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Short communication

Analysis of herpes simplex virus type 1 glycoprotein D nucleotide sequence in human herpes simplex encephalitis

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Viral factors responsible for HSV neurovirulence in humans are still unknown. The aim of this work was to investigate the hypothesis that viral variants might contribute to the specific neurovirulence of some HSV strains. HSV-1 DNA was recovered from cerebrospinal fluid (CSF) in ten patients with HSV encephalitis (HSE) and the regions of HSV-1 gD gene corresponding to known antigenic sites were analyzed by direct sequencing of PCR products. Twenty-two mutations were found among a total of 6580 bp analyzed over a portion of 1000 bp of gD gene, of which 20 were silent whereas two conferred amino acid substitution. One missense mutation (E117D) was found in two CSF samples as well as in two control laboratory strains. The other one (A269T) was found in a single CSF sample, and lies within a region corresponding to a functionally essential antigenic site. These are the first mutations of the gene encoding gD of HSV identified *in vivo* in human encephalitis samples. Overall, the results argue against the role of gD in neurovirulence in humans.

Keywords: HSV-1; human encephalitis; glycoprotein D; mutations

Herpes simplex virus type 1 (HSV-1) is a ubiquitous human pathogen, causing a variety of diseases. After the primary infection, HSV establishes latency in peripheral sensory neurons, and can then be subjected to episodes of reactivation. The far most severe complication of HSV infection is HSV encephalitis (HSE), a rare sporadic acute focal necrotizing encephalitis occurring in immunocompetent patients. Both primary and secondary HSV infection can cause HSE. Whereas the determinants of peripheral reactivatable HSV infections are currently being elucidated, the precise source of the virus and the factors responsible for the occurrence of HSV central nervous system (CNS) invasion in humans are still unknown. Host factors are difficult to investigate and have not been looked for intensively. In contrast, the existence of specific neurovirulent strains has been suggested. Indeed, increased-neurovirulent HSV variants have been obtained in experimental animal models. In such models, several genes governing neurovirulence have been investigated by the use of recombinant strains, or deletion mutants. The results of these studies have permitted us to differentiate viral genes conferring ability to replicate in the CNS, versus viral genes conferring ability to gain access to the CNS (reviewed in Roizman and Kaplan, 1992). Although the relevance of these data to human disease has not been established, it has been hypothesized that neuroinvasiveness might be one factor of interest in the pathogenesis of human HSE (Bergström *et al*, 1990). Among the viral genetic determinants of interest for a possible role in neuroinvasion, HSV glycoproteins are good candidates. At least eight virus-encoded glycoproteins have been identified hitherto, three of them (glycoproteins D, B, and H, or gD, gB, and gH, respectively) being essential for viral replication in culture, and penetration of cells. Whereas glycoproteins homologous to gB and gH have been identified in other human herpesviruses examined (Cranage *et al*, 1986, 1988), gD is not conserved while retaining the same properties. In particular, gD has focused interest as a major target for neutralizing antibodies (Eisenberg *et al*, 1985). Furthermore, gD plays an essential role in post-attachment entry of the virus into cells (Johnson and Ligas, 1988; Ligas and Johnson, 1988; Muggeridge *et al*, 1990), and has interference activity (Campadelli-Fiume *et al*, 1988; Brandimarti *et al*, 1994). Mutational analyses have

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allowed us to identify functionally critical regions, in terms of antigenic properties, attachment to cells, and replication of virus in infected cells (Cohen *et al*, 1988; Isola *et al*, 1989; Campadelli-Fiume *et al*, 1990; Feenstra *et al*, 1990; Dean *et al*, 1994; Chiang *et al*, 1994). Whereas amino acid sequence analysis has revealed that gD is a highly conserved protein, with >98% identity between strains, *in vitro* studies have led to the characterization of gD amino acid substitutions conferring resistance to neutralization or interference (Minson *et al*, 1986; Campadelli-Fiume *et al*, 1990; Dean *et al*, 1994; Brandimarti *et al*, 1994). Moreover in an *in vivo* study of experimental pathogenesis, a single amino acid substitution in the gene encoding gD was identified in a viral strain with enhanced neurovirulence (Izumi and Stevens, 1990). Although the role of this subtle modification in such drastic change of the neuroinvasive phenotype requires further studies, taken together, all these results support the hypothesis that gD is an important element of the virus neurotropic properties. To test this hypothesis *in vivo* in humans, we have undertaken a molecular analysis of HSV-1 gD nucleotide sequences derived from amplified products obtained directly from cerebrospinal fluid (CSF) DNA of 10 HSE patients prior to treatment.

