briefly, about 150 – 300 µl of CSF was incubated with proteinase K (10 µg/100 µl) for 60 mins at 56 °C. After phenol-chloroform extraction, the aqueous phase was precipitated with ethanol in the presence of 0.1 mol/l NaCl overnight at -20 °C. The DNA pellet was dissolved in 10 µl TE buffer. The medium from HSV-infected cells and the infected or control cell suspensions (10^6 cells per 100 µl) were deproteinized, their DNA was precipitated and redissolved in TE buffer to 10% of the original volume.

The reaction mixture was prepared using the GenAmp kit (Perkin Elmer Cetus, CT, USA) reagents to obtain 100 mmol/l Tris-HCl pH 8.3, 50 mmol/l KCl and 1.5 mmol/l $MgCl_2$, 200 nmoles of dATP, dTTP, dGTP, dCTP and 50 pmoles of each primer. The reaction mixture (90 µl) and 10 µl of DNA sample were mixed and covered with mineral oil. The sample was denatured for 10 mins at 94 °C, quickly chilled and Taq polymerase (2.5 U) was added to each vial. The reaction was run in Perkin Elmer thermal cycler for 40 cycles as follows: 100 secs at 94 °C, 75 secs annealing at 42 °C, and 90 secs elongation at 72 °C (during the last cycle the elongation was prolonged for up to 6 mins). To avoid false positive amplifications, DNA samples and the reaction mixture were prepared in separate hoods.

The amplified DNA (10 µl) was electrophoresed in 1.5% agarose gel in TAE, stained with ethidium bromide, viewed and photographed under UV light. The digestion of 20 µl amplification product with *Pvu*II or *Bgl*I was carried out using 2 U or 1 U of the enzyme, respectively, in a corresponding buffer for 2 hrs at 37 °C. The bands prepared from the amplified PCR product were identified by Southern blot hybridization and hybridization with the *Kpn*I H HSV-1 DNA fragment (MU 0.89 – 0.95) which covers the US6 region (kindly provided by dr. C.H. Preston, University of Glasgow, Scotland). The fragment cloned in pUC19 was labelled by nick translation according to Rigby *et al.* (1977) using ^{32}P-dCTP. The specific activity of the DNA probe was 2.5 x 10^8/µg; the hybridization procedure was performed as previously described (Kúdelová *et al.*, 1991).

