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DELETION OF THE HERPES SIMPLEX VIRUS 1 INTERNAL REPEAT SEQUENCES AFFECTS PATHOGENICITY IN THE MOUSE

Frank J. Jenkins,[1] Alyson M. Donoghue[2] and John R. Martin[3]

[1]Department of Pathology and Infectious Diseases and Microbiology, University of Pittsburgh, and Division of Behavioral Medicine, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213, [2]Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD. 20814, and the [3] Food and Drug Administration, Division of Antiviral Drug Products, Rockville, MD 20857.

ABSTRACT

We have isolated three different herpes simplex virus 1 (HSV-1) recombinant viruses, each frozen in either the P (prototype), I_S (inversion of S component), or I_{LS} (inversion of both components) genome arrangement. Common to all three recombinant viruses is the deletion of approximately 14 kilobases (kb) of viral DNA sequences representing greater than 95% of the internal repeat sequences and the insertion of a 9.6 kb mini-Mu genome containing a functional thymidine kinase gene. No unique DNA sequences were deleted from the viral genomes. Analyses of growth curves of the wild-type and recombinant viruses in cell culture has revealed that the recombinants grow somewhat more slowly, producing final titers within 1.5 logs of wild-type HSV-1(F). There is no discernible difference in plaque size or plaque morphology between the recombinant and wild type strains. Analysis of the recombinant viruses in mice reveals the following: I), the recombinant viruses are essentially avirulent, exhibiting drastically increased LD50 values as compared to the wild-type strain by intracerebral injection; ii), the recombinant viruses are not neuroinvasive in that they do not spread from the cornea to sensory ganglion; iii), the recombinant viruses exhibit minimal local replication both in the corneas of infected mice and in the brains of mice inoculated by intracerebral injection; and iv), the recombinant viruses do not establish a reactivable latent infection in the trigeminal ganglion following either intracerebral inoculation or inoculation of scarified corneas. These properties suggest a unique pattern of pathogenesis for HSV mutants in the mouse model.

INTRODUCTION

The herpes simplex virus 1 (HSV-1) DNA genome consists of two covalently linked components designated L and S which are bracketed on each end by inverted repeat sequences. The inverted repeats of the L component have been designated as ab and b'a', while those of the S component have been designated as ac and c'a' (1,2) (Fig 1A). An unusual property of the HSV-1 genome is the ability of the L and S components to invert relative to each other such that viral DNA isolated from either virions or infected cells consists of four equimolar populations each differing in the relative orientation of the L and S components (3).

Earlier studies from our laboratory described the characterization of three independently isolated HSV-1 recombinant viruses frozen in either the P (prototype), I_S (inversion of S component), or I_{LS} (inversion of both components) arrangement (4,5). These recombinants were generated by a target-specific mutagenesis procedure utilizing an TK mini-Mu derivative of the transducing bacteriophage Mu (Fig. 1C). Common to all three recombinant viruses is the deletion of approximately 14 kilobases (kb) of viral DNA sequences from the internal repeats and the insertion of a 9.6 kb mini-Mu genome containing a functional thymidine kinase gene under the control of the HSV ICP4 promoter (Fig. 1B). The DNA sequences deleted from each recombinant are not identical, but are very similar, differing by only a few hundred base pairs and represent greater than 95% of the internal repeat DNA sequences. The deletion of these sequences has resulted in the reduction of several HSV genes and DNA sequences from the normal diploid state to a unique haploid state. Thus, these recombinants contain only a single copy of the genes encoding the immediate early peptides ICP0 and ICP4 (6), a single copy of the gene designated ICP34.5 (7), and a single copy of the latency associated transcript (8)(LAT; Fig. 2).

Our initial studies indicated that these recombinant viruses were capable of independent replication in Vero, rabbit skin, and human 143 TK^- cells, producing viral titers comparable to that of the wild-type parent HSV-1(F) (4,5). The cytopathic effect (CPE), plaque size, and morphology of the recombinant viruses were indistinguishable from HSV-1(F). These studies clearly demonstrated that the sequences deleted in these recombinant viruses were not essential for viral replication in cell culture.