Virological diagnosis of HSV-induced encephalitis was performed as previously described (Rozenberg and Lebon, 1991) in patients whose CSF samples were referred to our laboratory to investigate an acute encephalitis. Briefly, 100–200 μl of CSF were analyzed for the presence of HSV DNA by PCR, using primers that allow the detection of a region of the DNA polymerase gene highly conserved among herpesviruses. Characterization of the amplified product was then achieved by restriction enzyme analysis. Cerebrospinal fluids containing detectable HSV DNA were stored at −20°C until further analysis. Ten CSFs from 10 different HSE patients obtained at the beginning of their neurological symptoms (i.e. prior to any specific anti-viral treatment) were further selected for gD sequence analysis. These patients were seven adults and three children (8 months, 7 and 14 years, respectively), originating from different regions of France. Clinical features of the disease in these 10 patients are summarized on Table 1. For amplification of gD, 200 μl of CSF were used. The total DNA extracted from these samples was used for PCR. A 1092 base pairs fragment of HSV-1 gD gene, from nucleotide or nt 268 to nt 1360 of the published nucleotide sequence (Watson *et al*, 1982), was amplified using two 20-base oligonucleotide primers, gD1 (5′-GCCGTGATTTTGTTTGTCGT-3′) and gD2 (5′-GCTTTGGGGCTTTCCGAGTG-3′), thus bracketing previously described antigenic sites (Eisenberg *et al*, 1994): continuous epitopes VII (nt 343–372), II (nt 1105–1152), XI (nt 1164–1218), and functional sites I (nt 394–444), II (nt 617–723), III (nt 979–1066) and IV (nt 1144–1245) (Figure 1). 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 (DMSO), 200 μM of each dNTP, 10 pmol of each oligonucleotide primer and 2.5 units of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT). 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 30 s of annealing at 56°C, and 2 min of elongation at 72°C, was repeated 40 times. The presence of the amplified product was verified by UV transillumination of ethidium-bromide agarose gels. A second round of PCR was performed, in which single-stranded DNA suitable for sequencing was generated by means of PCR

Table 1 Clinical presentation of the 10 HSE patients whose CSF was analyzed for HSV-1 gD sequence

				CSF			*Treatment***		
No	*Sex/Age*	*Geographic origin**	*Neurological symptoms*	*Cells†*	*Protein††*	*αIFN‡*	*Dosage*	*Duration in days*	*Evolution*
1	M/57	SW	F°, aphasia, meningism	130 (80%)	0.8	18	NA	15	recovery
2	M/48	SW	F, aphasia, altered consciousness	116 (100%)	1.67	50	45 mg/kg/d	15	recovery
3	M/46	P	F, confusion	180 (98%)	0.8	2	2 g/d	15	recovery
4	M/16	P	F, altered consciousness	650 (85%)	2.9	6	1.5 g/d	15	recovery
6	M/50	S	F, confusion	NA	NA	50	NA	NA	NA
7	M/50	P	F, seizures	NA	NA	200	NA	NA	NA
10	M/53	W	F, aphasia, seizure	242 (100%)	0.6	100	3 g/d	15	recovery
Children									
5	M/5	P	F, focal seizures	300 (75%)	0.46	400	45 mg/kg/d	15	recovery
8	F/14	P	F, chronic meningitis	1470 (90%)	0.78	18	30 mg/kg/d	2 (delayed)	deceased
9	M/8months	P	F, focal seizures	103 (96%)	0.97	50	45 mg/kg/d	21	recovery

*P: Paris or area; SW: southwest of France; W: West of France; S: South of France
**treatment consisted in intravenous aciclovir
† number of cells per mm^3 (% of lymphocytes)
†† protein concentration in g/l
‡ α-interferon level expressed in international units per ml
F°: Fever

Table 2 Summary of mutations identified in the different glycoprotein D regions from HSE patients CSF samples

