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Short Communication

Variations of HSV-1 glycoprotein B in human herpes simplex encephalitis

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The factors which cause herpes simplex encephalitis (HSE) to occur among herpes simplex virus type 1 (HSV-1) infected humans are not understood. In experimental models, HSV-1 neuroinvasiveness is influenced by amino acid changes in HSV glycoproteins D (gD) or B (gB), which are essential to the virus infectivity and to the induction of host immune responses. To test the possible involvement of these glycoproteins in human HSE, we compared CSF-derived sequences of these genes with those obtained from peripheral HSV-1 isolates. We have previously shown the conservation of gD in 10 HSE samples. Here, we show that the functional domains of gB involved in cell penetration and cell fusion, and the major antigenic domains D2a, D2b and Dd5a were highly conserved. In the gB amino-terminal domain, we distinguished several alleles that were common to HSE and peripheral isolates, and identified in only three out of fifteen HSE cases, a variation that was not encountered in 20 control strains. Overall, there were no striking differences between peripheral and HSE gBs. These results suggest that gB alone may not be responsible for neuroinvasiveness nor human neuropathogenicity.

Keywords: HSV-1; human herpes simplex encephalitis; glycoprotein B; variations

Among the large number of individuals infected with herpes simplex virus type 1 (HSV-1), 1/250 000/year develop herpes simplex encephalitis (HSE), a severe disease that leads to death if left untreated. The reasons that HSE rarely occurs despite the endemic spread of HSV-1 infections, remain undefined. HSE appears sporadically in adults, children and infants who do not have any obvious immunological deficiency. Moreover, it may occur either as the consequence of primary infection, or in patients previously exposed to the virus, as shown by a history of recurrent cold sores or the presence of serum specific antibodies prior to the onset of HSE. The search for factors responsible for HSE in humans has proven puzzling in view of the multigenic nature of HSV-1 virulence. Among the hypotheses that have been advanced to explain increased virulence to the central nervous system (CNS), we have focused our attention on those which involve viral neuroinvasion. In one of these hypotheses, infection of the brain is seen as the consequence of rare mutations that change the ability of the virus to spread to, infect and replicate in relevant neural cells (Roizman and Kaplan, 1992; Yuhasz and Stevens, 1993). Precedents for these hypotheses exist in the case of unrelated infectious viruses, such as rabies. In fact, replacement of a single amino acid residue in the envelope glycoprotein of rabies virus is critical for neurovirulence of the mutant virus, perhaps by modifying its tropism to specific nerve endings (Tuffereau *et al*, 1989). The second hypothesis proposes that precise mutations in HSV-1 surface components could cause escape of the virus from the host response towards infection, rendering it more infectious to the CNS. Indeed, in the mouse system, a single amino acid change in glycoproteins D (gD) or B (gB), two major membrane glycoproteins of HSV-1, is responsible for the non-neuroinvasiveness of strains ANG and KOS, respectively (Izumi and Stevens, 1990; Yuhasz and Stevens, 1993). This limitation in neuroinvasiveness is related to an increased immune response of the host (Mitchell and Stevens, 1996).

Since both hypotheses postulate amino acid substitutions in surface glycoproteins of HSV-1 as the cause for neuroinvasiveness, we thought of obtaining evidence for such substitutions by directly comparing glycoprotein sequences of viral

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isolates from HSE patients to corresponding sequences of clinical peripheral isolates. However, as virus isolation from HSE patients is most often impossible due to the necessity for brain biopsies (Whitley, 1996), we resorted to sequencing viral DNA extracted from small specimens of CSF, employing PCR technology. In a previous study we applied this approach to the sequence comparison of glycoprotein D, which is of crucial importance in the penetration of the virus into host cells. Furthermore, it is also a potent inducer of humoral and cellular immune responses in the infected individual. We showed that gD sequences were conserved in 9 out of 10 cases, and identified a single amino acid substitution specific to a unique HSE sample (Rozenberg & Lebon, 1996). We thus decided to extend our investigation to glycoprotein B (gB), which also plays a major role in viral infectivity, and is highly immunogenic to the infected host (Pereira, 1994). In addition, gB has been shown to be involved in neuropathogenicity in animals (Engel *et al*, 1993; Goodman and Engel, 1991; Kostal *et al.*, 1994; Weise *et al.*, 1987; Yuhasz and Stevens, 1993). Here, we compare the results of the analysis of HSV-1 gB sequences derived from the CSF of 20 HSE patients to 23 clinical isolates of peripheral origin.

