Neurovirulence of Herpes Simplex Virus Types 1 and 2 Isolates in Diseases of the Central Nervous System

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Herpes simplex virus (HSV) isolates derived from the central nervous system of ten patients with HSV-1-induced encephalitis, one patient with multiple sclerosis, and 14 patients with HSV-2-induced meningitis were investigated for neurovirulence by assaying the LD_{50} after nose and intracerebral (i.c.) inoculation of mice. HSV-1 encephalitis strains were significantly more virulent after nose inoculation (i.e. neuroinvasive) when compared with HSV-1 isolates from patients with oral lesions only, whereas HSV-2 meningitis strains were significantly more virulent after i.c. inoculation when compared with HSV-2 isolates from patients with genital lesions only. No correlation between high neurovirulence (defined as low LD_{50} for both routes of infection) and replication in cell cultures of neuronal and non-neuronal cell lines was found, but the weakly neurovirulent HSV-1 strain isolated from a patient with multiple sclerosis gave low replication yields. After nose inoculation, a highly neuroinvasive HSV-1 laboratory reference strain replicated to high titers in nose tissue, the trigeminal ganglia and brainstem, while a strain with low neuroinvasiveness but high i.c. virulence replicated less well in the brainstem. Neuroinvasiveness of the virus strain might be one factor of relevance in the pathogenesis of HSV-1 encephalitis in man.

When infecting the central nervous system (CNS) herpes simplex virus type 1 (HSV-1) can cause focal, necrotizing encephalitis, this virus being considered the most important cause of acute encephalitis in immunocompetent patients (1–3). The sporadic occurrence of this disease and the fact that as yet no pathogenetic factor has been singled out as playing a decisive role might indicate that in a particular patient a number of unfortunate circumstances concurr. Of relevance to the pathogenesis could be predisposing factors in the patient himself, the virus dose, or the site of replication at primary infection, as well as neurovirulent properties of the virus.

Data have been presented in support of HSV-1 strain variability of neurovirulence in mouse models (4–7) and in rabbits (8). Attempts have been made to pinpoint the HSV-1 genes regulating neurovirulence by the use of recombinant strains (9–12) or deletion mutants (13), but results indicate that several genes might be involved. However, whether the pathogenesis in HSV-1 encephalitis in man is influenced by the neurovirulence of HSV-1 strains is still not known.

Herpes simplex virus type 2 (HSV-2) is a rare cause of encephalitis in man (2), and in most cases reported the patients have been immunocompromised (14, 15). Instead, HSV-2 may sometimes induce a serous meningitis when HSV-2 infection spreads from the genital tract to the CNS (16, 17). Symptoms of meningitis are far more common in primary HSV-2 infection, where the surface area of involvement is larger and the duration of virus shedding longer, as compared with secondary HSV-2 infection (i.e. the first episode of HSV-2 infection preceded by an HSV-1 infection) (18). These findings indicate possible relevance of virus load from the periphery in the development of HSV-2 meningitis, but the potential virulence properties of HSV-2 strains causing meningitis have not yet been described.

We used a mouse model to assay the LD_{50} after infection by different routes with CNS-derived HSV-1 isolates from ten patients with encephalitis and one patient with multiple sclerosis, and HSV-2 isolates from 14 patients with meningitis, in comparison with clinical reference strains isolated from oral lesions (HSV-1) and genital

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lesions (HSV-2) in patients without neurological symptoms, and with known laboratory reference strains. The mouse is permissive to CNS infections with both HSV-1 and HSV-2, also by peripheral routes (19). It has been reported that the neurovirulence of recombinant mouse-adapted poliovirus strains in this animal is in concordance with neurovirulence in man (20).

Materials and Methods

Virus Isolation. Clinical samples of brain material, cerebrospinal fluid (CSF), and secretion from oral and genital lesions were inoculated in cell cultures of green monkey kidney (GMK AH-1 cells) or Vero cells. Isolates were typed as HSV-1 or HSV-2 by EIA using monoclonal antibodies (21, 22).

