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Comparative Neurovirulence of Herpes Simplex Virus Type 1 Strains After Peripheral or Intracerebral Inoculation of BALB/c Mice

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Twenty-three strains of herpes simplex virus type 1 were compared for their pathogenicity in 4-week-old BALB/c mice after peripheral (footpad) or intracerebral inoculation. Among those strains examined were (i) six clinical isolates of brain or cerebrospinal fluid origin, (ii) seven clinical isolates of oral or genital origin, (iii) five prototype laboratory strains that have been passaged numerous times in culture, and (iv) five syncytial variants capable of producing cell fusion in culture. Based on comparative 50% lethal dose values, the strains appeared to segregate into one of three classes of neurovirulence. Class I strains were highly virulent by both the peripheral and intracerebral routes of inoculation, class II strains were highly virulent by the intracerebral route only, and class III strains were highly attenuated by both routes of inoculation. In vivo growth curves for whole brain homogenates infected with class III strains revealed titers of infectious virus approaching those found in the brains of animals infected with class I or II strains. These results would therefore suggest that (i) a strain-dependent variation in neural spread exists that may influence the ability of the virus to cause acute neurological disease and (ii) the amount of infectious virus present within an infected brain does not necessarily determine or reflect the clinical status of the animal. Of the clinical isolates examined, the strains recovered from brain tissue of humans after fatal episodes of encephalitis were found to be no more neurovirulent in mice than the strains isolated from nonneural sites. However, although syncytial variants were found to be highly attenuated by the peripheral route, as a group these strains proved to be among the most virulent when inoculated directly into the central nervous system.

After being inoculated into peripheral sites, herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) localize within nervous system tissue and produce a wide spectrum of neurological disease. Recurrent eruptions on ocular, oral, genital, or cutaneous surfaces stem from the reactivation of latent virus harbored within the neurons of sensory ganglia (1, 3, 37). Direct involvement of the central nervous system (CNS) ranges from an acute necrotizing encephalitis, which is accompanied by severe mortality and morbidity (41, 42), to a benign self-limited meningitis (2).

Previous animal studies investigating herpesvirus pathogenicity have been directed mainly at type-specific differences (21, 28) and showed that HSV-2 was generally more virulent than HSV-1 (19). Paradoxically, cases of fatal encephalitis in adults are usually produced by HSV-1 (22), whereas HSV-2 is usually associated with the more benign meningitis (2). Since it is conceivable that unusually neurovirulent strains of HSV-1 are responsible for adult encephalitis (40), we initiated studies to compare 23 strains of HSV-1 for their ability to induce acute neurological disease in BALB/c mice after either peripheral or intracerebral inoculation. Among those strains examined were (i) clinical isolates recovered from the brain or cerebrospinal fluid (CSF) of patients suffering from acute CNS disease, (ii) clinical isolates of oral or genital origin, (iii) high-passage prototype laboratory strains, and (iv) syncytial variants that induce cell fusion in culture.

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MATERIALS AND METHODS

Cell culture and media. Serially propagated human embryonic lung (HEL) fibroblasts (cell line MRC-5)

and African green monkey kidney (Vero) cells (Flow Laboratories, Inglewood, Calif.) were cultured in Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum, 0.225% $NaHCO_3$, and antibiotics. Cells were maintained in MEM supplemented with 2% fetal calf serum, 0.225% $NaHCO_3$, and antibiotics.

Virus strains. All clinical isolates, laboratory strains, and syncytial variants used in this investigation are listed in Table 1.

Preparation and titration of virus stocks. Before animal inoculation, stocks of all virus strains were prepared by infecting cultures of HEL cells with virus at an input multiplicity of 0.01 to 0.1 PFU/cell. After adsorption for 1 h at 37°C, maintenance medium was added, and the cultures were incubated at 37°C. When the infected monolayers showed greater than 90% cytopathic effect, the cultures were frozen at −80°C, thawed, and centrifuged at 4°C for 10 min at 2,000 rpm in a clinical centrifuge. The supernatant fluid was transferred in 1.0-ml volumes to vials and stored at −80°C. The virus infectivity of all virus stocks was assayed at 37°C in cultures of Vero cells by the plaque method, using a 2% methylcellulose overlay (10). Results for a single virus strain throughout this investigation were obtained with the same virus stock preparation.

Typing of virus strains. To confirm the identity of all clinical isolates, laboratory strains, and syncytial variants as HSV-1, we examined the pattern of polypeptide synthesis within cultures of infected HEL cells by electrophoresis of sodium dodecyl sulfate-polyacrylamide slab gels as described previously (29, 36).

Animals. Four-week-old male BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were used throughout this study.