Viral gene products or DNA sequences that are not essential for viral replication in cell culture may, nevertheless, still have an important function in either viral replication or pathogenicity in animals. In the

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[1] To whom correspondence should be addressed at: Division of Behavioral Medicine and Oncology, University of Pittsburgh Cancer Institute, 3600 Forbes Ave, Suite 405, Pittsburgh, PA 15213, Tel #: (412) 624-4630, FAX #: (412) 647-1936, E-mail: jenkins@pcicirs.pci.pitt.edu

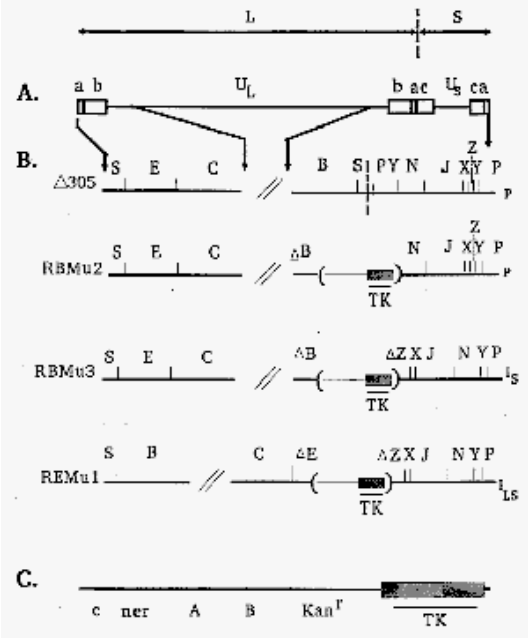

Figure 1. Sequence arrangement of the wild-type and recombinant HSV-1 virus genomes and of the TK-mM bacteriophage genome. (A) Sequence arrangement of HSV-1(F) DNA. The open boxes represent terminal sequences (ab and ca) of the L and S components, respectively that are inverted internally (bac). (B) BamHI restriction endonuclease map of the termini of HSV-1 305(F), and the recombinant viruses. The location and orientation of the TK-mM insertion in the recombinant viruses is shown within the parentheses. The dashed line represents the junction of the L and S components. The parentheses delineate the location of the deleted sequences in the recombinant viruses. The hatched box represents the TK gene located in the TK-mM insertion. (C) Schematic of the TK-mM bacteriophage genome. c, ner, A and B represent Mu genes; Kan represents the kanamycin resistance marker; TK represents the thymidine kinase gene under the control of the ICP4 promoter.

animal, HSV infects cells at a peripheral site where it replicates locally, infects local nerve endings and spreads via axons to sensory ganglia establishing a latent state. At later times, the virus can be reactivated whereby it travels back down the axon to produce a recurrent infection at or near the area of the initial primary infection. Several aspects of HSV pathogenesis including neurovirulence, neuroinvasiveness, and the establishment and maintenance of a latent state in neural tissues has been studied for a number of years using a mouse model system.

In this study, we have analyzed the pathogenicity of the recombinant viruses RBMu2, RBMu3 and REMu1 in mice. We show that deletion of the internal repeats from the HSV genome has resulted

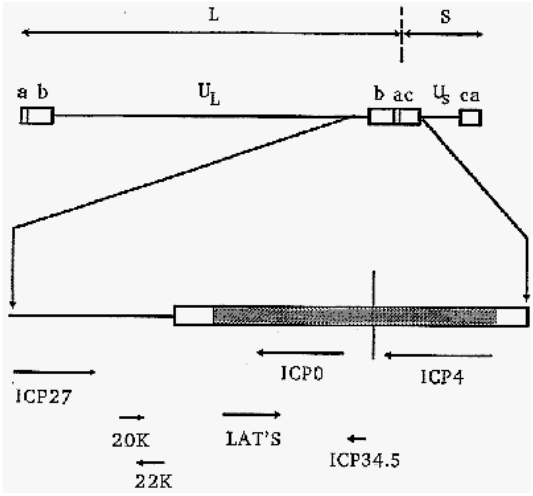

Figure 2 Schematic of the HSV-1 genome showing an enlarged internal repeat region. The arrows indicated the direction of transcription of the indicated genes. The hatched box represents the region of the internal repeats that are deleted in the recombinant viruses.

in a pronounced effect on neurovirulence, neuroinvasiveness and in the ability to establish a reactivable latent infection in mice. Our findings strongly suggest a potential role for the presence of diploid regions and/or inversion of the HSV genome in viral pathogenicity.