Virus Strains. Ten HSV-1 strains were isolated from brains of patients with encephalitis (HSV-1 ENC). Eight of these strains were derived from biopsies (patients no. 1–8, Table 1) performed during a trial of antiviral treatment of HSV encephalitis (3). All biopsies were taken before the commencement of antiviral treatment. In three of these patients (no. 1, 2 and 3), serological data were consistent with a probable primary HSV-1 infection (low level or absence of HSV-1 IgG antibodies and presence of HSV IgM antibodies in the first serum sample) at the time of the encephalitis, while four had serologic evidence of reactivated HSV-1 infection. In one patient, serological data were missing. Two HSV-1 strains were isolated from brain material obtained at autopsy of two patients who died of encephalitis before receiving antiviral treatment (patients no. 8 and 10, Table 1) and in whom serologic data (high levels of HSV-1 IgG antibodies at onset) indicated reactivation. As controls, we used ten HSV-1 strains isolated from oral lesions in patients without neurological symptoms seen at the Department of Dermatology, University of Göteborg. These strains are referred to as HSV-1 REF strains. Also included is an HSV-1 strain which we isolated from the CSF of a patient during the first bout of multiple sclerosis. The isolate was designated HSV-1 BAN and was recently described in more detail (23). Laboratory reference strains (HSV-1 LABREF) used were F (B. Roizman, Chicago), McIntyre (A. Nahmias, Atlanta) and our own strain KJ 502 (24).

In fourteen patients with meningitis, HSV-2 was isolated from CSF (HSV-2 MEN) at either the Department of Virology, University of Göteborg (six strains) or at the Department of Virology, Stockholm County Central Microbiological Laboratory (eight strains). HSV-2 clinical reference strains (HSV-2 REF) were isolated from genital lesions in ten patients with HSV-2 infection without neurological symptoms seen at the Department of Dermatology, University of Göteborg. HSV-2 laboratory reference strains (HSV-2 LABREF) used were B4327UR and 7875 (S. Jeansson, Göteborg).

Preparation and Titration of Virus Stocks. In all experiments, low (second or third) passages of virus were used, and virus stocks were prepared by limited dilution of HSV strains in GMK AH-1 cell-tube cultures followed by low multiplicity infection of monolayers of GMK AH-1 cell in 200 cm^2 glass flasks with Eagle's medium and incubated at 37 °C. Flasks were frozen at – 70 °C when an almost confluent cytopathic effect was observed. The HSV-infected cell cultures were thawed, homogenized, centrifuged at low-speed, aliquoted in 2 ml vials, and stored at – 70 °C. In connection with the experiments, plaque titrations of the virus strains were performed on GMK AH-1 cells grown in 5 cm plastic dishes with 1 % methyl cellulose as overlaying medium, and the virus strains were quantified by counting plaque forming units (PFUs). HSV titers were then expressed as PFUs/ml.

LD_{50} in Mice. Outbred Swiss albino mice were used. We preferred an outbred strain, since clear differences in susceptibility of several inbred mouse strains to HSV have been reported (25). Groups of five mice of 3 to 4 weeks of age were infected by intracerebral (i.c.) injection of 0.025 ml in the left temporal region or, when assessing neuroinvasiveness by nose inoculation, by scraping the region of the virbrissae bilaterally, and inoculation (2 × 0.05 ml) of 10 log dilutions in Hank's medium of the HSV strains. Virus quantities per mouse given were for i.c. infection 10^{-1}–10^5 PFUs for HSV-1 and 10^{-2}–10^4 PFUs for HSV-2, and for

Table 1: Patient data for CNS-derived HSV-1 encephalitis strains.

Patient no.	Strain no.	Sex	Age	Outcome	Source of strain	Status of infection[a]
1	7682	M	8	death	biopsy	primary
2	3355	F	19	death	biopsy	primary
3	25	M	27	mild sequelae	biopsy	primary
4	47	M	48	mild sequelae	biopsy	reactivated
5	164	M	52	death	biopsy	reactivated
6	2762	M	58	death	biopsy	?[b]
7	1666	M	59	severe sequelae	biopsy	reactivated
8	E4	F	61	death	autopsy	reactivated
9	274	F	62	death	biopsy	reactivated
10	E5	M	76	death	autopsy	reactivated

[a]Encephalitis caused by probable primary or reactivated HSV-1 infection according to serological data.
[b]Serological data not available.

nose infection 10^2–10^6 PFUs for both HSV-1 and HSV-2. As negative control, medium alone was used. The groups of mice were coded and observed daily for two weeks after i.c. and for three weeks after nose infection for signs of neurological involvement and the number of deaths were recorded. The LD_{50} was calculated for each strain.