CSFs obtained from patients with encephalitis of presumed viral origin were referred to our laboratory for virological diagnosis. In all CSFs, alpha-interferon (IFN-alpha) level was measured by a biological assay (Lebon *et al*, 1979). Direct diagnosis of HSE was performed using HSV-1 DNA detection by PCR as previously described (Rozenberg and Lebon, 1991). Samples were stored at -20°C until further analysis. When serum was available, the detection of anti-HSV antibodies and specific IgM was performed by ELISA tests (Enzygnost anti HSV/IgG, and Enzygnost anti HSV/IgM, Behring). Twenty CSF samples from HSE patients originating from different regions of France were selected on the basis of sufficient volumes for molecular analysis. They were 17 adults (37–86 years old) and three infants (3–7 months old).

Twenty-three HSV-1 strains were obtained from the oropharynx or from broncho-alveolar lavages of nine adults (16–74 years old) and 14 children (6 days to 16 years old). Viruses were isolated by culture on Vero cell lines, and identified by type-specific monoclonal antibodies (Imagen™ Herpes simplex virus type 1 and 2, Dako). The HSV-1 reference strain F was used as a control.

HSV DNA was recovered from the CSF samples and cell cultures as previously described (Rozenberg and Lebon, 1991). In order to avoid contamination, precautions were taken such as the use of positive displacement pipettes, and separate rooms for pre and post-PCR work. In particular, the CSF samples were extracted in series of less than 4 or 5, and multiple negative controls (distilled water) were included in all experiments. The total DNA obtained from 100–200 μl of CSF was used for each PCR assay. Oligonucleotide primers were designed for amplification of the gB gene (Table 1). Reaction mixtures contained 10 mM Tris-HCl pH 8.9 or 8.3 depending on the primer sets, KCl 50 mM, $MgCl_2$ 1.5 mM, glycerol 5%, 200 μM of each dNTP (PCR Nucleotide Mix™, Boehringer Mannheim), 20 pmol of each primer, and 1.5 U Taq polymerase (Boehringer Mannheim), in a 50 μl total volume. Amplification was performed in an automated thermal cycler (Perkins-Elmer Cetus) After a first step of denaturation at 94°C for 2 min, 40 cycles consisted in 1 min of denaturation at 94°C, 1 min of annealing at 64 or 66°C according to the primers Tm, 1 min of elongation at 72°C, and were followed by a 10 min final elongation at 72°C. For the longest amplified product (1230 bp), the annealing duration was 2 min and the elongation was prolonged to 3 min+15 s incrementation per cycle. After UV transillumination of ethidium-bromide stained agarose gels, amplified products were purified using either microcolumns (QIA Quick PCR Purification kit™, QIAGEN), or by adsorption on silica particles after excision of the expected band in agarose gel (QIAex™, QIAGEN). The amount and purity of the amplified DNA recovered for sequencing was checked on ethidium-bromide agarose gel. Purified PCR products were sequenced directly, using the same primers than for amplification. Reactions were performed with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit™ (Applied Biosystems), and AmpliTaq® polymerase, FS (Perkin-Elmer), as described by the manufacturer, in an automated thermal cycler (Perkin-Elmer Cetus 9600): 25 cycles consisted in 30 s of denaturation at 96°C, and 4 min of annealing and elongation at 65°C. Fluorescent products were purified on Quick Spin™ columns (Boehringer Mannheim) before loading on a 6% polyacrylamide gel. Sequence analysis were performed on an Applied Biosystem 373A sequencing automate. All sequences data

Table 1 Oligonucleotide primers selected for PCR and sequencing of HSV-1 gB gene.

Primers	*Orientation*	*Sequence (5′→3′)*	*Localization*[a]
gB1	sense	CCGATTCTCCTCCGACGCCA	1149–1168
gB2	anti-sense	TACCGAAAGCTGACCAGGGG	1795–1814
gB3	sense	ACGCCAACGCCGCCATGTTC	2174–2193
gB4	anti-sense	GAGCAGCGCGCTCGTGCCC	2595–2613
gB5	sense	TTGGGGTTGACGCTGGGGGT	52–71
gB6	anti-sense	CCTGCGGGCGAAGATGCGGT	1262–1281
gB7	sense	CACCGGGACGACCACGAGAC	667–686
gB8	anti-sense	TTGGGGGTCGTGAGCAGGTTC	1023–1043
gB9	sense	GGTTCGTCGTATGGGCGCTCT	32–52
gB10	anti-sense	CTTGATGTCCCGCAGGTGCTC	292–312
gB11	sense	GGTCGCCGCGGACAACGTGA	1722–1741
gB12	anti-sense	TGAAGGAGGACACGCCCGACA	2288–2308

[a]Nuclotide positions refer to strain F gB coding sequence (GenBank M14164).

were then verified by one or two investigators. The alignment of the amino acid sequences obtained was performed using the Clustal W multiple sequence alignment program (Higgins D, EMBL, Germany).