METHODS

Cells and viruses.

Vero cells (American Type Culture Collection) were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% Serum-Plus (SP; JRH, Rockville, MD) and 50 µg/ml gentamycin (USB, Inc. Cleveland, OH). LT$^-$A$^-$ cells were kindly provided by M.J. Tevethia (M.S. Hershey Medical Center, Pennsylvania State University, Hershey, PA) and grown in EMEM supplemented with 10% horse serum and gentamycin. Human TK$^-$ 143 cells were obtained from Bernard Roizman (University of Chicago, Chicago, IL) and grown in EMEM supplemented with 10% SP and gentamycin. Every fifth passage, the 143 cells were passaged in medium containing 50 µg/ml 5-bromodeoxyuridine (BUdR).

The properties of HSV-1(F), HSV-1(F) 305, and the recombinant viruses RBMu2, RBMu3 and REMu1 have been described elsewhere (4,5,9,10). Viral stocks were prepared and titered on Vero cells as previously described (9).

Viral growth curves

Confluent monolayers of Vero cells grown in six well dishes were infected with $1x10^3$ pfu and incubated at 37°C. At various times post infection, the cells were scraped into cell culture medium, frozen and

thawed two times, and the viral titers determined on confluent Vero cell monolayers.

Mouse inoculations

Six week old male BALB/c mice (NCI, Bethesda, MD.) were inoculated intracerebrally (i.c.), intraperitoneally (i.p.), or onto scarified corneas with varying amounts of virus. All viral dilutions were made in cell culture medium as described above, with the control groups receiving cell culture medium alone.

Intracerebral and interperitoneal inoculations were performed on mice anesthetized by brief exposure to metofane followed by injection of 0.03 ml and 0.1 ml of virus sample, respectively. For corneal inoculations, the corneas of mice anesthetized by i.p. injections of 2 mg pentobarbital were scarified by 10 passes of a hypodermic needle, followed by the placement of 0.01 ml of virus sample on the scarified corneas. The virus samples were allowed to absorb for 30 minutes while the mice were under anesthesia. For neurovirulence studies, the inoculated mice were observed daily for three weeks, and the number of deaths recorded. PFU/LD50 ratios were determined by the procedure of Reed and Muench (11). To detect acute ocular infection and both acute and latent virus in the trigeminal ganglion, the tissues were removed aseptically from exsanguinated mice and processed as described below. All animal experiments met the standards for humane animal care and use as set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

Analysis of lytic and latent virus

For detection of lytic virus, the tissue samples were homogenized using a Tissumizer (Tekmar Corp., Cleveland, OH), subjected to three rounds of freeze/thaw and plated on confluent monolayers of Vero cells. For detection of latent virus, the tissues were first incubated for 5 days in 1.0 ml cell culture medium at 37°C in an atmosphere of 5% CO_2 in order to reactivate the latent virus. Following incubation, the tissue samples were homogenized as described above, alternately frozen and thawed three times and cocultivated with confluent monolayers of Vero cells. The Vero cells were monitored daily for 8 to 10 days for the appearance of HSV plaques.

RESULTS

The construction, isolation and characterization of the viral recombinants RBMu2, RBMu3 and REMu1 have been described previously (4,5). Figure 1 illustrates the genomic structure of the recombinants and the TK^- parent HSV-1(F) 305. The deletion of the inverted repeat sequences, and specifically, the a repeat sequences (12,13) has resulted in the viral genomes becoming 'frozen' in a single isomeric form. These genomes are unable to invert and thus viral DNA isolated from either virions or infected cells contains only the single isomeric arrangement.