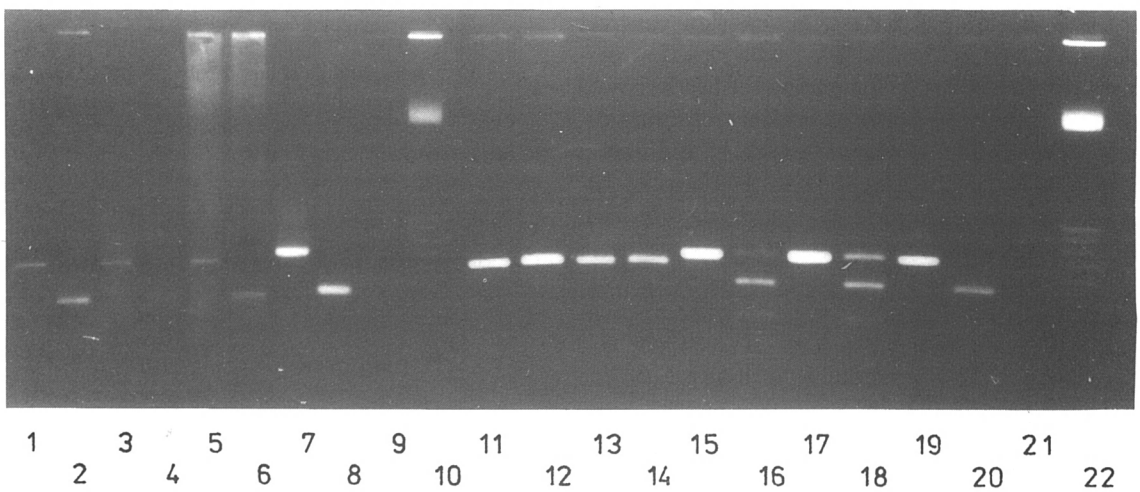

Fig. 2
The relative diagnostic significance of *Pvu*II cleavage
Fragments were amplified from strains KOS (lanes 1-2, 11-12), 17 (lanes 3-4, 13-14) and HSZP (lanes 5-6) DNA using the gD primer-set on one hand, and from strain KOS (lanes 7-8, 15-16), 17 (lanes 17-18) and HSZP (lanes 19-20) DNA using the primer set flanking a portion of UL41 (early shutoff) gene (lanes 7-8, 15-20) on the other hand. The gD (421 bp, lanes 1, 3 and 5) and UL41 amplimers (488 bp, lane 7) were digested with *Pvu*II (lanes 2, 4, 6 and 8) into 2 identical subfragments. In contrast, *Bgl*I did not cleave the gD amplicons (lanes 11-14) but digested the UL41 bands (here we show partial digestion) into 2 uneven fragments, 162 bp and 324 bp, respectively (lanes 16, 18 and 20); lanes 9 and 22: marker DNA (100 bp ladder).

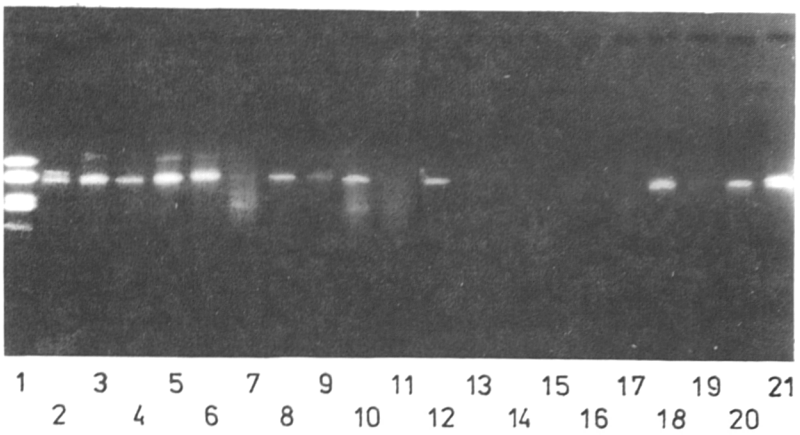

Fig. 3
The type-common nature of the gD primers
Supernatant fluids from cells infected with HSV-1 (lanes 2-11) and HSV-2 (lanes 12-21) clinical isolates selected at random were amplified. Lane 1: *Hae*III-digested pUC18.

Results

The ability of the gD primers to amplify DNA of type 1 laboratory strains KOS, 17 and HSZP is shown in Fig. 2. The gD amplimer (amplification product) was cleaved with *Pvu*II but not with *Bgl*I. Under given conditions, clearly visible bands in agarose gel were obtained when the threshold concentration of amplified viral DNA was at least 1 pg (data not shown). After deproteinization and precipitation as described above, amounts of HSV DNA corresponding to 10 µl medium harvested from Vero cell cultures with CPE were detected in 9 of 10 HSV-1 wild type (low passage) clinical isolates, and in 5 of 10 HSV-2 wild type (low passage) clinical isolates (Fig. 3). Some amplified bands showed a slightly slower mobility in the gel, possibly reflecting minor differences in the sequence length of individual amplimers. On repeated testing by PCR, there was no difference between the medium samples handled in a fresh state or after freezing and thawing. The PCR was positive in 5 of 8 CSF samples from 4 patients (Fig. 4) who had indication of intrathecal HSV IgG production (Table 2); in Southern blot hybridization the signal was positive with the half amount of amplification products normally used for electrophoresis (Fig. 5).

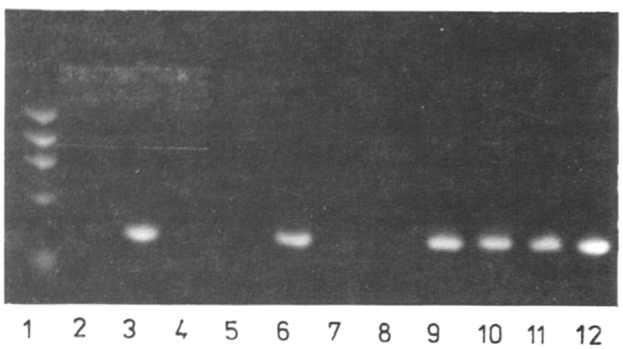

Fig. 4
Electrophoresis of amplified CSF DNA samples
Lane 1: *Hae*III digested øX174 DNA; lane 2: 500 ng human lung embryo cell DNA; lane 3 and 12: 500 ng total DNA from strain KOS-infected human embryo lung cells; lanes 4-5: CSF samples from patients showing negative results; lanes 6 and 7: samples from patient No. 2 taken on days 2 and 11 after onset of symptoms; lanes 8 and 9: CSF samples from patient No. 3 taken on day 42 and 56 after onset of symptoms; lanes 10 and 11: CSF samples from patient No. 4 taken on day 4 and 10 after onset of symptoms.