To test the possible involvement of HSV glycoproteins in human neuropathogenicity, we sought for unique amino acid substitutions in HSV-1 gB sequences from patients diagnosed with HSE, that would be absent in strains from patients with non neurological infections. To this aim, we compared sequences derived from the DNA of HSE patients' CSF samples to viral DNA sequences of HSV-1 clinical isolates obtained from peripheral infections. We also compared sequences from both sources to all available published sequences, i.e. laboratory strains F (GenBank M14164), Patton (K03541), 17 McGeoch (D10879), KOS (K01760), tsB5 (K02720), ANG (S65444) and ANGpath (U49121).

A total of 20 HSE patients were studied. All patients had an intra-thecal synthesis of IFN-alpha, indicative of CNS viral infection (Lebon *et al*, 1979). Diagnosis of HSE was assessed by PCR for HSV-1 DNA polymerase gene (Rozenberg and Lebon, 1991) which was positive in the first CSF sample drawn from all the patients. The presence of HSV-1 specific IgG was observed in nine out of the nine adult patients and in all three children whose serum had been drawn concomitantly with the CSF.

In order to analyse the HSV-1 gB gene, we used primers that would amplify short overlapping fragments corresponding to previously described antigenic sites and functional domains of the glycoprotein. The relative positions of these primers and the functional or antigenic significance of the corresponding gB domains are shown in Figures 1A and Figure 2A. As we were limited by the CSF volumes available and the small amounts of DNA recovered, we decided not to investigate the whole gB gene in all samples, but instead to conduct our screening prospectively with respect to the results of the conservation or variability of each fragment. The exact portion of the gB gene that was sequenced