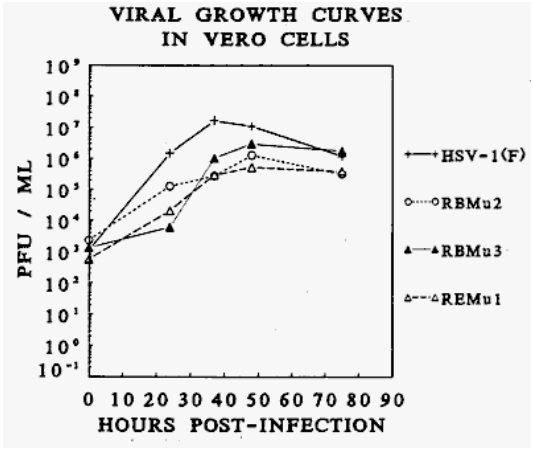

Figure 3. Growth curves of HSV-1(F) and the recombinant viruses in Vero cells.

Each recombinant was independently isolated and differs from the others in both the extent of the internal repeat sequences that have been deleted and in the isomeric form in which the viral genomes are frozen. Thus RBMu2 is frozen in the prototype form while RBMu3 and REMu1 are frozen in the inverted S and inverted LS forms respectively. All three recombinant viruses are missing approximately 14 Kbp of the internal repeats with each deletion differing by only a few hundred base pairs (4,5).

***In vitro* growth curves**

Our earlier studies (4,5) indicated that the recombinant viruses RBMu2, RBMu3, and REMu1 produced viral titers in Vero and rabbit skin cells comparable to those obtained with HSV-1(F). These results were based on the analysis of viral titers from infected cell cultures. To more carefully examine whether the deletion of the internal repeat sequences has any effect on viral replication in cell culture, growth curves of HSV-1(F) and the recombinant viruses were determined in Vero cells. Analysis of the growth curves demonstrated that the recombinant viruses grow somewhat more slowly than HSV-1(F) (Fig. 3). In addition, the recombinant viruses produced viral titers within 1.5 logs of HSV-1(F) in both Vero and primary mouse embryo fibroblast cells (Jenkins and Martin, unpublished observations). There were no detectable differences in the appearance or size of the plaques produced by these viruses as compared to the parental viruses in any of the cell culture lines tested (data not shown).

All three recombinant viruses were selected and isolated by their ability to grow on human 143 TK^- cells in the presence of HAT medium (100 µM hypoxanthine, 400 µM aminopterin, 16 µM thymidine), and should be expressing a functional TK gene product (contained within the mini-Mu insertion). Previous reports from other laboratories have demonstrated that a functional TK gene plays an important role in HSV

Table 1 Determination of pfu/ld50 ratios for hsv-1(f)

ROUTE	AMOUNT (PFU)	# KILLED/ # INOCULATED	% MORTALITY	PFU/LD50*
	0	0/5	0	
	$2.3x10^4$	1/5	20	$>2.3x10^6$
I.P.	$2.3x10^5$	0/5	0	
	$2.3x10^6$	0/5	0	
Corneal	0	0/5	0	
	$1.7x10^5$	0/4	0	$>1.7x10^7$
	$1.7x10^6$	0/5	0	
	$1.7x10^7$	1/5	20	
I.C.	0	0/5	0	
	8	1/5	20	
	39	3/5	60	$3.1x10^1$
	78	3/5	60	

* PFU/LD50 ratios determined by the procedure of Reed and Muench (11).

induced pathogenicity in the mouse (14-16). Cells infected with the viral recombinants expressed greater than 93% of the TK activity found in cells infected with HSV-1(F) at 6 hours post-infection (hpi)(data not shown).