HSV Replication in Nose Tissue, Trigeminal Ganglia and Brainstem of Mice. Groups of 40 mice were nose inoculated (see above) with HSV-1 strains F (dilution 10^7 PFUs/ml), McIntyre and KJ 502 (dilutions 10^5, 10^6 and 10^7 PFUs/ml). On day 1 to 7, five mice from each group were sacrificed and, after perfusion through the left heart chamber with Eagle's medium, nose tissues, trigeminal ganglia and brainstems were collected and frozen at $-70°C$. The samples were thawed simultaneously, homogenized in 1.5 ml of Eagle's medium containing 1 % penicillin-streptomycin, and centrifuged for 10 minutes at 2500 rpm. Supernatants were inoculated on plastic dishes with GMK AH-1 cells for plaque titration as described above.

Replication Assays. Twenty-four hour replication of all HSV strains was assayed by inoculating strains on GMK AH-1 cells in plastic dishes (inoculum 0.1 PFU/cell), and on C1300 cells, a mouse neuroblastoma cell line (inoculum 1.0 PFU/cell). After adsorption for 1 h, cell cultures were rinsed four times with Eagle's medium and 2 ml Eagle's medium was added to each culture. After incubation at 37 ° for 24 h, the cultures were frozen for later quantification in 10 log dilutions by plaque titration on GMK AH-1 cells as described above.

Statistical Methods. The nonparametric Wilcoxon's rank sum test was used throughout for comparisons between the CNS-derived HSV strains and the oral or genital HSV strains except for the analysis of time to death after nose inoculation of the HSV-1 strains, and HSV replication in cell cultures, where Student's t-test was used.

Results

Neurovirulence in Mice after CNS and Peripheral Infection. The HSV-infected mice died of encephalitis with seizures and/or paresis after both i.c. and nose inoculation of the HSV strains and the log PFU/LD_{50} could be calculated for each strain. By definition, death after nose inoculation was characterized as neuroinvasiveness, since this route constitutes replication and transport along the trigeminal pathway before entrance to the CNS. Neurovirulence for each strain was defined based on the combination of results of neuroinvasiveness and LD_{50} after i.c. inoculation (see below). The results from mice infected with HSV-1 strains are shown in Figure 1. After i.c. inoculation, mean log PFU/LD_{50} for HSV-1 ENC strains did not differ significantly from mean log PFU/LD_{50} for HSV-1 REF strains. The HSV-1 BAN strain isolated from a patient with multiple sclerosis showed a higher PFU/LD_{50} ratio than did any of the HSV-1 ENC or REF strains.

After nose inoculation, one HSV-1 ENC strain and four HSV-1 REF strains were non-lethal with the inocula used (Figure 1). Seven of the
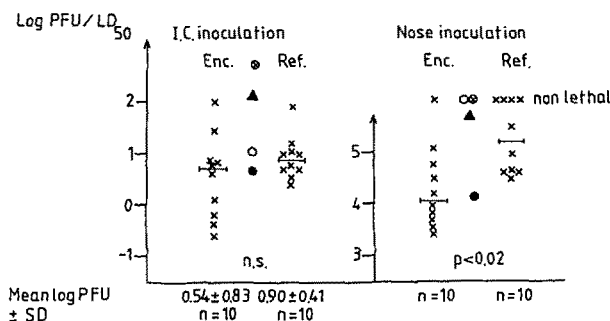

Figure 1: Log PFU/LD_{50} in Swiss albino mice i.c. or nose inoculated with 10-fold dilutions of 10 HSV-1 strains isolated from brains of patients with encephalitis (Enc.), 10 HSV-1 clinical reference strains (Ref.) obtained from patients with oral lesions only, the HSV-1 BAN strain isolated from the cerebrospinal fluid of a patient with multiple sclerosis (▲), or HSV-2 laboratory reference strains F (⊗), McIntyre (O) and KJ 502 (●). Wilcoxon's rank sum test was used for statistical comparisons. Horizontal bars indicate median values.

HSV-1 ENC strains showed a lower log PFU/LD_{50} than did any of the HSV-1 REF strains and, as a group, the HSV-1 ENC strains were significantly ($p < 0.02$) more neuroinvasive when compared with the HSV-1 REF strains. Also, in the mice who died, the time to death after nose inoculation of 10^6, 10^5 and 10^4 PFUs of HSV-1 ENC strains was significantly ($p < 0.01$) shorter: 7.54 ± 1.41, 8.14 ± 1.88 and 8.78 ± 2.11 days (mean $\pm$ SD), respectively, as compared with 8.77 ± 1.44, 10.5 ± 1.67 and 10.33 ± 0.58 days (mean $\pm$ SD) for the same inocula of HSV-1 REF strains.