	Continuous epitopes[a]			*Functional regions*[a]				
	VII aa[e] 1–19	*II* aa 264–279	*XI* aa 284–301	*I* aa 27–43	*II* aa 125–161	*III* aa 227–246	*IV** aa 277–310	*Other domains*
No cases[b]	1,4,7,8,9	1,2,3,4,5	1,2,3,4,5	1,4,6,7,8,9,10	all ten	1,2,3,4,5	1,2,3,5	all ten
Silent mutations[c]	C342→G_1 C372→$T_{1,8,9}$	G1146→T_2	A1188→$G_{1,2,5}$ C1205→A_4 G1209→T_2	A447→G_2	C717→$T_{2,8,9}$		C1146→T_2 A1188→$G_{1,2,5}$ C1205→A_4 G1209→T_5	G384→A_8 C474→A_9 C591→A_2 C597→T_5 T609→C_2 T756→$C_{2,8,9}$ G771→C_2 C777→T_5 G912→A_2 A915→G_2 C924→G_6 C973→$T_{1,3}$ A666→$C_{8,9}$
Missense mutations[d]	G1120→A_2 **A269T**							**E117D**

* Note the overlap between continuous epitope XI and functional region IV, which are described separately
[a] The antigenic sites and functional regions are defined by their residue number as previously described by Chiang *et al* (1994)
[b] The case number of each CSF sample analyzed corresponds to that indicated in Figure 1
[c] Mutations are defined by the universal code letter and nucleotide number of the reference sequence followed by the mutated nucleotide, and in index, the case number of the CSF sample
[d] Missense mutations are designated in bold at the protein level by the wild-type residue (single letter amino acid code) followed by its sequence number and the mutant residue
[e] aa: amino acid

using unequal amounts of the two primers (50 pmol/1 pmol) as previously described (Gyllenstein and Ehrlich, 1988). Then, the total amplification reaction mixture was mixed with 2 ml of distilled water, applied to a Centricon 100 microconcentrator (Amicon), and spun at 3000 × g to remove excess dNTPs and buffer components. Approximately 7 μl of the final retenate was used for sequencing with the dideoxy-nucleotide chain termination method (DNA sequencing kit, United States Biochemical). Sense primer gD1 and seven antisense primers gD4: 5′-TCCAACACGGCGTAGTAAAC-3′, gD6; 5′-GCGTACTTACAGGAGCCCTT-3′, gD8: 5′-GGACCCCCGGAGGGTCGGTC-3′, gD10: 5′-ATGACCGTGATGGGGATAGC-3′, gD12: 5′-GGCGTGCATCAGGAACCCCAG-3′, gD14: 5′-GCCACCCGGCGATCTTCAAGC-3′, gD16: 5′-CAGTTGGTGG GATTGCGGC-3′, were used for sequencing. As shown in Table 2, the sequence corresponding to epitope VII, was analyzed in five patients (CSFs no 1, 4, 7, 8, 9), and those corresponding to continuous epitopes II and XI were analyzed in five other patients (CSF no 1, 2, 3, 4, 5). In addition, the sequences corresponding to functional region I were analyzed in seven cases (CSFs no 1, 4, 6, 7, 8, 9, 10), to functional region II in all 10 cases, to functional region III in five cases (CSFs no 1, 2, 3, 4, 5), and to functional region IV in four cases (CSFs no 1, 2, 3, 5). Last, the region known to contain one experimental neuroinvasiveness associated mutation, A84G (Izumi and Stevens, 1990) was analyzed in nine cases (all except case 10). The nucleotide changes with reference to the strain HSV F are shown in Table 2. Changes were found at 22 positions, 20 of which being conservative. However, two changes were found to lead to amino acid substitutions: mutation GAA→GAC (E117D) was found in cases 8 and 9, and mutation GCC→ACC (A269T) was found in case 2 in a viral strain that differed from the reference strain by 11 silent mutations over 862 bp analyzed.

To ascertain the specificity of these results, we then selected 20 peripheral HSV-1 strains obtained from various clinical lesions in different patients, isolated in our laboratory by means of routine culture procedures on Vero cell lines, and identified by monoclonal antibodies (Syva-Mérieux, Paris, France). We focused on the regions surrounding the two missence mutations found in HSE samples. As seen in Table 3, among 12 strains sequenced between nt 630 and 820 of gD, five silent mutations were found, clustered in four strains. Moreover, four out of these five silent mutations were identical to those found in HSE strains. In two out of these four strains, the missence mutation A666→C leading to substitution E117D was also present. In contrast, among eight strains sequenced between nt 850 and 1170 of gD, only four silent mutations were found, clustered in four strains, while four other strains displayed a completely conserved sequence with regard to the F reference strain. Moreover, three out of these five silent mutations were identical to those observed in HSE strains. As detailed in Table 3, no mutation was specific for a particular site of infection.