Peripheral inoculation. Peripheral virus infection was accomplished by inoculation into the right rear footpad of mice with a 30- to 40-μl volume containing approximately 10^4, 10^5, 10^6, or 10^7 PFU of virus. Appropriate dilutions of virus stocks were prepared in MEM, and the virus inocula were titrated as described above to define the dose of virus administered. Groups of mice were coded and examined daily for 2 weeks for the appearance of acute ascending neurological disease, characterized by monoplegia, paraplegia, ascending myelitis, and death. No change in the incidence of morbidity or mortality was observed in animal groups surviving longer than 2 weeks. For each strain, the virus dose producing death in 50% of infected animals (LD_{50} value) was calculated by the method of Reed and Muench (30).

Intracerebral inoculation. Virus was introduced directly into the CNS by inoculation into the right hemisphere of the brain with a 10-μl volume containing approximately 10, 10^2, 10^3, 10^4, or 10^5 PFU of virus. Appropriate dilutions of virus stocks were prepared in MEM, and the virus inocula were titrated as described above to define the dose of virus administered. Groups of mice were coded and examined daily for 2 weeks for the appearance of encephalitis and death. Animals surviving longer than 2 weeks remained free of acute disease. LD_{50} values were calculated for each strain by the method of Reed and Muench (30).

Growth curves. In vivo growth curves were developed after the intracerebral inoculation of mice with approximately 10^4 PFU of virus. At daily intervals

TABLE 1. HSV-1 strains

Group	Strain	Site of isolation	Disease	Year of isolation	Passage no.	Reference
CNS clinical isolates	H129	Brain	Encephalitis	1977	3	
	H144	Brain	Encephalitis	1979	3	
	H186	Brain	Encephalitis	1981	3	8
	H193	Brain	Encephalitis	1982	3	*[a]
	H166+	CSF	Meningitis	1980	2	14
	H168+	CSF	Meningitis	1980	3	36
Oral and genital clinical isolates	DAB	Lip	Labialis	1979	2	
	KOS-79	Lip	Labialis	1979	3	
	HL4	Saliva	Labialis	1974	4	26
	HL17	Saliva	Labialis	1974	4	26
	EKN	Saliva	Gingivostomatitis	1980	1	
	HTZ	Pharynx	Pharyngitis	~1974	7	20
	E377	Penis	Genitalis	1965	~8	
Laboratory strains	F	Lip	Labialis	~1968	Numerous	11
	KOS-63	Lip	Labialis	1963	Numerous	34
	−GC Miyama	Lip	Labialis	1958	Numerous	24, 33
	mP	Skin	Eczema Herpeticum	1957	Numerous	15
	RE	Cornea	Keratitis	~1967	Numerous	16
Syncytial variants	MP	In vitro		1957	Numerous	15
	+GC Miyama	In vitro		1958	Numerous	24, 33
	CGA3	In vitro		1958	Numerous	12, 32
	H166Syn	CSF	Meningitis	1980	2	14
	H168Syn	In vitro		1980	2	

[a] R. D. Dix, S. Lukes, L. Pulliam, and J. R. Baringer, submitted for publication.

postinoculation, three to five brains per virus strain were removed and individually homogenized in MEM to yield 10% (wt/vol) homogenates. Plaque assays were performed in duplicate for each homogenate and expressed as PFU per gram (wet weight) of brain tissue.

RESULTS

Selection of HSV-1 strains. Factors considered during the selection of the HSV-1 strains used in this investigation included (i) the clinical source, (ii) the degree of passage in culture, and (iii) the cytopathic effect produced in culture. The source and history of the 23 strains of HSV-1 selected for this study are summarized in Table 1.

Four groups of HSV-1 strains were examined. The first group consisted of six low-passage clinical isolates of CNS origin. Four strains (H129, H144, H186, and H193) were recovered from brain tissue obtained from cases of adult encephalitis, and two strains ($H166^+$ and $H168^+$) were recovered from the CSF of patients suffering from meningitis.

The second group consisted of seven clinical isolates of oral (strains DAB, KOS-79, HL4, HL17, EKN, and HTZ) or genital (strain E377) origin. Of these, the EKN and E377 strains were representative of primary HSV-1 infections.

To assess the effect of extensive passage in vitro on pathogenesis in vivo, we included in the third group five prototype laboratory strains (F, KOS-63, -GC Miyama, mP, and RE) that have been passaged numerous times in culture. It is interesting to note that the prototype laboratory strain KOS-63 and the low-passage clinical isolate KOS-79 possess a common origin and were isolated 16 years apart from recurrent lip lesions in the same individual. However, biochemical analysis of these strains revealed significant differences in the patterns of virus-induced polypeptides as well as differences in DNA restriction endonuclease cleavage patterns (data not shown). By definition, therefore, KOS-63 and KOS-79 represent unique and unrelated strains of HSV-1 (4).