Data from i.c. and nose inoculation in mice were combined to classify all HSV-1 strains into three classes of neurovirulence (Table 2, see legend for limit values). Of the HSV-1 ENC strains, six were classified as highly neurovirulent (Class I), three as intermediately neurovirulent (Class II), and one as weakly neurovirulent (Class III). Of the HSV-1 REF strains, none qualified as Class I, seven belonged to Class II and three to Class III, as did the isolate HSV-1 BAN derived from a patient with multiple sclerosis.

The highly neurovirulent (Class I) HSV-1 LABREF strain KJ 502 and the Class II HSV-1 LABREF strain McIntyre differed in their neuroinvasiveness (a_3 versus a_1, Table 2). The nature of this difference was further investigated by monitoring virus replication at three levels (skin, trigeminal ganglia and brainstem) after nose inoculation with these two strains and the Class III (a_0) HSV-1 LABREF strain F. As shown in Figure 2, HSV-1 replication reached its maximum after 2 to 4 days in the periphery, after 3 to 5 days in ganglia, and after 4 to 7 days in the brainstem with all three strains. However,

Table 2: Classes of neurovirulence in mice of HSV-1 strains.

Class	Encephalitis		Laboratory		Reference	
	\[HSV-1 strains[a]\]					
I	7682[b]	a_3b_3				
	3355[b]	a_3b_3				
	E4	a_3b_3				
	25[b]	a_3b_3				
	2762	a_3b_2	KJ 502	a_3b_2		
	E5	a_2b_3				
II	1666	a_3b_1			90395	a_2b_2
	47	a_2b_2			90355	a_2b_2
	274	a_2b_2			90132	a_2b_2
					90147	a_2b_2
					90579	a_2b_2
			McIntyre	a_1b_2	90602	a_1b_2
					90270	a_0b_3
III					90238	a_0b_2
					94783	a_0b_2
					HSV-1 BAN	a_1b_1
	164	a_0b_1	F	a_0b_1	90237	a_0b_1

[a]HSV-1 strain characteristics expressed as a: neuroinvasiveness after nose inoculation (a_0 indicates $> 10^6$; $10^6 \geq a_1 > 2 \times 10^5$; $2 \times 10^5 \geq a_2 \geq 2 \times 10^4$; and $a_3 < 2 \times 10^4$ PFU/LD$_{50}$) and b: neurovirulence after intracerebral inoculation (b_1 indicates > 20; $20 \geq b_2 \geq 2$; and $b_3 < 2$ PFU/LD$_{50}$). Class I (highly neurovirulent) is defined as: a + b = 5 or 6; Class II (moderately neurovirulent) as: a + b = 3 or 4; and Class III (weakly neurovirulent) as: a + b = 1 or 2.
[b]Serological data indicating a probable primary HSV-1 infection as the cause of the encephalitis.

two different patterns of progression of HSV-1 infection were found: strain F (Class III) replicated well at the periphery but to low titers at both the ganglionic and brainstem levels, whereas strains McIntyre (Class II) and KJ 502 (Class I) both replicated to relatively high titers in ganglia and brainstems after high-dose inoculation peripherally. When the infectious dose was lowered, although KJ 502 replicated better at all levels, strain McIntyre barely replicated in the brainstem, while the highly neuroinvasive strain KJ 502 readily progressed to the brainstem level.

In Figure 3, results from the assessment of virulence after i.c. or nose inoculation of HSV-2 strains are depicted. Generally, log PFU/LD$_{50}$ values were lower for the HSV-2 strains than for the HSV-1 strains. After i.c. inoculation, HSV-2 meningitis strains were significantly ($p < 0.01$) more virulent as compared with the HSV-2 reference strains, but no difference was found after nose inoculation.