Although much work has been accomplished on animal models of HSV CNS infection, the pathogenetic mechanisms leading to HSE in humans are still not elucidated. In particular, there is little information on HSV strains responsible for HSE in humans. While the virus must be present in CSF, isolation by culture is almost always impossible, thereby hampering the analysis of viral CNS strains unless a brain biopsy is performed. The recent use of PCR has allowed not only to detect and characterize viral DNA in this biological compartment, but also to gain access to genetic analysis. The aim of this work was to investigate the hypothesis that viral variants might contribute to the specific neurovirulence of some HSV strains. Although in a previous work, restriction enzyme analysis of a few HSV isolates obtained concomitantly from the brain and from oral or labial sites in HSE patients revealed that patients may be infected by the same as well as by two different viruses (Whitley *et al*, 1982), to our knowledge, further detailed molecular analyses have never been performed.

Whereas HSE occurs commonly in immunocompetent patients, it has been shown that the local immune reaction to HSV primary infection plays a central role in the control of neuroinvasion, in as much as in B-cell suppressed mice, passively administered HSV specific neutralizing antibodies mediate protection by decreasing spread of virus to

Table 3 Summary of mutations identified in the 2 glycoprotein D regions from peripheral HSV-1 strains

	*Region A**	*Region B***
No of strain and site of infection	oropharynx: 1,2,3,4 respiratory tract: 5,6,7,8,9 skin: 10,11 genital tract: 12	oropharynx: 13,14,15 eye: 16 skin: 17,18,19 genital tract: 20
Missense mutations	A666→$c_{2,8}$**(E117D)**	
Silent mutations	C717→T_2 T729→C_7 T756→C_2 G771→C_5 C777→T_6	A915→$C_{13,16}$ C924→$G_{17,18}$ C1146→$T_{13,16}$ G1170→C_{17}

* Region A surrounds the missense mutation E117D found in 2 HSE samples: from nucleotide 620 to 820.
** Region B surrounds the missense mutation A269T found in 1 HSE sample: from nucleotide 850 to 1170
legend as in Table 2.