Studies on neurovirulence

To determine the effect of the deletions in the recombinant virus genomes on neurovirulence in the mouse, it was first necessary to determine the PFU/LD50 ratios for the wild-type parent HSV-1(F). We initially used three separate routes of inoculation (i.p., i.c., and scarified corneas) to measure neurovirulence since it has been reported that different strains of HSV vary greatly in their neurovirulence depending upon the route of inoculation (17-20). As shown in Table 1, while HSV-1(F) is quite avirulent by i.p. and corneal inoculation with PFU/LD50 ratios greater than $2.3x10^6$ and $1.7x10^7$ plaque forming units (pfu) respectively, i.c. inoculation demonstrated a PFU/LD50 ratio of 31, a value which agrees with previously published reports (21). Based on these results, neurovirulence studies with the recombinant viruses were confined to i.c. inoculations.The PFU/LD50 ratios for the recombinant viruses inoculated i.c. are shown in Table 2. The PFU/LD50 ratio for RBMu2 was $1.38x10^6$, while RBMu3 and REMu1 exhibited ratios of $1.06x10^5$ and $>2x10^6$ respectively. Thus, deletion of the internal repeat sequences has resulted in a dramatic increase, ranging from three to four logs, in the PFU/LD50 ratios of these three recombinants compared to that of the parental wild-type HSV-1(F) strain.

Studies on neuroinvasiveness

We define neuroinvasiveness as the ability of the virus to spread from a local site of infection (*e.g.* cornea) to the peripheral nervous system (trigeminal ganglion). To determine if the recombinant viruses were neuroinvasive, scarified corneas of 10 six week old BALB/c male mice were inoculated with $1x10^6$ pfu of either HSV-1(F), RBMu2, or RBMu3. Each day for 5 days following inoculation, two mice from each group were anesthetized and the eyes and trigeminal ganglia harvested. The tissue was processed for acute virus as described in Methods. Plaque assays were performed on each tissue sample with the results presented in Figure 4. The parental wild-type strain HSV-1(F) demonstrated a biphasic growth pattern in the eyes of the mice which agrees with previous reports using different strains of HSV-1 (22,23). Analysis of the trigeminal ganglion from these mice demonstrated the presence of infectious HSV-1(F) as early as day 2, reaching its peak titer by day 4. In contrast, the recombinants RBMu2 and RBMu3 demonstrated a greatly reduced amount of virus in the eyes, with a slight increase in viral titer past day 1. Since virus was detected through day 4, and a slight increase in viral titer was observed between days 1 and 4, we believe that the recombinants exhibit a limited replication in the corneas of infected mice. However, analysis of the trigeminal ganglia from mice inoculated with the recombinants failed to detect any infectious virus (data not shown), indicating that the recombinants were not transported, at least in an infectious form, to the trigeminal ganglion. Therefore, the deletion of the internal repeat sequences results in the virus becoming non-neuroinvasive. Further, while corneas inoculated with HSV-1(F) would demonstrate a severe keratitis by day 5 - 6 which would gradually heal by day 14, corneal inoculations with the recombinant viruses failed to show any visible signs of keratitis at any time after inoculation (data not shown).

Table 2. Determination of pfu/ld50 ratios for hsv-1 recombinant viruses by intracerebral inoculation

VIRUS	AMOUNT (PFU/BRAIN)	# KILLED/ # INOCULATED	% MORTALITY	PFU/LD50[1]
Control	0	0/5	0	
RBMu2	10	0/5	0	
	50	0/5	0	
	100	0/5	0	
	250	0/5	0	
	500	0/5	0	$1.38x10^6$
	$3.9x10^3$	0/5	0	
	$3.9x10^4$	0/5	0	
	$3.9x10^5$	0/5	0	
	$3.9x10^6$	9/10	90	
RBMu3	$4.5x10^3$	0/5	0	
	$4.5x10^4$	1/5	20	
	$4.5x10^5$	5/5	100	$1.06x10^5$
	$4.5x10^6$	10/10	100	
REMu1	$2.0x10^6$	1/5	20	$>2.0x10^6$

[1] PFU/LD50 ratios determined by the procedure of Reed and Muench (11).