Although syncytial variants have been observed to arise spontaneously in vitro (12, 15, 24), the potential contribution of these virus strains to clinical disease remains unclear. For this reason, the fourth group consisted of five strains of HSV-1 (MP, +GC Miyama, CGA3, H166Syn, and H168Syn) capable of inducing cell fusion in culture. Of these, H166Syn may represent a true syncytial clinical isolate (14).

Peripheral inoculation of HSV-1 strains. All virus strains were initially compared by the footpad model of peripheral inoculation. Previous work has shown that virus spread is neural and not viremic by this route (6, 17, 25). In addition, the evaluation of acute virus-induced neurological disease in this murine model has been shown to be very consistent (6, 9, 19). After footpad inoculation with appropriate virus doses, mice develop a dramatic ipsilateral monoplegia on day 5 postinoculation. Over the next 2 days, the disease progresses to flacid paraplegia with ascending myelitis, which results in death usually within 7 to 10 days postinoculation. Thus, this animal model not only allowed for the clinical scoring of neurological illness and death in a blinded fashion, but has been shown previously to provide a reliable index of neurovirulence (19).

For each strain, animal groups were inoculated with approximately 10^4, 10^5, 10^6, or 10^7 PFU of virus, coded, and examined daily for 2 weeks for the appearance of acute virus-induced neurological disease and death (Table 2). Of the CNS isolates examined, five proved to be highly virulent and displayed LD_{50} values between $10^{5.3}$ and $10^{6.0}$ PFU. At 10^6 PFU, mean survival times ranged from 6.3 to 9.5 days (mean, 8.0 days) (Fig. 1A). The single exception was the CSF isolate $H168^+$, which displayed a marked attenuation. Whereas all brain isolates and the CSF isolate $H166^+$ produced an incidence of mortality ranging from 50 to 92% at 10^6 PFU, 100% of the animals inoculated with strain $H168^+$ remained free of illness and survived. Only at 10^7 PFU did this virus strain produce significant neurological disease and death.

Although a spectrum of virulence was also observed among oral and genital HSV-1 isolates, most displayed LD_{50} values similar to those found for CNS isolates. In addition, mean survival times were also comparable with those calculated for CNS isolates and ranged from 7.2 to 11.3 days (mean, 8.8 days) (Fig. 1A). Thus, isolates originating from cases of adult encephalitis or meningitis appeared to be no more virulent than clinical isolates originating from peripheral sites.

Unlike most clinical isolates, most prototype laboratory strains were weakly virulent, producing little mortality and only moderate morbidity. Even at an input dose of 10^7 PFU, the incidence of mortality was not sufficient to establish LD_{50} values. However, the high-passage RE strain displayed a pattern of virulence similar to that of several low-passage strains. Thus, although clinical isolates were generally found to be highly virulent by the peripheral route and laboratory strains were generally found to be highly attenuated, exceptions were noted within each groups and overlaps occurred.

All syncytial variants examined failed to produce significant neurological illness or death. As for the laboratory strains, LD_{50} values could not be established. However, unlike the clinical and

TABLE 2. Incidence of mortality after peripheral inoculation

Group	Strain	No. of mice dead/total (%) after inoculation with (PFU):				LD_{50}
		10^4	10^5	10^6	10^7	
CNS clinical isolates	H129	1/6 (17)	2/6 (33)	12/13 (92)	—[a]	$10^{5.3}$
	H144	0/6	0/6	6/7 (86)	—	$10^{5.6}$
	H186	0/9	0/8	7/10 (70)	—	$10^{5.6}$
	H193	0/9	1/8 (13)	4/8 (50)	—	$10^{6.0}$
	$H166^+$	—	3/8 (37)	7/8 (87)	—	$10^{5.3}$
	$H168^+$	—	—	0/8	5/8 (63)	$10^{6.8}$
Oral and genital clinical isolates	DAB	0/8	0/8	3/8 (37)	6/8 (75)	$10^{6.3}$
	KOS-79	0/8	11/16 (69)	18/18 (100)	—	$10^{4.7}$
	HL4	—	8/24 (33)	14/24 (58)	—	$10^{5.7}$
	HL17	0/8	0/8	6/8 (75)	—	$10^{5.7}$
	EKN	—	2/8 (25)	7/8 (87)	—	$10^{5.4}$
	HTZ	—	1/6 (17)	28/34 (82)	—	$10^{5.5}$
	E377	—	7/20 (35)	25/26 (96)	—	$10^{5.3}$
Laboratory strains	F	0/6	0/20	1/27 (4)	2/14 (14)	$>10^{7.0}$
	KOS-63	—	0/16	0/27	2/28 (7)	$>10^{7.0}$
	−GC Miyama	—	0/16	0/16	1/8 (13)	$>10^{7.0}$
	mP	0/10	0/10	0/10	4/16 (25)	$>10^{7.0}$
	RE	0/8	2/8 (25)	4/8 (50)	—	$10^{6.0}$
Syncytial variants	MP	0/10	0/12	1/18 (5)	2/8 (25)	$>10^{7.0}$
	+GC Miyama	—	0/16	1/16 (6)	3/8 (38)	$>10^{7.0}$
	CGA3	0/8	0/8	0/8	1/8 (13)	$>10^{7.0}$
	H166Syn	—	—	1/8 (13)	3/8 (38)	$>10^{7.0}$
	H168Syn	—	0/6	0/10	1/8 (13)	$>10^{7.0}$