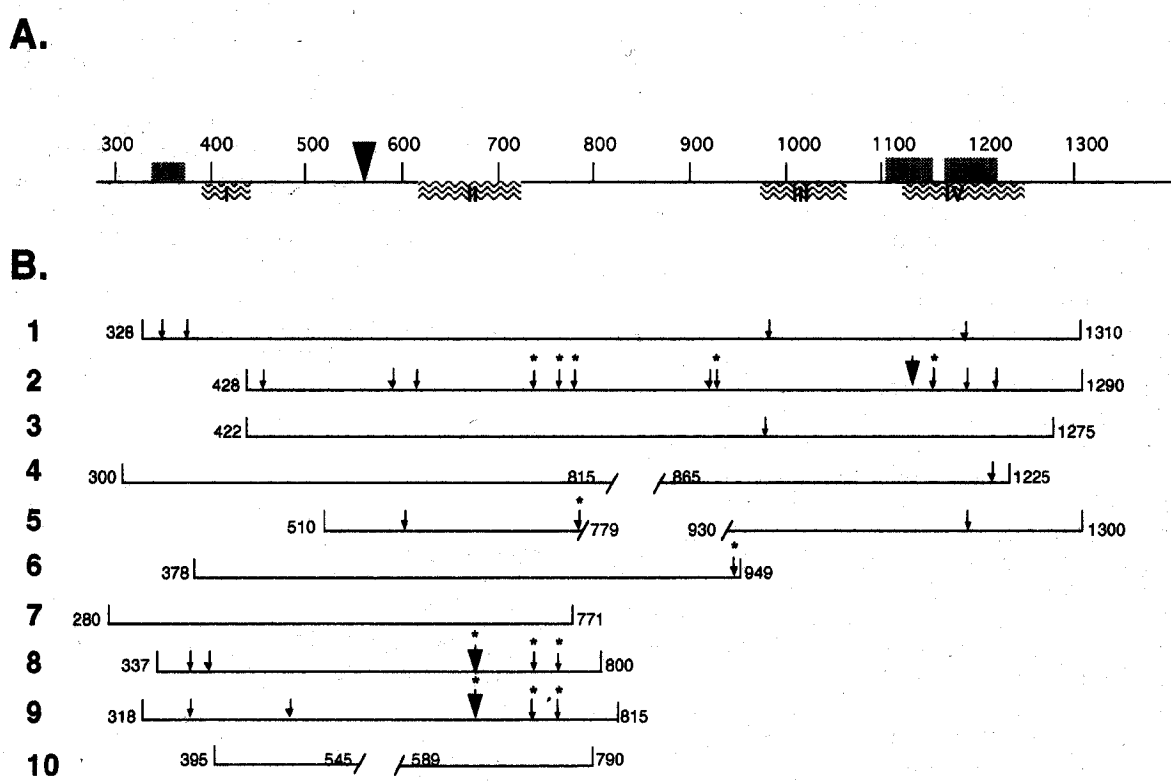

Figure 1 HSV-1 glycoprotein D nucleotide sequence analysis in 10 PCR products obtained from HSE patients' CSF samples. **(A)** Diagrammatic representation of the main functionally characterized domains of HSV-1 glycoprotein D gene: continuous epitopes ▒ and functional regions ▒ are located as described by Chiang *et al* (1994). The bold arrow defines the mutation previously described in an experimental neurovirulent strain (Izumi and Stevens, 1990). **(B)** Representation of the nucleotide sequences analyzed in the 10 encephalitis patients' CSF samples. Small arrows locate silent mutations, bold arrows define the missense mutations. Asterisks mark the mutations also found in the control peripheral HSV-1 strains (cf Table 3.)

and within the nervous system (Mester *et al*, 1991). It is also of note that HSE occurs frequently in HSV seroconverted patients, suggesting that encephalitis may be caused either by reactivation of a latent infection or by reinfection with an exogenous strain (Whitley, 1990). We focused our interest on glycoprotein D because of two complementary reasons. First, gD is a major target of the immune humoral response, and this property has even made it a candidate for a sub-unit vaccine (Mishkin *et al*, 1991). Glycoprotein D antigenic domains have been predicted by computer analysis (Becker, 1991) and precisely defined by reaction with monoclonal antibodies (Eisenberg *et al*, 1985; Isola *et al*, 1989). Recently a model of the folded structure of the protein has been proposed (Long *et al*, 1992). Since several *in vitro* gD variants produce resistance to neutralization (Minson *et al*, 1986), one can hypothesize that similar *in vivo* naturally occurring mutations could make the virus able to escape from the immune humoral response and account for increased virulence. Such a mechanism has been described in another model of viral infection of neurons (Griffin *et al*, 1994). This hypothesis should be kept in mind if a HSV sub-unit gD vaccine was to be administered for protection against HSV. Second, gD has been shown to play an essential role in viral penetration into susceptible cells, and in the intercellular spread of the virus *in vitro*. (Brandimarti *et al*, 1994; Burioni *et al*, 1994; Campadelli-Fiume *et al*, 1990; Dean *et al*, 1994; Johnson and Ligas, 1988; Muggeridge *et al*, 1990). It has been concluded that gD structure could be a determinant of cell tropism. In the CNS, HSV migrates by transneuronal transfer, establishing anatomical pathways along synaptically connected neurons (Ugolini *et al*, 1989). However, the specificity and the directions of this transfer seem to differ among viral strains (Zemanick *et al*, 1991). The same observations have already been made in other models of neurotropic virus infections (Barnett *et al*, 1993; Babic *et al*, 1993; Card *et al*, 1991; Lafay *et al*, 1991). It has been thus hypothesized that during reactivation, the existence of mutations in viral structural proteins might allow the virus to be preferentially sorted to the CNS instead of the periphery (Roizman and Kaplan, 1992). Last, a single mutation (A84G) in gD has been described in a viral strain modifying the phenotype of infection and causing lethal neurologic disease in mice (Izumi and Stevens, 1989).