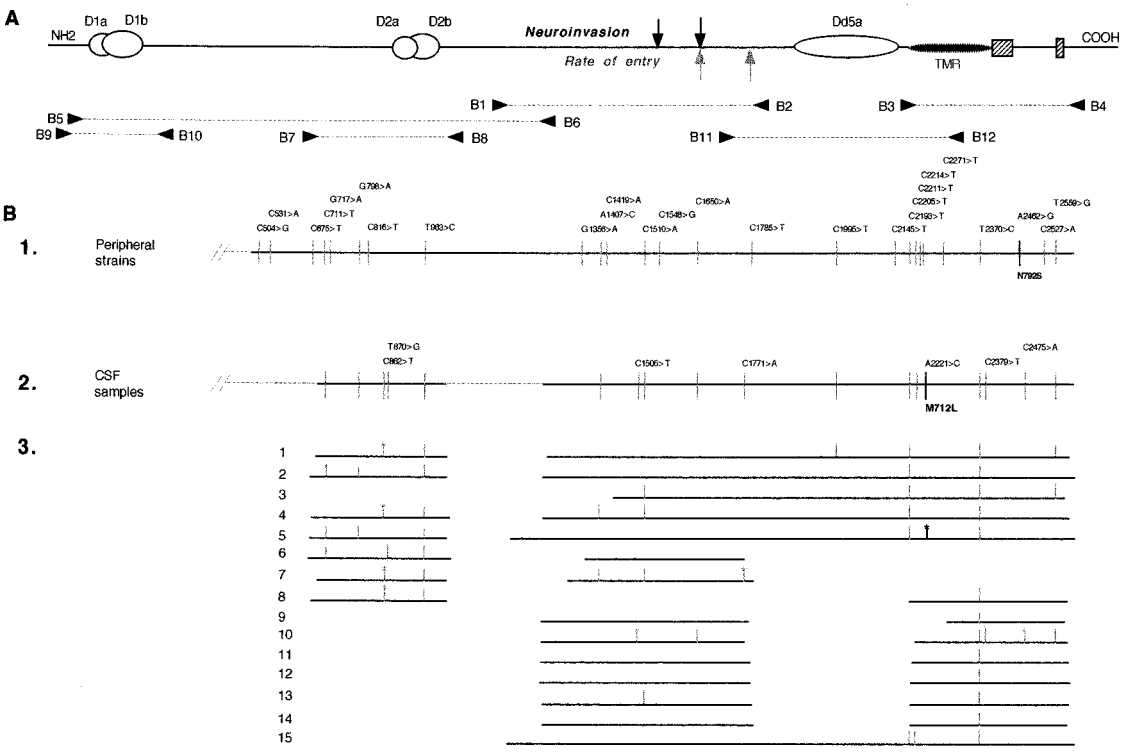

Figure 1 Sequence variations of the gB gene in 15 CSF samples and 15 peripheral strains. (**A**). Linear representation of gB polypeptide depicting as empty ellipses or circles the antigenic domains D1a, D1b, D2a, D2b, and Dd5a, which are the main binding sites recognized by neutralising antibodies. Previously identified amino acid residues involved in cell penetration are pointed by grey arrows, and those associated with neuroinvasion in mice by black arrows. The transmembrane sequence (TMR) is represented as a dark ellipse, and syncytial loci as shaded boxes. Primers used for PCR are indicated by arrows at the ends of broken lines below the representation of the complete polypeptide. (**B**) Linear representation of the nucleotide sequence of the gB gene, corresponding to the portion of the polypeptide schematised above. On line (1) are compiled all nucleotidic changes found in 15 peripheral strains, and on line (2), all those found in 15 CSF samples. Nucleotide substitutions and their position with reference to strain F gB coding sequence (GenBank M14164), are indicated above the horizontal lines, and the corresponding amino acid substitutions are indicated below the lines. Sequence variations are detailed in (3) for each CSF specimen. Light vertical lines define silent nucleotidic variations, and heavy ones locate missense variations. Variations which are specific to CSF samples are indicated with an asterisk.

in each HSE case is depicted in Figures 1 and 2. For each CSF PCR fragment analysed, at least an equal number of peripheral controls was tested. In this study, the strain F gB gene was used as a reference, and all the sequence variations depicted thus refer to this sequence.

Overall, among 23 peripheral strains a total of 26 000 nucleotides was sequenced over a portion of 2500 bp of the gB gene. This was compared to a total of 21 000 nucleotides sequenced in the 20 HSE samples. The variations relative to strain F sequence found in the 23 peripheral strains consisted in 45 nucleotide changes located at 45 positions on a portion of the gene starting approximately 50 nucleotides into the 5′ end of the genome and ending at nucleotide 2580 (Figure 1 B1; Figure 2 B1). Out of these, 18 were identical to those previously described for the published laboratory strains. Out of the 13 observed amino-acid differences, three were uniquely present in the peripheral strains, whereas 10 were also present in the CSF samples. In the HSE derived sequences, a total of 37 nucleotide changes was observed at 35 positions (Figure 1 B2 and B3; Figure 2 B2 and B3). Of these, 23 were silent, thus without consequence on the gB structure. However, 14 others led to 13 amino acid substitutions. Of these nine were also present in the peripheral strains' sequences, whereas four were unique to the CSF samples.

The gB fragment bracketed by primers gB1-gB2 (Figure 1) encompasses a region of the protein essential for the rate of HSV entry into cells, as judged from mutations that alter viral entry *in vitro*, such as single amino acid substitutions at position 564 or 523 (Bzik *et al*, 1984; Highlander *et al*, 1989). Recombinant virus studies have further emphasised the role of residues 523 and 485 in neuroinvasion *in vivo* Kostal *et al*, 1994; Yuhasz and Stevens, 1993). However, the analysis of 14 CSF samples and 15 peripheral strains derived gB1-gB2 PCR products revealed the high conservation of this domain.

We then analysed the carboxy-terminal region involved in fusion as determined by *in vitro* studies (Cai *et al*, 1988). Genetic mapping has indicated that single mutations resulting in the syncytial phenotype were located in two cytoplasmic regions: region I is proximal to the TMR and spans residues 766 to 786–787 (Gage *et al*, 1993), region II is more central in the cytoplasmic domain and includes residues 824, 825, and 828 (Bzik *et al*, 1984; Cai *et al*, 1988; Gage *et al*, 1993; Walev *et al*, 1994). In addition, amino acid 821 influences the rate of virion entry into cells (Gage *et al*, 1993). *In vivo*, the study of recombinant viruses has shown that amino