Replication Assays. The replication yields in GMK-AH1 (mean log PFU/ml $\pm$ SD) and C1300 neuroblastoma cells (mean log PFU/ml $\pm$ SD) of HSV-1 ENC strains (7.68 ± 0.36 [5.04 ± 0.86], respectively) did not differ significantly from that of the HSV-1 REF strains (7.66 ± 0.44 [5.18 ± 0.66]). When these strains were grouped according to neurovirulence, replication yields were for Class I: 7.89 ± 0.27 [5.40 ± 0.61], for Class II: 7.58 ± 0.47 [5.18 ± 0.67], and for Class III: 7.58 ± 0.21 [4.53 ± 0.70]. Strain HSV-1 BAN, grouped as Class III in neurovirulence, displayed a markedly low replication capacity in both cell lines tested (6.79 ± 0.11 [4.41 ± 0.20]). Results for HSV-1 LABREF strains were for strain F: 7.61 ± 0.04 [4.96 ± 0.26], for strain McIntyre 8.45 ± 0.02 [6.32 ± 0.12] and for strain KJ502 8.15 ± 0.02 [not tested].

Likewise, the HSV-2 MEN strains gave mean replication yields (6.26 ± 0.19 [5.37 ± 0.25]) similar to that of the HSV-2 REF strains in both cell types used (6.31 ± 0.11 [5.17 ± 0.46]). Results for HSV-2 LABREF strains were for strain B4327UR: 6.47 ± 0.07 [5.07 ± 0.09] and for strain 7875: 6.59 ± 0.07 [5.50 ± 0.11].
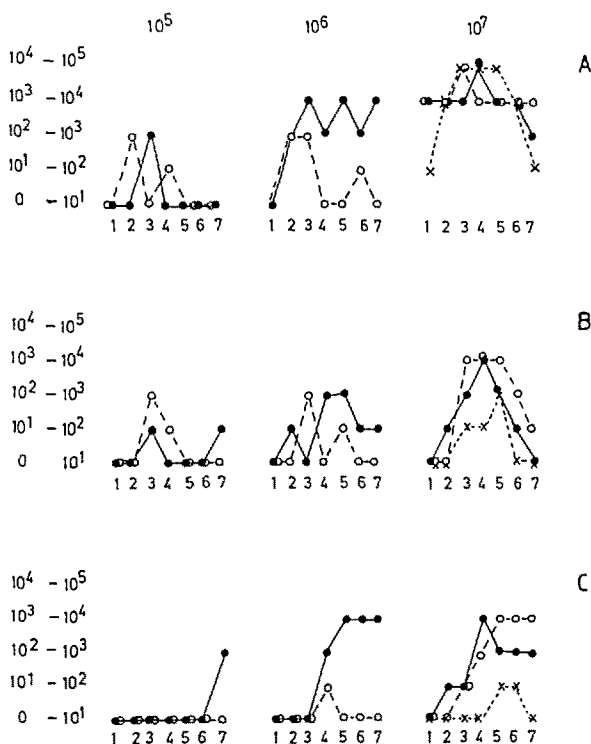
Discussion

The pathogenesis of encephalitis caused by HSV-1 is largely unknown. The sporadic occurrence and scarcity of underlying immunocompromising conditions found in patients (26) may suggest pathogenetic relevance of viral alteration by recombination (27) or mutation. Although the existence and genomic locations of HSV neurovirulence have been well documented in experimental studies (5, 10, 12, 13), the link between neurovirulence in humans in the form of encephalitis and neurovirulence in animal models has hitherto not been established. The HSV-1 brain isolates used in this study were all proven to be neuropathogenic to man by causing encephalitis with either a fatal outcome or sequelae in spite of antiviral treatment.

The peripheral route of nose infection, where virus particles reach the brain via the trigeminal pathway by neuron to neuron transmission (28), was chosen to imitate the probable route that HSV-1 travels when this virus causes encephalitis in humans (29). The virulence to mice displayed by encephalitits-inducing HSV-1 strains by this route of infection suggests that invasiveness is a property of relevance for HSV-1 neurovirulence. Furthermore, we have shown that the same encephalitis-inducing strains also were significantly more invasive as compared with the HSV-1 REF strains in an in vitro model using cultured rat sensory neurons in a dual-chamber system permitting infection of neuritic extensions only (30).

In experimentally infected animals, spread along sensory neurons has been found to be the main route by which HSV enters the CNS (31, 32). HSV strain variability in capacity to invade the CNS has been described (4, 6), and non-neuroinvasive strains have been found to be unable to progress from sensory ganglia to the CNS (33, 34). In our study, results of viral replication at peripheral, ganglionic and brainstem levels after nose inoculation of mice indicate that the neuroinvasive HSV-1 LABREF strain KJ502 differs from the non-neuroinvasive strains McIntyre and F by its greater ability to reach and to replicate in the brainstem.