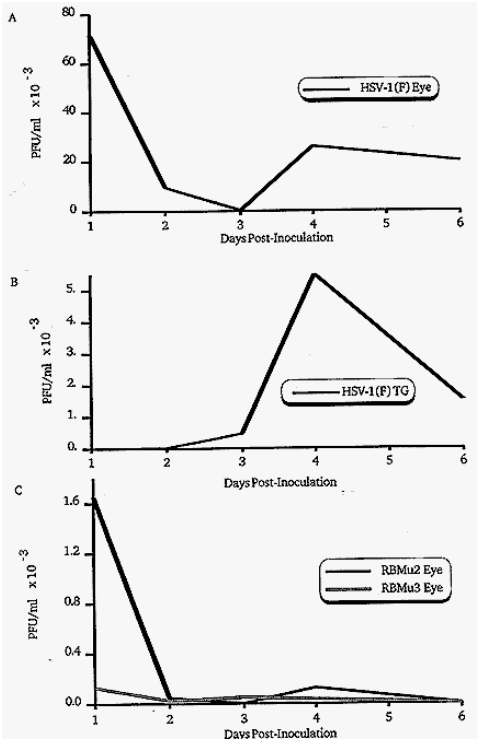

Figure 4. Growth of HSV-1(F) and the recombinant viruses RBMu2 and RBMu3 in the eye and HSV-1(F) in the trigeminal ganglion of mice.

Studies on latency

Two methods for studying HSV-induced latency involve either corneal or i.c. inoculations followed by the reactivation of latent virus from the trigeminal ganglia. Since the recombinant viruses were found to be non-neuroinvasive by corneal inoculation, it is unlikely that the viruses would establish a latent infection in trigeminal ganglia following corneal inoculation. This hypothesis is supported by the results shown in Table 3. Scarified corneas of mice were inoculated with $1x10^6$ pfu of either HSV-1(F) or each of the recombinant viruses. The trigeminal ganglion were harvested 21 days post inoculation and processed for the detection of latent virus as described in Methods. As shown in Table 3, HSV-1(F) was detected in 94% of the trigeminal ganglia, while no latent virus was rescued from mice inoculated with the recombinant viruses. As a more direct approach, we analyzed trigeminal ganglia for latent virus following sublethal i.c. inoculations. As seen in Table 4, while latent virus was readily detected from the trigeminal ganglion of mice inoculated with HSV-1(F), we were again unable to recover latent virus from mice inoculated with either of the three recombinant viruses. These results are particularly noteworthy since both HSV-1(F) and the recombinant viruses reach the trigeminal ganglion following i.c. inoculations. As shown in Table 5, acute virus can be detected through day 3 in the trigeminal ganglion of mice inoculated i.c. with either HSV-1(F) or any of the three recombinant viruses. Recent reports have shown that the use of either

Table 3. Determination of virus latency following corneal inoculation

VIRUS	# DAYS POST-INOCULATION	# POSITIVE GANGLIA/ # TESTED	% POSITIVE
HSV-1(F)	21	17/18	94.4
RBMu2	21	0/10	0
RBMu3	21	0/10	0
REMu1	21	0/18	0

Table 4. Detection of latent virus in trigeminal ganglion following i.c. inoculations

VIRUS	AMOUNT (PFU/BRAIN)	# LATENT GANGLIA/ # TESTED	% POSITIVE
CONTROL	0	0/10	0
HSV-1(F)	8	5/8	62.5
	39	4/4	100
	78	4/4	100
RBMu2	10	0/10	0
	50	0/9	0
	100	0/9	0
	250	0/7	0
	500	0/10	0
	$3.9x10^3$	0/10	0
	$3.9x10^4$	0/10	0
	$3.9x10^5$	0/10	0
	$4.7x10^4$ (22mM DMSO)	0/6	0
	$1x10^5$ (50µm AZC)	0/4	0
RBMu3	$4.5x10^3$	0/10	0
	$4.5x10^4$	0/8	0
	$4.5x10^4$ (22mM DMSO)	0/4	0
REMu1	$2.0x10^6$	0/2	0
	$4.3x10^4$ (22mM DMSO)	0/10	0

Table 5. Detection of acute virus in trigeminal ganglion following i.c. inoculations