[a] —, Not done.

laboratory strains, the decreased virulence displayed by syncytial variants in vivo was apparently independent of passage in vitro; recent isolates (strains H166Syn and H168Syn) were found to produce an incidence of mortality identical to that of variants passaged over 20 years in culture (strains MP, +GC Miyama, and CGA3) (Table 1).

Intracerebral inoculation of HSV-1 strains. The failure of a virus strain to produce significant mortality after peripheral inoculation might reflect its inability to successfully travel to components of the CNS and initiate an acute neurological illness. To examine more fully the role of virus spread in HSV-1 neurovirulence, we compared all strains for their ability to produce neurological disease after direct CNS inoculation. For each strain, animal groups were inoculated by the intracerebral route with approximately 10, 10^2, 10^3, 10^4, or 10^5 PFU of virus, coded, and examined daily for the appearance of encephalitis and death (Table 3). When the LD_{50} values for all clinical isolates were compared, most of the strains proved to be highly virulent and produced significant illness and death even at 10 PFU. Morever, CNS isolates did not display enhanced virulence. This conclusion was strengthened when mean survival times were compared at 10^4 PFU (Fig. 1B). Although values of 4.4 to 6.4 days (mean, 4.95 days) were calculated for brain and CSF isolates, oral and genital isolates produced values ranging from 3.3 to 5.3 days (mean, 4.4 days). Indeed, the CSF isolate $H168^+$ proved to be the most attenuated virus of these groups and produced a protracted course of clinical disease.

Prototype laboratory strains displayed a wide spectrum of virulence when compared by the intracerebral route; LD_{50} values ranged from less than 10 to greater than 10^4 PFU. Similarly, mean survival times at 10^4 PFU ranged from 2.9 to 8.5 days (mean, 5.1 days) (Fig. 1B). Three laboratory strains (RE, KOS-63, and mP) were found to be as virulent as low-passage clinical isolates. Of these, the KOS-63 and mP strains were shown previously to be weakly pathogenic by the footpad route (Table 2). The remaining two laboratory strains (F and −GC Miyama) proved to be dramatically attenuated. Even at an input dose of 10^4 PFU, the F strain produced a mortality rate of only 38%. In a similar manner, the −GC Miyama strain produced encephalitis and death in only 50% of infected animals.

Although syncytial variants were found to be highly attenuated after peripheral inoculation (Table 2), all were among the most virulent strains when examined by the intracerebral route. Producing 100% mortality at 10 PFU, all