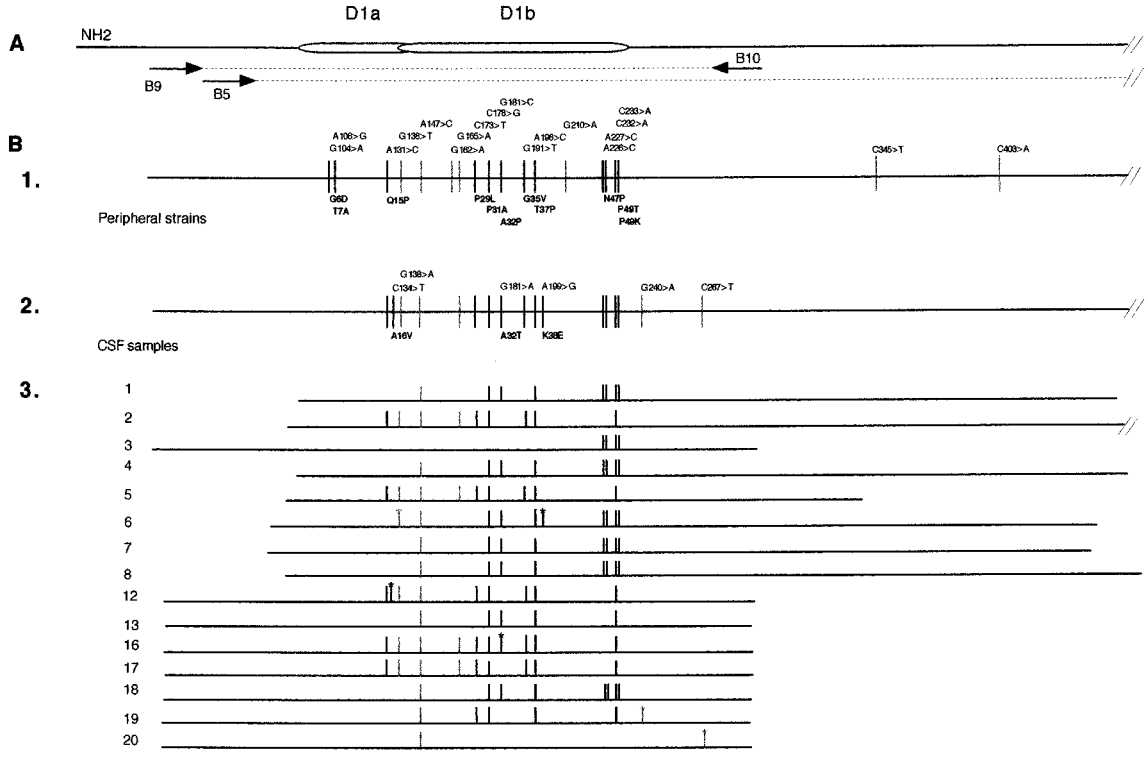

Figure 2 Sequence variations of the 5′ portion of the gB gene in 15 CSF samples and 20 peripheral strains. (**A**) Same as in Figure 1(A): the amino-terminal domain of the gB polypeptide has been magnified, and antigenic domains D1a and D1b appear as extended ellipses. (**B**) Same as in Figure 1(B): all the nucleotidic variations found in 20 peripheral strains and in 15 CSF samples are summarised in (1) and (2) respectively, and variations are detailed for each CSF specimen in (3).

acid residues 825 and 787 are crucial to neuropathogenicity upon peripheral inoculation, without affecting viral growth (Engel *et al*, 1993). This domain was analysed by amplifying the gB fragment gB3-gB4, and was highly conserved in the 13 CSF samples studied. In one out of 15 peripheral samples, we observed a single nucleotide substitution leading to the replacement of Asn 792 by Ser, both neutral and polar amino acids. However, no unusual cytopathogenic effect of the related viral strain was observed in cell culture.

Finally, the gene fragment coding for the transmembrane region (TMR) was entirely analysed in six samples of each panel (HSE and peripheral), and partly sequenced in seven more CSF samples and nine controls (Figure 1), using two overlapping primer pairs gB3-gB4, and gB11-gB12. In one CSF (no 5), we found a single substitution (A2221→C) which led to the replacement of methionine 712 by a leucine residue, both neutral and hydrophobic amino-acids.

We then studied gB functional antigenic domains (Figure 1A and 2A). Glycoprotein B is a major target of both humoral and cellular immune responses. HSV-1 gB antigenic domains have been mapped precisely (Kousoulas *et al*, 1988; Navarro *et al*, 1992; Pellett *et al*, 1985; Pereira *et al*, 1989; Qadri *et al*, 1991), and continuous and discontinuous epitopes have been defined (Chapsa and Pereira, 1988). The specific epitopes involved in virus neutralisation and antibody dependent cellular cytotoxicity (ADCC), which are two mechanisms that affect the outcome of HSV infection *in vivo* in animals, have been delineated in mice and more recently in humans (Sanchez-Pescador *et al*, 1992, 1993). Almost all neutralising activity is accounted for by epitopes in the 298 amino acids N-terminal domain of the molecule. This includes the antigenic domains D1, D2a and D2b, with one exception in antigenic domain Dd5a, which overlaps a region of gB required for oligomerization (Laquerre *et al*, 1996). The sequence of antigenic domain Dd5a was analyzed using primers gB11-gB12, and was highly conserved in 6 CSF samples and 6 peripheral strains. The region surrounding the gB major antigenic domains D2a and D2b was then studied in 7 CSF specimens and seven peripheral isolates using primers gB7-gB8. All the mutations observed in this region were silent. Lastly, we studied the amino-terminal domain containing the continuous epitopes D1a and D1b, with primer sets gB5-gB6 and gB9-gB10. As this was the only region where significant variations in the amino acid sequence of gB could be detected, we extended the analysis of this domain to 15 CSF samples and compared them to 20 peripheral HSV samples. The resulting amino acid sequences were compared to those of reported laboratory strains. The amino-terminus of gB contains the residues responsible for type-specific and strain-specific antigenicity (Pereira *et al*, 1989). In this region, we indeed found 14 amino acid changes at 13 nucleotide positions, including substitutions at the key positions 32 and 47 of the protein, previously described as cognitive sites of D1b epitope (Kousoulas *et al*, 1988). The presence of amino acid changes that were common to the two panels of samples (CNS and peripheral) allowed us to distinguish several alleles of gB (Figure 3). When compared to available published sequences, it appeared that five peripheral strains and one CSF displayed the same amino-terminal sequence as strain F (type I). Three peripheral strains and one CSF-derived sequence were similar to strain Patton's (type III). The pattern IV was present in five CSFs and six peripheral strains, and the pattern V in 1 CSF and 1 peripheral strain. The pattern II was encountered in a single CSF. In six CSFs and five peripheral strains, the amino-terminal domain was comparable to that of ANGpath and strain 17 (pattern VI).