Therefore, in this study, starting from CSF obtained in human HSE patients, we analyzed 10 HSV gD PCR fragments and performed direct sequencing. We chose this procedure instead of cloning PCR products, because one significant advantage using this technique is that erroneous PCR products generated by misincorporation will not interfere with the sequence determination since it is a population of amplified products that is analyzed (Gyllenstein and Erlich, 1988). As shown in Table 2, we analyzed sequences corresponding to continuous epitopes II, VII and XI in five cases. Then, we analyzed functional regions I, II, III and IV in seven, ten, five and four cases, respectively. Last, we analyzed the region surrounding the mutation Ala84→Gly, previously described to be associated with neuroinvasiveness in an experimental model, in nine cases. The first striking feature of this study is the high conservation of HSV-1 gD sequence, a result in agreement with previous reports (reviewed in Minson *et al*, 1986). Among four strains, amino acid differences had been found at seven positions, concentrated in the signal peptide, transmembrane region, and carboxyterminal region; only three more positions had been found, at which nucleotide changes were silent. Interestingly, even silent nucleotide changes seem to be much less frequent in gD gene compared to thymidine kinase gene. Our results show that this conservation is equally present in human HSE strains. First, we always amplified a PCR product of expected size, ruling out a gross variation of this sequence due to major deletion or rearrangement. Second, in 10 cases, obtained in patients from various geographic origins in France, analysing a total of 6580 bp, we found a low rate of silent mutations. However, the presence of these mutations allowed us to discriminate the strains studied, a result demonstrating the heterogeneity of strains responsible of HSE in humans (Hammer *et al*, 1980). We also found two mutations conferring amino acid substitutions. The first one, the replacement of the glutamate residue 117 by an aspartate, lies near a cysteine known to be essential for the folding of the protein (Long *et al*, 1992). The second one, the alanine to threonine substitution at nucleotide position 1120 (Figure 2), was found in a strain that exhibited 11 silent mutations among 862 bp analyzed. Furthermore, this missense mutation lies within continuous epitope II, and very

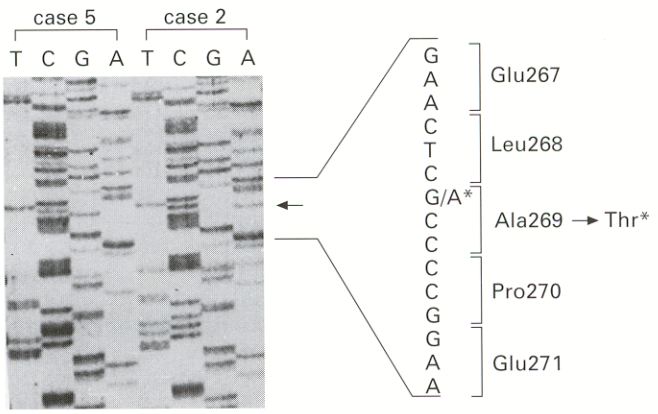

Figure 2 Direct sequencing of HSV-1 gD PCR products obtained from CSF in HSE patients 2 and 5.

close to an essential functional region. However, despite the heterogeneity of patients due to age, geographical diversity, and variations in treatment dosage or duration, there was no correlation between one of the mutations observed and the clinical presentation, severity of evolution of the disease. To further attest to the relevance of these results, we looked for mutations in peripheral strains, by focusing on the regions surrounding these two missense mutations. The mutation E117D was found in two strains out of 12 analyzed. In contrast, mutation A269T was not found in eight strains analyzed. Moreover, it is of interest to notice that in these 20 control strains, 10 mutations were found among which eight had already been characterized in HSE strains. As depicted in Table 3, no relationship between the site of infection and a specific sequence could be shown.

This is the first study to our knowledge aimed to analyze HSV sequences obtained from CSF in HSE patients. It involves 10 clinical samples, representative of specifically 'neurovirulent' strains in humans, compared to 20 peripheral HSV-1 strains. Overall, our results further underline the conservation of gD sequence in neurovirulent as well as in peripheral strains, and would argue against the role of gD in neurovirulence. However, in a single case, we found a surprisingly higher rate of silent mutations, together with a missense mutation (Ala629→Thr) lying in an antigenic site, which was not detected among the control strains. *In vitro* studies are needed to test the functional relevance of this latter substitution. Interestingly, the mutation known to be associated with an increase in neuroinvasiveness (Izumi, 1991) was not present in our CSF samples, thereby suggesting that the relevance of animal models to human pathology has to be carefully evaluated. Nevertheless, whereas the mechanisms underlying neurovirulence are probably heterogeneous and multifactorial, involving host as well as viral factors, it is of interest to test for the clinical relevance of experimental results. Further similar studies are currently underway, aimed to analyze the genes of HSV previously shown to be involved in animal pathogenesis (Yuhasz and Stevens, 1993), and that could be relevant to human pathology.

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