One of the HSV-2 isolates in this study was derived from a patient who was part of an epidemiological chain of three subjects who all suffered from meningitis after genital herpes infection. This might suggest the existence of HSV-2 strains that are more prone to induce meningitis, but studies on virulence characteristics of isolates from patients with meningitis are lacking. The finding in this study that CNS-derived meningitis-inducing HSV-2 strains showed enhanced neurovirulence after i.c. inoculation in mice might indicate the existence of such a

Figure 2: Replication yields in PFU of HSV-1 laboratory reference strains consisting of the highly neurovirulent (Class I, see Table 2) strain KJ 502 (●—●), moderately neurovirulent (Class II) strain McIntyre (O— O), and weakly neurovirulent (Class III) strain F (×— ×) in A) nose tissue, B) trigeminal ganglia, and C) brainstem of mice 1 to 7 days after nose inoculation of 0.1 ml of the HSV strains diluted to 10^5 and 10^6 PFU/ml (KJ 502 and McIntyre), and 10^7 PFU/ml (KJ 502, McIntyre and F). Values are given as intervals, within which mean values ± SD of each group of five mice are found.
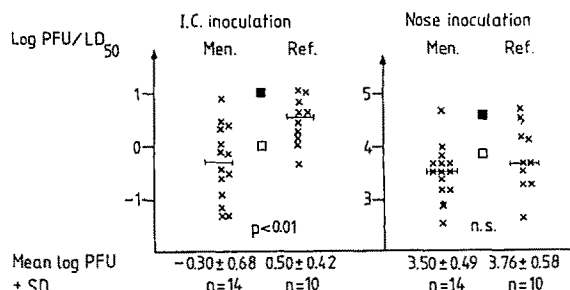

Figure 3: Log PFU/LD$_{50}$ in Swiss albino mice i.c. or nose inoculated with 10-fold dilutions of 14 HSV-2 strains isolated from the cerebrospinal fluids of patients with meningitis (Men.), 10 HSV-2 clinical reference strains isolated from patients with genital lesions only (Ref.), or HSV-2 laboratory reference strains B4327UR (■) and 7875 (□). Wilcoxon's rank sum test was used for statistical comparisons. Horizontal bars indicate median values.

strain characteristic. This characteristic was not revealed by the peripheral route of nose inoculation used here. The clinical picture of HSV-2 induced meningitis described earlier (16, 17) with local neurological signs in the lumbosacral region but without spread to the brain or higher regions of the spinal cord may suggest that HSV-2 is a weakly neuroinvasive virus in adults. However, studies on HSV-2 invasiveness after genital inoculation in animals with meningitis-inducing strains are needed to further address this question, since a difference exists in tropism between HSV-1 and HSV-2 (1).

A connection between HSV-1 neurovirulence in vivo and replicative capacity has been suggested (11), but conflicting data have been presented. In later studies, neither an avirulent strain (34) nor a virulent strain (10) showed any correlation between virulence and replication in different cell lines. The indications of HSV-1 and HSV-2 neurovirulence properties of CNS-derived isolates found in vivo in our study were not paralleled by higher replication yields in either a highly permissive non-neuronal (GMK-AH-1) or a mouse neuroblastoma (C1300) cell line giving low replication yields. On the other hand, a tendency towards lower replication yields in C1300 cells was seen for the Class III non-neurovirulent strains in comparison with the Class I strains (t = 2.15, Student's t-test). Likewise, the strain HSV-1 BAN from a patient with multiple sclerosis, which displayed weak neurovirulence in vivo, gave a markedly low replication yield in both cell types.

Restricted viral replication as a pathogenetic factor in demyelination was found in a study of mice infected with mutants of the coronavirus mouse hepatitis virus (35), and has earlier been suggested for HSV in humans in a hypothesis on multiple sclerosis (36). However, we have not been able to test this hypothesis properly, due to the lack of additional viral isolates from the CNS of patients with multiple sclerosis.

In man, HSV-1 induced CNS infections such as encephalitis are rare events although this virus is neurotropic and commonly recovered from sensory ganglia at autopsy (37). The suggestion derived from our data that the HSV-1 strain characteristic of neuroinvasiveness is linked to encephalitis indicates the usefulness of well-defined clinical isolates in the further study of HSV neuropathogenesis.

Acknowledgements

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