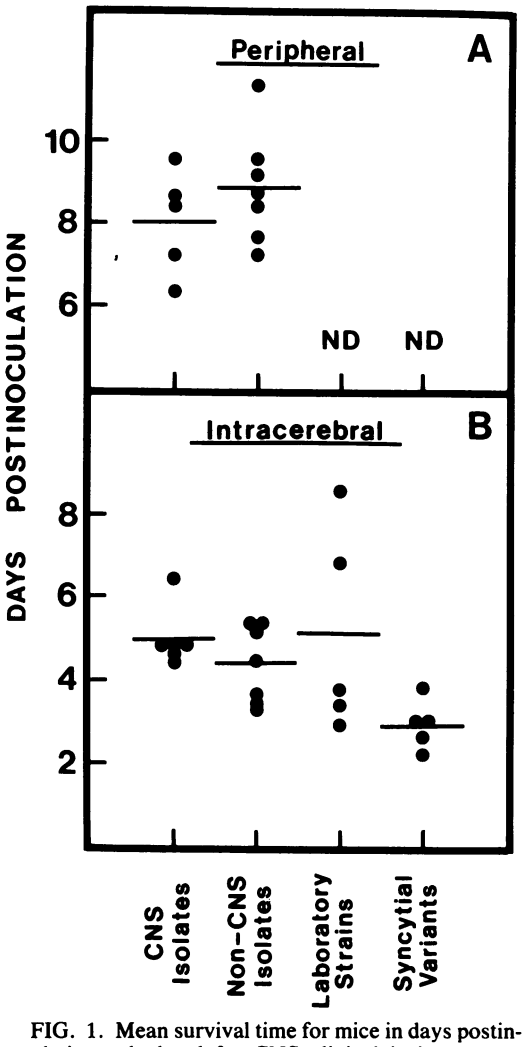

FIG. 1. Mean survival time for mice in days postinoculation calculated for CNS clinical isolates, non-CNS clinical isolates, prototype laboratory strains, and syncytial variants of HSV-1 after (A) peripheral (footpad) inoculation with 10^6 PFU of virus or (B) intracerebral inoculation with 10^4 PFU of virus. Horizontal bars indicate average mean survival times for each virus group. Note that laboratory strains and syncytial variants produced limited mortality at 10^6 PFU after peripheral inoculation (Table 2), and mean survival times were not determined (ND) for these groups.

syncytial variants had LD_{50} values of less than 10 PFU. Furthermore, as a group these strains had generally decreased mean survival times at 10^4 PFU which ranged from 2.2 to 3.8 days (mean, 2.9 days) (Fig. 1B). It must be emphasized, however, that the apparent increased virulence displayed by syncytial variants was not unique to this group. Several nonsyncytial strains (mP, RE, KOS-79, HL4, and HL17) also produced similar patterns of virulence.

In vivo replication of HSV-1 strains within brain tissue. Since previous intracerebral inoculation studies had shown the F and −GC Miyama strains to be weakly virulent and the syncytial variants to be among the most virulent (Table 3 and Fig. 1B), it was of interest to compare the replication of these strains within CNS tissue. To this end, in vivo growth curves were constructed. Included in this study were a clinical isolate (strain E377), three prototype laboratory strains (F, −GC Miyama, and mP), and four syncytial variants (strains CGA3, H166Syn, +GC Miyama, and MP). After intracerebral inoculation with approximately 10^4 PFU of virus, three to five brains per virus strain were removed at daily intervals postinoculation and individually homogenized and titrated for determination of infectious virus levels (Fig. 2).

Analysis of the production of infectious virus in strain E377-infected brains revealed a daily increase in virus titer until day 4 postinoculation. The titer remained relatively constant until day 6, when peak mortality was observed. In comparison, in vivo growth curves for animals inoculated intracerebrally with strain F or −GC Miyama revealed titers of infectious virus approaching those found in strain E377-infected brains. These results were surprising, since these laboratory strains failed previously to produce 100% mortality at an input dose of 10^4 PFU (Table 3), and a majority of animals within the strain F- or −GC Miyama-infected groups in this study remained free of clinical illness. Virus titers eventually decreased within these groups of infected animals, presumably through clearance by immunological mechanisms.

Consistent with the apparent increased virulence displayed by the syncytial variant strains CGA3, H166Syn, and +GC Miyama and the nonsyncytial mP strain after intracerebral inoculation (Table 3), high titers of infectious virus were found within 24 h postinoculation in the brain tissue of animals infected with these strains. A similar growth curve was obtained from brain tissue recovered from strain H168Syn-infected animals (data not shown). The single exception was the MP strain: although highly virulent by the intracerebral route (Table 3), less infectious virus was apparently recovered from brains infected with this strain than with the other syncytial variants.

Classes of neurovirulence. When compared by peripheral and intracerebral routes of inoculation (Tables 2 and 3), the 23 strains of HSV-1 examined in this investigation appeared to segregate into one of three classes of neurovirulence (Table 4): (i) those which were virulent by both peripheral and intracerebral routes (class I), (ii) those which were virulent by the intracerebral route only (class II), and (iii) those which were