In addition to this variability, a few specimens contained specific changes. Two amino-acid substitutions were found in three out of 20 peripheral strains: these were a G6D change (G104→A) in strains 1 and 23, and a T7A substitution (A106→G) in strain 8. More interesting, three other amino acid substitutions were observed in three out of the 15 CSFs analysed. An Ala to Val change at residue 16 (C134→T) was observed in CSF no 12. A more striking change was observed in CSF no 6. This change (A199→G) results in the substitution of a basic residue (lysine) to an acidic residue (glutamate) at position 38. Finally in CSF no 16, the alanine to threonine substitution at position 32 (G181→A) both replace a hydrophobic residue by a polar one and creates an important modification of the size of the residue.

HSV human neurovirulence and pathogenicity are as yet unsolved issues. Animal models of experimental infection have helped to decipher host and viral parameters that influence pathogenesis. In these models, host factors include age, immunity and genetic background; however, human HSE occurs at all ages, and is seldom related to well-recognized immune defects. On the other hand, viral molecular genetics has identified numerous genes that confer virulence, depending on the route of inoculation and the species investigated (Roizman and Kaplan, 1992). The virus ability to replicate in the brain at high titers and cause disease defines neurovirulence, but replication in the brain is not constantly related to animal pathogenesis (Dix *et al*, 1983). The experimental investigation of neuroinvasion appears relevant to human pathology in as much as the peripheral route of infection and the intra-axonal transfer of the virus through synaptically connected nerve fibers very closely mimics the human mode of infection. In humans, natural access of the virus to the CNS involves necessarily a trans-synaptic step (Davis