Three patients listed in Table 2 (No. 1, 3 and 4) developed classical signs of HSV encephalitis as detected by EEG and CT. Patient No. 1 showed a dramatic rise of serum antibody levels to HSV-1 and an increase of both IgM and IgG antibodies to HSV-1 in CSF. Low-density lesions appeared in CT scan on day 6 after onset of symptoms along with diffuse abnormal focal waves in EEG in temporal lobe area. The PCR was positive on day 8 from the onset of symptoms but became negative 28 days later, when in turn, the antibody index increased. Acyclovir therapy was started on day 4 allowing the patient to recover with slight loss of memory. Patient No. 3 was admitted to the clinic relatively late (on day 42 after the onset of symptoms) and no previous serum or CSF samples were available. Extremely high HSV-specific antibodies in CSF (both IgG and IgM) and low-density lesions in the temporal lobe found by CT scan were suggestive of HSV encephalitis. Oral acyclovir treatment was combined with steroid therapy from day 48. The diag-

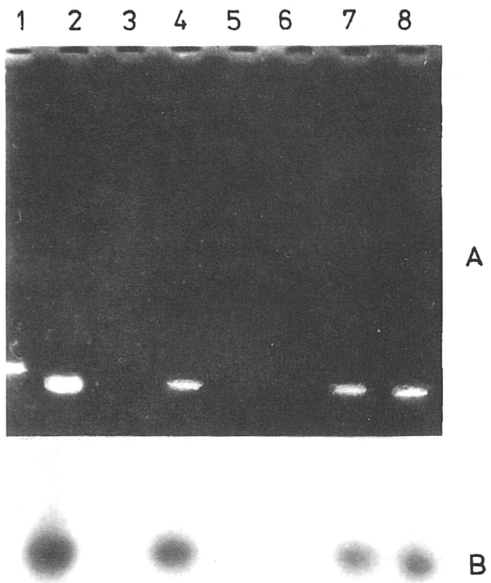

Fig. 5
Electrophoresis (A) and Southern blot hybridization (B) of amplified CSF samples
Lane 1: positive control phage DNA from the Perkin-Elmer GeneAmp kit; lane 2: control DNA from strain KOS-infected LEP cells; lane 3: negative control DNA from non-infected LEP cells; lanes 4 and 5: CSF samples from patient No. 2; lanes 6 and 7: CSF samples from patient No. 3; lane 8 : CSF sample from patient No. 4 taken on day 4. Southern blot was prepared from a gel loaded with 5 µl PCR product only with ^{32}P-labelled *Kpn*I fragment H. Positive lanes: 2,4,7 and 8.

nosis of HSV encephalitis with prolonged course was confirmed on day 56, when PCR became positive. Acyclovir therapy was continued for 10 days followed by recovery albeit with more severe mental sequelae. Patient No. 4 had extremely high serum HSV antibody levels and a clearcut increase of HSV-specific IgG in CSF along with PCR positivity of both acute CSF samples (days 4 and 10 after onset). CT scan showed temporal oedema on day 3 and low-density lesions on day 10. Intravenous ACV therapy proceeded since day 3, and the patient recovered with severe residual symptoms.

Patient No. 2 had relatively low HSV antibody levels in the CSF but PCR was positive on day 2 after the onset of meningeal symptoms. Acyclovir therapy was immediately started and the patient recovered without any sequelae.

Another l4 CSF samples from 11 patients with aseptic meningitis showed negative PCR and had no HSV antibody in their CSF (Table 1). No ACV therapy was started in these patients and they all recovered.