TABLE 3. Incidence of mortality after intracerebral inoculation

Group	Strain	No. of mice dead/total (%) after inoculation with (PFU):					LD_{50}
		10	10^2	10^3	10^4	10^5	
CNS clinical isolates	H129	3/8 (38)	6/8 (75)	8/8 (100)	8/8 (100)	—[a]	$10^{1.4}$
	H144	6/8 (75)	8/8 (100)	8/8 (100)	8/8 (100)	—	<10
	H186	5/8 (63)	6/8 (75)	8/8 (100)	8/8 (100)	—	<10
	H193	6/8 (75)	7/8 (87)	8/8 (100)	8/8 (100)	—	<10
	$H166^+$	3/8 (38)	6/8 (75)	8/8 (100)	8/8 (100)	—	$10^{1.4}$
	$H168^+$	—	3/8 (38)	7/8 (88)	8/8 (100)	—	$10^{2.2}$
Oral and genital clinical isolates	DAB	2/8 (25)	5/8 (63)	8/8 (100)	8/8 (100)	—	$10^{1.7}$
	KOS-79	8/8 (100)	8/8 (100)	8/8 (100)	19/19 (100)	—	<10
	HL4	8/8 (100)	8/8 (100)	8/8 (100)	18/18 (100)	—	<10
	HL17	8/8 (100)	8/8 (100)	8/8 (100)	18/18 (100)	—	<10
	EKN	3/8 (38)	6/8 (75)	8/8 (100)	8/8 (100)	—	$10^{1.4}$
	HTZ	5/8 (63)	8/8 (100)	8/8 (100)	8/8 (100)	—	<10
	E377	3/8 (38)	6/11 (55)	8/11 (73)	11/11 (100)	—	$10^{1.7}$
Laboratory strains	F	—	—	3/10 (30)	4/11 (36)	11/11 (100)	$10^{4.2}$
	KOS-63	1/8 (13)	5/8 (63)	8/8 (100)	8/8 (100)	—	$10^{1.7}$
	−GC Miyama	—	—	1/8 (13)	4/8 (50)	8/8 (100)	$10^{4.0}$
	mP	2/8 (25)	8/8 (100)	8/8 (100)	8/8 (100)	—	$10^{1.3}$
	RE	8/8 (100)	8/8 (100)	8/8 (100)	10/10 (100)	—	<10
Syncytial variants	MP	8/8 (100)	8/8 (100)	8/8 (100)	8/8 (100)	—	<10
	+GC Miyama	8/8 (100)	8/8 (100)	8/8 (100)	8/8 (100)	—	<10
	CGA3	8/8 (100)	8/8 (100)	8/8 (100)	10/10 (100)	—	<10
	H166Syn	8/8 (100)	8/8 (100)	8/8 (100)	8/8 (100)	—	<10
	H168Syn	8/8 (100)	8/8 (100)	8/8 (100)	10/10 (100)	—	<10

[a] —, Not done.

weakly virulent by both routes (class III).

A majority of the strains examined were class I viruses and included most of the CNS, oral, and genital clinical isolates as well as a single laboratory strain. Class II was composed of a single oral clinical isolate and two laboratory strains, KOS-63 and mP. It is also of interest that all syncytial variants segregated together as class II viruses. Finally, exhibiting weak virulence by both peripheral and intracerebral routes were the CSF isolate $H168^+$ and the laboratory strains F and −GC Miyama.

DISCUSSION

Previous studies have shown that strains of HSV-1 differ in (i) the cytopathic effect produced in culture (11), (ii) the location of restriction endonuclease cleavage sites within the virus genome (4), and (iii) the electrophoretic mobility of certain structural virus-induced polypeptides (27). The results of the present investigation provide evidence that strains of HSV-1 also display a wide spectrum of virulence in BALB/c mice when compared by peripheral and intracerebral routes of inoculation.

Differences in the pathogenicity of ocular HSV-1 isolates (23) and groups of oral, genital, and laboratory strains of HSV-1 (31) have been reported. However, these studies failed to include clinical isolates of CNS origin. Although HSV-1 has been recovered infrequently from the CSF of patients suffering from benign self-limited meningitis (5, 13, 14, 36), it has been isolated from the brains of approximately 96% of adults suffering from severe necrotizing encephalitis (22). Displaying a strong predilection for the temporal lobes (22), it is conceivable that the HSV-1 strains responsible for herpes simplex encephalitis in adults are unique, possessing a special affinity for CNS tissue and increased neurovirulence (40). The results of the present investigation, however, would speak against this possibility and suggest that strains isolated from brain tissue after a fatal episode of encephalitis are no more neurovirulent in mice than are strains isolated from nonneural sites. It is interesting to note, however, that strain $H168^+$, isolated from the CSF of a patient with a history of recurrent (Mollaret) meningitis (36), was found to be highly attenuated. In comparison, strain $H166^+$, isolated from the CSF of a patient suffering from a single nonrecurrent episode of meningitis (14), proved to be as virulent as the brain isolates. Why this strain apparently produced a benign meningeal irritation and failed to invade brain tissue to produce severe encephalitis remains an enigma.