VIRUS	INOCULUM TITER[1]	DAY[2]							
		1		2		3		4	
		Gang.[3]	Mice	Gang.	Mice	Gang.	Mice	Gang.	Mice
HSV-1	100	1/4	1/2	2/4	2/2	2/4	2/2	0/4	0/2
RBMu2	$1x10^6$	2/4	2/2	1/4	1/2	2/4	1/2	0/4	0/2
RBMu3	$1x10^6$	4/4	2/2	3/4	2/2	0/4	0/2	0/4	0/2
REMu1	$1x10^6$	2/4	2/2	2/8	2/4	0/4	0/2	0/4	0/2

[1] PFU inoculated per brain
[2] No. Positive/No. Tested
[3] gang. = ganglion

50µM 5-azacytidine (AZC) (24) or 22mM dimethylsulfoxide (DMSO) (25) can increase the reactivation of HSV from latently infected trigeminal ganglia. Attempts to reactivate recombinant virus from trigeminal ganglia following sublethal i.c. inoculations with either 50µM AZC or 22 mM DMSO were also unsuccessful (Table 4). Therefore, the recombinant viruses either do not establish a latent infection in trigeminal ganglion or latent virus is not reactivable by the procedures used in this study.

Viral replication in brain

The studies on neuroinvasiveness indicated that the recombinant viruses may be capable of a limited replication in the corneas of mice, since infectious virus could be isolated 4 - 5 days post inoculation (Fig. 4). To determine if the recombinants were capable of replicating in other tissues of the mouse, we determined viral growth curves from the brains of BALB/c mice following sublethal i.c. inoculations. As shown in Figure 5, the recombinant viruses were capable of a minimal replication, maintaining viral titers of approximately $1x10^3$ PFU/brain up to 4 days post inoculation.

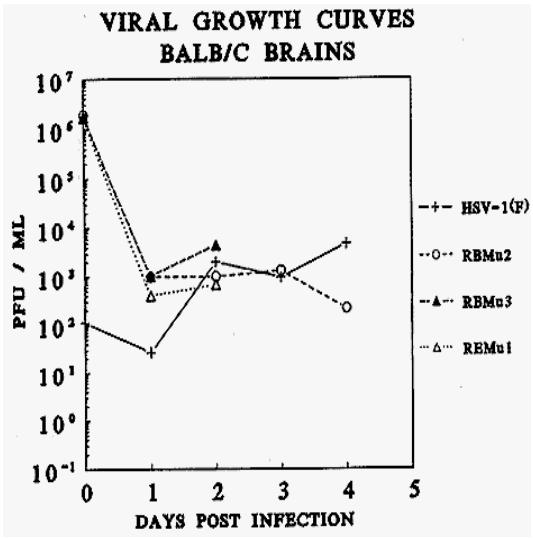

Figure 5. Growth curves of HSV-1(F) and the recombinant viruses in Balb/C brains following intracerebral inoculation.

DISCUSSION

In the course of a natural infection, herpes simplex virus encounters a number of different cell types and cellular environments. The virus infects animals at a peripheral site where it replicates locally before traveling through neural tissue to a sensory ganglion. Once at the ganglion the virus establishes a latent state which can, at a later date, become reactivated causing a recrudescent disease. Therefore several areas require investigation in the understanding of viral pathogenicity in the animal, including local replication, neuroinvasiveness (the spread of virus from the local site of replication to sensory ganglion), neurovirulence, and the establishment and maintenance of a latent state.

Studies designed to identify specific viral genes involved in these areas have demonstrated a complex involvement of several different regions of the HSV DNA genome. Centifanto-Fitzgerald *et al.* (26) reported that the region of the HSV genome defined by map units 0.70-0.83 was responsible for producing a stromal disease in the eyes of rabbits, while Thompson and coworkers have also reported that this region is involved in allowing HSV to replicate in neural tissue in the mouse (27-29). A subset of this region defined by map units 0.761 to 0.796 has recently been reported to be involved in intraperitoneal virulence in mice (17). Day *et al.* (30) have reported that the 0.40-0.44 region of the viral genome is involved in the spread of virus from corneas to the central nervous system. In addition, Meignier *et al.* (31) have shown that the deletion of several genes from the unique short region of the HSV genome results in a marked decrease in neurovirulence and latency. Based on these studies, it is clear that HSV neurovirulence, neuroinvasiveness and the ability of HSV to establish and maintain a latent state in animals is notdue to a single HSV gene product but can be affected by several viral gene functions.