We tested another 22 CSF samples from 15 patients with unconfirmed HSV encephalitis; all the samples were negative (Table 1). These patients had HSV antibodies in their CSF, but intrathecal HSV antibody production was not proven. In 6 of these patients no paired CSF-serum samples were available. One of the cases in question was a 16-month-old child who had extremely high anti-HSV IgG levels in CSF (IgG 1 024-16 000) as well as in serum (up to 640 000) throughout the observation period, namely from day 18 to 72 of the disease. The amplification results in all his 4 successive CSF samples remained consistently negative. Nevertheless, ACV therapy was applied in this particular pa-

Table 2. Results of ELISA and PCR in 4 cases of intrathecal HSV-1 antibody synthesis in suspected cases of herpetic encephalitis

Patient no.	Age/ /sex	Acyclovir therapy	Days after onset of disease	ELISA titers antibody				Index[a]	PCR results
				CSF		Serum			
				IgG	IgM	IgG	IgM		
1	43/m	from day 4	8	64	4	2 560	640	2	+
		(intraven.)	36	>512	128	81 920	<40	78	–
2	10/f	from day 2	2	<4	<4	ND	ND	8[b]	+
		(intraven.)	11	16	8	10 240	128	61	–
3	59/m	from day 48	42	12 800	512	ND	ND	18[b]	–
		(oral)	56	12 800	160	81 920	<40	20	+
4	51/m	from day 3	4	16	<4	81 920	<40	8	+
		(intraven.)	10	256	4	81 920	<40	30	+
			32	ND	ND	5 120	160	ND	ND

[a]Values above 1.91 indicate intrathecal antibody production.
[b]Calculated as HSV IgG to albumin ratio in CSF (see Methods). ND – not done.

tient similarly as in another 12 suspect but unconfirmed cases.

Discussion

We used a newly designed primer set for PCR amplification of HSV DNA in 44 CSF samples from 29 patients with viral meningoencephalitis or meningitis. The specificity of reaction product was verified by Southern blot hybridization using a DNA probe (*Kpn*I H fragment) spanning the gD gene (US6). This primer set amplified unknown amounts of HSV DNA present in about 10 µl of medium from HSV-1 infected cell cultures with a probability of 90% and that from HSV-2 infected cultures with a probability of 50%. Negative results with some clinical materials might be caused by concentrations of viral DNA below the observed sensitivity of PCR (1 pg DNA). The amplified sequence codes for the type-common main antigenic domain of gD, which reacts with neutralizing antibody. The sequence in question showed no homology with 48 of 50 mammalian genes when aligned by computer analysis.

HSV DNA was successfully amplified from 5 CSF samples of 4 suspect patients at early stages (up to day 10) of their disease. The positive PCR results were consistent with the diagnosis of HSV encephalitis based on increased antibody index (> 2.1) indicating intrathecal antibody production (Bos *et al.*, 1987; Klapper *et al.*, 1981; Koskiniemi and Vaheri, 1982). PCR was negative in 10 cases of serous meningitis with no HSV antibody in CSF as well as in 15 patients with medium or high HSV-1 antibody levels who lacked evidence of intrathecal antibody production. However, in one case of meningitis the PCR was positive on day 2 of the disease and later on this patient really developed intrathecal antibody production (No. 2 in Table 1). In one case of the 15 suspect patients, which showed unusually high HSV antibody levels in serum and CSF but no intrathecal antibody production as calculated from the antibody index, CSF remained consistently negative when tested by PCR for HSV-1 DNA.

It possible that the PCR positivity disappeared with the rise of HSV antibodies (Pohl-Koppe *et al.*, 1992), and that PCR was repeatedly negative in those patients who had high HSV antibody levels in CSF. Another factor to be considered is the duration of the ACV treatment at the time of investigation as well as its timing after the onset of clinical symptoms. HSV DNA was regularly found in CSF when amplified up to 5 days after the start of the ACV therapy (Aurelius *et al.*, 1991; Pohl-Koppe *et al.*, 1992). Among 433 samples tested by Aurelius *et al.* (1991), there were no positive CSF sample after day 30 from the beginning of symptoms. Though our group of positive patients was small, our data (Table 1) seem to be consistent with these descriptions; namely, our amplifications were mostly positive up to day 10 from the onset of symptoms and to day 9 from the beginning of the ACV therapy. However, we had a patient (No. 3) whose CSF was PCR-positive on day 56 from the start of disease, probably due to a low efficiency of his oral ACV therapy. Pohl-Koppe *et al.* (1992) described 2 patients with positive PCR on day 36 indicating that subacute course of the disease may depend on many factors including the efficiency of therapeutic measures and the delay of their start.

We conclude that PCR is a powerful tool for diagnosis of HSV encephalitis and its positivity in the absence of other classical symptoms may be an indication for ACV therapy. On the other hand, a negative PCR result in the presence of other symptoms of HSV encephalitis may not be the reason for deferring such therapy.

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