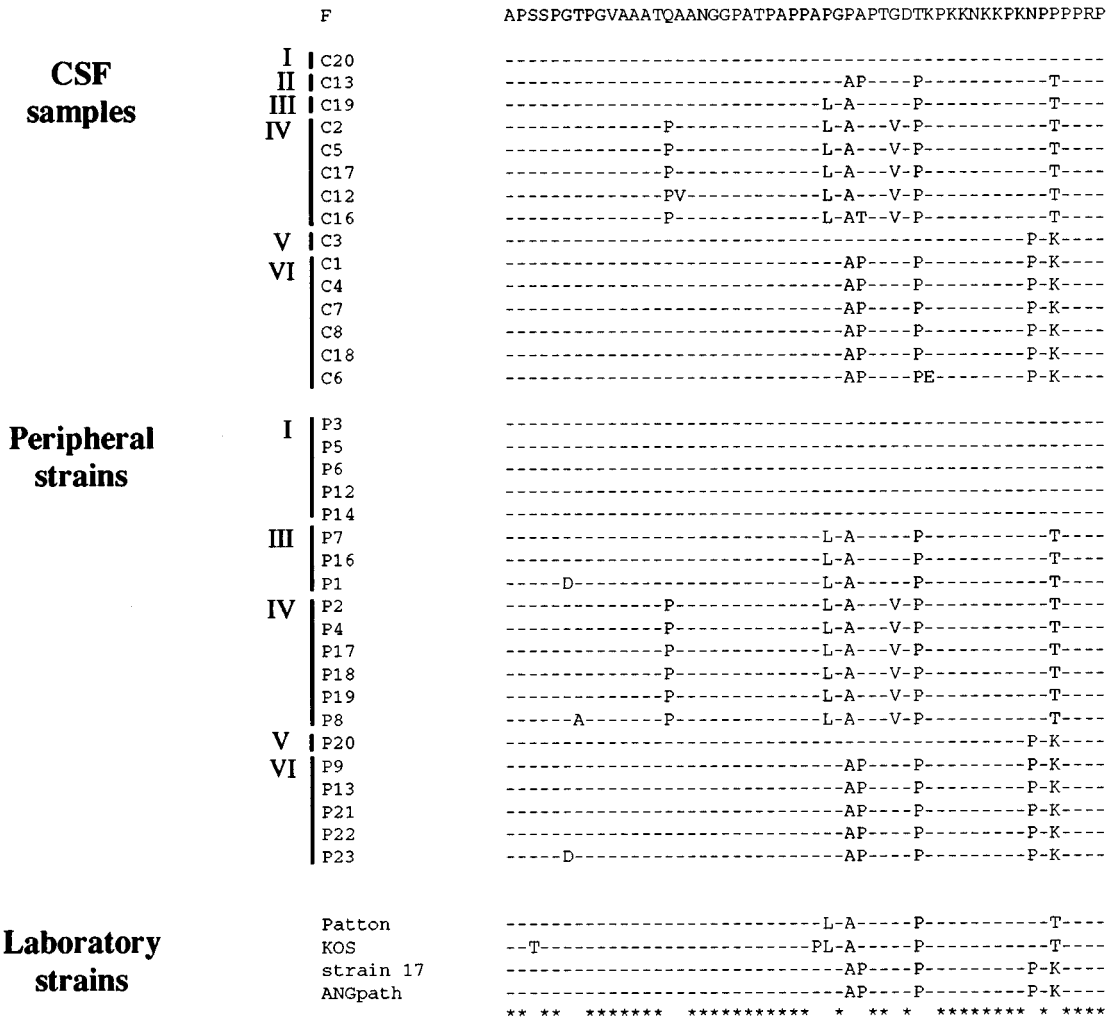

Figure 3 Alignment of the NH2-terminal amino-acid sequences of HSV-1 gB (first 53 residues of mature protein): comparison of viral DNA obtained from 15 CSF, 20 peripheral strains and four laboratory strains [F (M14164), Patton (K03541), KOS (K01760), 17 [McGeoch] (D10879) and ANGpath (U49121)]. Only substituted residues with reference to strain F are shown.

and Johnson, 1979; Esiri, 1982; Ojeda *et al*, 1983), even though the precise source of brain infection is still a subject of debate. Second, when human HSV strains were compared for pathogenicity in mice, encephalitis strains were significantly more invasive after nose inoculation when compared with HSV-1 isolates from patients with oral lesions only (Bergström *et al*, 1990a), supporting a strain-dependent variation in neural spread (Bergström and Lycke, 1990b). Nevertheless, other studies did not find any difference between encephalitis versus peripheral strains (Dix *et al*, 1983; Roizman and Sears, 1996) so that these findings still warrant confirmation. Last, in experimental studies single amino acid changes in HSV-1 gD or gB modify access of the virus to the CNS and pathogenicity by modifying the host immune response (Izumi and Stevens, 1990; Mitchell and Stevens, 1996). In these models, the restriction of passage in the nervous system is manifested at the level of peripheral ganglia. Interestingly, in humans, the regular presence of lymphocytes in sensory ganglia has been interpreted as reflecting the surveillance role of these cells to herpes viruses (Esiri and Reading, 1989).

Whether HSE is caused by increased neuroinvasiveness during primary infection or increased neurovirulence of reactivating viruses still remains unknown. In humans, most HSV strains seem restricted to the peripheral ganglia (Simmons *et al*, 1992), considering the rarity of HSE among HSV seropositive people. On the other hand, the recent detection of limited amounts of the viral genome in the CNS of patients dying of non-neurological

disease (Baringer and Pisani, 1994) led to the conclusion that HSV is able to gain access to the brain without leading to overt infection, and that HSE could be due to local reactivation. We hypothesised that in either case, subtle changes in HSV-1 might modify the virus interaction with the host, rendering it more neuroinvasive. We therefore decided to compare viral DNA retrieved from the CSF of HSE patients with viral DNA from cutaneo-mucous isolates. As HSE due to HSV-1 mostly occurs in adult patients, and peripheral HSV-1 lesions are more often investigated for diagnosis in children, we compared 17 adults and three infants in the HSE group, *versus* nine adults and 14 children in the control group.