Two strains (F and −GC Miyama) consistent-

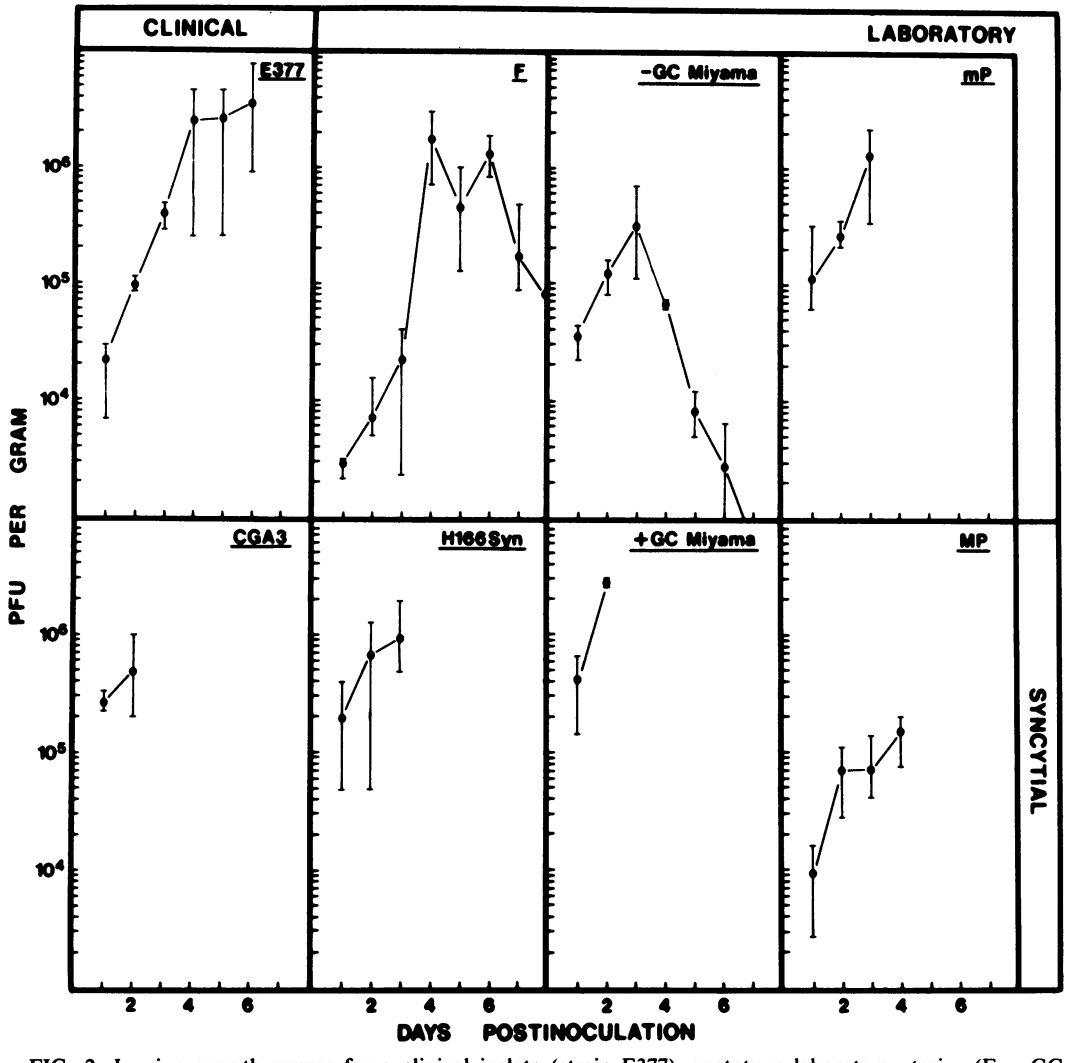

FIG. 2. In vivo growth curves for a clinical isolate (strain E377), prototype laboratory strains (F, −GC Miyama, and mP), and syncytial variants (strains CGA3, H166Syn, +GC Miyama, and MP) after intracerebral inoculation of 4-week-old BALB/c mice with 10^4 PFU of virus. At daily intervals postinoculation, three to five brains per virus strain were removed and individually homogenized in MEM to yield 10% (wt/vol) homogenates. Plaque assays were performed in duplicate for each homogenate and expressed as PFU per gram (wet weight) of brain tissue. Error bars indicate the range of values obtained for each time point.

ly failed to produce clinical encephalitis and death in 100% of infected animals when inoculated intracerebrally at an input dose of 10^4 PFU. Failure to successfully replicate to high titers within CNS tissue would provide an explanation for the significantly decreased virulence displayed by these strains. However, since high titers of infectious virus were indeed recovered from the brains of asymptomatic animals inoculated with strain F or −GC Miyama, strain-dependent, site-specific replication of the virus within brain tissue would provide an alternative explanation. Whereas highly virulent strains may rapidly invade and destroy areas of the brain necessary for the maintenance of life functions (e.g., brainstem), weakly virulent strains may spare these areas and instead replicate to high titers within less-critical brain sites (e.g., cortex). Animals inoculated intracerebrally with weakly virulent strains would therefore develop a subclinical encephalitis and appear to be asymptomatic. Virus clearance within CNS tissue would then presumably proceed by immunological mechanisms.