The results presented in this paper demonstrate that the internal repeat sequences play an essential role in the replication and pathogenesis of HSV in the mouse. The use of three independently isolated recombinant viruses each containing similar but not identical deletions of the internal repeats strengthens our findings. The viral recombinants have a dramatically increased PFU/LD50 ratio by i.c. inoculation and do not establish a reactivable latent infection in the trigeminal ganglion even when inoculated directly into the brain. In addition, the recombinants are not neuroinvasive since they do not spread from the cornea to the trigeminal ganglion, and viral replication in mouse tissues is severely affected. The dramatic decrease in viral titer in both the corneas and brains of infected mice indicates that the recombinants exhibit at best, a limited replication.

The precise function of the internal repeat sequences in HSV pathogenesis is obscure. All of the viral genes and DNA sequences contained within these repeats are also located at the termini of the genome. Therefore deletion of these sequences decreases the copy number of the viral genes and sequences from diploid to haploid without completely removing any known viral gene from the genome. The internal a repeat sequences which have also been deleted in these recombinants are required for the inversion of the viral genome producing the four isomeric forms of viral DNA. However, the construction of the frozen recombinants used in this study clearly demonstrates that inversion *per se* and the

presence of diploid genes is not required for viral replication in cell culture. These results have recently been supported by Harland and Brown (32) who reported the construction of an HSV-2 deletion mutant which is missing the majority of the internal repeat structure and which grows well in cell culture.

The requirement for the internal repeats, therefore, is not obvious from *in vitro* studies of these recombinant viruses. However, since naturally occurring mutants with large deletions in these sequences or mutants that are frozen and unable to invert have never been described, it would seem likely that the internal repeat sequences are important in the natural environment of the virus.

The recombinant viruses used in this study exhibit high levels of TK activity even though the TK gene is not located in its normal position on the viral genome. Meignier *et al.* (31) reported that a HSV-1(F) recombinant containing a functional TK gene inserted into the coding region of the immediate early gene ICP47 was capable of establishing latency. Therefore the unusual location of the TK gene in the recombinants used in this study is not likely to be a factor in the decreased pathogenicity.

The DNA sequences deleted in RBMu2, RBMu3 and REMu1 correspond to map units 0.78 - 0.86 on the HSV genome in the prototype arrangement. This region would include some of the sequences identified by Thompson and coworkers (27-29) that are involved in the ability of HSV to replicate in neural tissue. It is possible that the frozen recombinants share defect(s) with the RE6 mutant of Thompson *et al*. but also must contain additional defects, since the RE6 mutant replicates well at peripheral sites and establishes a latent state in sensory ganglion, while the frozen recombinants used in this study do not replicate well peripherally and do not establish reactivable latent infections.

The work of Leib *et al.* (25) has indicated roles for ICP0 in viral latency and ICP4 and ICP27 in viral replication and neuroinvasiveness. Since both ICP4 and ICP27 have been shown to be essential for virus replication in cell culture (33-35) it is not surprising that viruses containing mutations in these genes are unable to replicate or exhibit neuroinvasiveness in the mouse. An appealing hypothesis for the results presented in this study is that the reduction of several viral genes, namely ICP0 and ICP4, from diploid to haploid in the viral genome are collectively responsible for the observed effects on pathogenicity. The potential role of inversion of the HSV genome also cannot be ruled out as a factor in the observed decrease in pathogenicity. The important role of the internal repeats in HSV pathogenicity may also explain why naturally occurring mutants containing deletions of the internal repeats have not been described.

ACKNOWLEDGMENTS

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