It would certainly have been interesting to compare neurovirulent strains with peripheral strains obtained in the same patients. However, 1/3 of HSE cases are caused by HSV primary infection (Whitley, 1996). A previous molecular study based on RFLP analysis has shown that in these cases the same virus was present at the two sites of infection (Whitley *et al*, 1982), although this technique does not provide precise sequence information. Furthermore, even in the case of a secondary reinfection/reactivation, such a comparison would not necessarily be adequate since we assume that a mutated virus able to gain access to the CNS would nevertheless be able to cause infection at the peripheral level. Finally, only 15% of HSE patients have a peripheral lesion at the time of brain infection (Whitley, 1996). Obviously, the number of HSE patients necessary to perform such a comparison analysis would be far more than one hundred. In this study, we compared strains obtained from 20 HSE patients with those from 23 controls. Our aim was to look for the existence of variants of gB in both groups, a study that had never been performed before. To this aim, we amplified the viral DNA directly obtained from the patients' CSF, and from control isolates cultured on Vero cells. We resorted to the analysis of CSF derived viral DNA, because brain biopsies are no more performed since the wide use of diagnostic PCR and the availability of antiviral drugs. On the other hand, the peripheral control strains were cultivated on Vero cells before viral DNA was purified. We assume that one or two (at most) passages of HSV strains on these cells would probably not be sufficient to select mutants. Even if this had occurred, we would have amplified a mixed population of HSV strains. However, the use of direct sequencing of PCR products allows to detect such an event. On the contrary, we obtained in all cases pure PCR-derived sequences, from homogenous viral populations. Last, the fact that different alleles of gB were observed among the different controls further argues against the hypothesis of a selection/modification of gB on Vero Cells.

We examined 2500 bp of the gB gene out of the 2700 bp of the gB gene coding sequence. In addition, we looked at all the gB domains previously identified as involved in neuropathogenicity in animal models. The risk of sequence analysis errors introduced by the process of PCR is negligable when using the direct sequencing technique (Gyllensten and Erlich, 1988). However, to further attest to the validity of this technique, the gB gene from reference strain F DNA was amplified as a control and its sequence revealed concordant with the published one over a total of 2376 bp of the gB gene, except for a unique silent nucleotide variation (nucleotide 1929 T→C) which is present in most other HSV-1 gB published sequences.

The weak overall percentage of mutations observed is in agreement with previous studies (Rojas *et al*, 1993; Sakaoka *et al*, 1994), and underlines the high conservation of the glycoprotein, probably linked to its essential role in the viral replication cycle. In particular, the regions involved in the rate of entry and syncytium formation *in vitro* were found to be conserved among peripheral and CSF samples. It is interesting to note that none of the mutations described in experimental models of neuroinvasion was observed in human samples. However, significantly more variations were clustered in the N-terminal antigenic domain of the protein than in the rest of the molecule. This variability may account for the low percentage of human sera exhibiting antibodies towards strain F-derived D1 epitopes in contrast with a higher rate of antibodies towards the more conserved epitopes D2a and Dd5a (Sanchez-Pescador *et al*, 1993). Nevertheless, the striking result was that in this D1 domain, several alleles of gB were common to HSE, peripheral and laboratory strains. The location of the three HSE specific mutations within the variable amino-terminus of gB suggests that these substitutions most likely represent strain variation. The first one, A16V is conservative, both amino acids being neutral and hydrophobic. The second change (K38E) modifies the amino acid charge in a domain of gB that is particularly rich in basic residues, and might alter the protein conformation. Finally, the A32T substitution observed in one HSE case had been previously described in a naturally occuring mutant virus resistant to a monoclonal antibody (Kousoulas *et al*, 1988). However, these changes are located in a continuous epitope which is target to rather weakly neutralising antibodies, and we speculate that loss of this epitope would not adversely compromise the immunogenicity of gB. Nevertheless, only functional studies could address the role of these modifications in pathogenesis.

The concept that mutations in viral surface proteins may modify the phenotype of CNS viral infections is well established. Many examples have been depicted in rabies, alpha-virus, and LCMV experimental infections (Tuffereau *et al*, 1989; Salvato *et al*, 1991; Tucker *et al*, 1993), and more recently in HSV infection (Mitchell and Stevens,

1996). However, how such mutations relate to the course of human disease is still unknown. In this study, in spite of the characteristics of the patient/control matching, the striking observation that various alleles of gB were present in human HSE as well as in peripheral strains, argues against the existence of a link between a particular aspect of infection and HSV gB.

Overall, our results further underline the molecular heterogeneity of human neuropathogenic HSV-1 strains (Hammer *et al*, 1980; Whitley *et al*, 1982). The impressing absence of difference between peripheral and HSE alleles of gB in most cases strongly suggests that gB alone does not participate in HSV neuroinvasion in humans, or that HSE could be caused by peripheral strains, due to other viral or host factors.

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