The finding that HSV-1 strains segregate into groups based on routes of inoculation relative to the CNS suggests that virus spread plays a critical role in determining the ability of a virus

TABLE 4. Classes of neurovirulence

Class	Virulent by:		Strains included in class from group:			
	Peripheral route (LD_{50}, 10^4 to 10^6)	Intracerebral route (LD_{50}, <10 to 10^2)	CNS clinical isolates	Oral and genital clinical isolates	Prototype laboratory strains	Syncytial variants
I	+	+	H129 H144 H186 H193 H166$^+$	KOS-79 HL4 HL17 EKN HTZ E377	RE	
II	−	+		DAB	KOS-63 mP	MP +GC Miyama CGA3 H166Syn H168Syn
III	−	−	H168$^+$		F −GC Miyama	

strain to produce acute neurological disease. Although several laboratory and syncytial strains proved to be highly virulent when inoculated directly into brain tissue, these viruses apparently lacked a strain-dependent factor(s) necessary for successful spread from peripheral sites of inoculation to CNS tissue. Factors involved in virus spread would include successful replication at peripheral sites (e.g., footpad), neural entry, neural spread, and escape from immunological surveillance.

Although syncytial variants have been observed to arise spontaneously in culture (12, 15, 24), the frequency and potential contribution of these virus strains during clinical disease remains largely unknown. Early work examining the virulence of syncytial strains in animals was performed by Wheeler (39). When equivalent titers of virus were inoculated intracerebrally into mice, the syncytial variant proved to be less virulent than the nonsyncytial parent strain. In a more recent study, however, Shimizu et al. (33) compared the virulence of the −GC and +GC Miyama strains in mice after intraperitoneal inoculation. By this route, the −GC Miyama strain was found to be highly attenuated, whereas the +GC Miyama strain was found to be highly virulent and produce significant mortality. In the present investigation, all syncytial variants proved to be highly attenuated when examined by the peripheral route. In contrast to the work of Wheeler (39), however, these strains were among the most virulent when examined by the intracerebral route. This observation was reinforced when three of the syncytial variants (strains MP, +GC Miyama, and H168Syn) were compared at equivalent input doses with the corresponding nonsyncytial parent strains (mP, −GC Miyama, and H168$^+$). However, it must be emphasized that several nonsyncytial virus strains also displayed comparable virulence within CNS tissue. Thus, the syncytial phenotype may represent an epiphenomenon, and additional strain-dependent factors are probably involved.

The immunological and biochemical bases of HSV-1 neurovirulence remain obscure. Examining the role of humoral and cell-mediated defense mechanisms, Shimizu et al. (33) reported that the replication of the highly attenuated −GC Miyama strain in vitro was suppressed more effectively by antibody, interferon, and antibody-dependent cell-mediated cytotoxicity than was the highly virulent +GC Miyama strain. Furthermore, immunosuppression of infected animals by cyclophosphamide was found to convert a normally nonlethal infection with −GC Miyama into a fatal infection. Additional studies by Lopez and co-workers (18) examining the genetics of resistance to HSV-1 in mice provided evidence suggesting that resistance to infection is a marrow-dependent function, allowing for rapid virus clearance, which prevents the spread of virus to CNS tissue.

The results of the present investigation would suggest that the major envelope glycoprotein gC plays no significant role in the pathogenesis of HSV-1. Although the syncytial MP strain lacks the ability to produce gC (35), this strain displayed a virulence comparable to the other syncytial variants, which produce normal amounts of this glycoprotein (R. Dix, unpublished data). However, when the in vivo growth curves for the syncytial variants were compared, reduced amounts of infectious virus were apparently recovered from strain MP-infected brains. Since

glycoprotein gC represents a major envelope component, virions lacking this glycoprotein may exhibit increased fragility and a concomitant loss of infectivity. Thus, the apparent decreased titers consistently expressed within strain MP-infected brains may be artifactual and not reflect the true production of infectious virus within brain tissue.

The complex issue of herpesvirus neurovirulence no doubt involves numerous type- and strain-specific functions. The finding that thymidine kinase-negative (TK^-) mutants of HSV-1 display decreased pathogenicity in vivo (7, 38) represents the identification of one such function. Ultimately, a genetic approach through marker transfer studies may prove to be the only viable means of determining the molecular basis of HSV-1 neurovirulence.

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