

Replication, Establishment of Latency, and Induced Reactivation of Herpes Simplex Virus γ_1 34.5 Deletion Mutants in Rodent Models

Richard J. Whitley,* Earl R. Kern,* Subhendra Chatterjee,* Joany Chou,† and Bernard Roizman‡

*The Departments of Pediatrics and Microbiology, The University of Alabama at Birmingham, Birmingham, Alabama 35294; and †The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, Chicago Illinois 60637

Abstract

Previous studies have shown that a gene mapping in the inverted repeats of the L component of herpes simplex virus, type 1 DNA, designated as γ_1 34.5, was dispensable for growth in cells in culture but that the deletion mutant (R3616) and a mutant containing a stop codon (R4009) in each copy of the gene were incapable of replicating in the central nervous systems (CNS) of mice. Restoration of the deleted sequences restored the wild type virus phenotype. We report here that the γ_1 34.5 mutant viruses (R3616 and R4009) replicated in the vaginal tract of two different strains of mice and guinea pig, although both viruses were shed at lower titer and for fewer days than the wild type and restored viruses. Both R3616 and R4009 failed to replicate or cause significant pathology in the cornea of Balb/C mice or following intranasal inoculation of Swiss Webster mice. Analyses of sensory trigeminal and dorsal root ganglia innervating the site of inoculation indicated that the incidence of establishment of latency or reactivation from latency by R3616 and R4009 viruses was significantly lower than that determined for mice infected with wild type or restored virus. Thus, selective deletion of γ_1 34.5 gene abolished the capacity of the virus to spread from peripheral mucosal sites to the CNS or replicate in the CNS, and diminished the capacity of the virus to replicate at mucosal sites and, subsequently, establish latency, or be able to be reactivated *ex vivo*. The results of our studies may have direct implications for the development of genetically engineered herpes simplex virus vaccines. (*J. Clin. Invest.* 1993. 91:2837–2843.) Key words: herpes simplex virus deletions • latency • rodent models

Introduction

The herpes simplex virus, type-1 (HSV-1)¹ genome consists of a linear double stranded DNA of 150 kilobase pairs (kbp) (1, 2). The genome consists of two covalently linked components, L and S. Each component consists of unique sequences (U_L or U_S) flanked by inverted repeats. The inverted repeats of the L

component designated as *ab* and *b'a'* are each approximately 9 kb in length whereas those of the S component designated as *a'c'* and *ca* are each approximately 6.5 kbp in length (3, 4). The two components invert relative to each other and one of the four equimolar isomers was designated as the prototype (5). The shared *a* sequence in the HSV-1 (F) strain is approximately 500 bp in length (6). Interest in the function of the *a* sequence led to the discovery that in one specific orientation it functions as a promoter (7). Subsequent studies revealed that the *a* sequence promotes the transcription of a RNA that encodes a protein 263 amino acids in length (7–9). The gene, designated as γ_1 34.5, is located in the *b* sequence of the inverted repeats flanking U_S sequences, between the *a* sequence and the transcription initiation of the $\alpha 0$ gene and, therefore, it is present in two copies per genome (4).

Interest in this gene stems from numerous observations that genomic sequences encoding factors or *cis*-acting sites associated with virulence map at the right terminus of the L component of HSV DNA when this DNA is displayed in the prototype arrangement. Centifano and colleagues utilized marker transfer experiments to move a fragment of HSV DNA from a virulent to an avirulent strain of HSV-1 (10). Removing genes located at the terminus of the U_L sequence of HSV DNA, a prototype HSV vaccine strain could be rendered avirulent (11). Other investigators, using HSV-1 X HSV-2 recombinants, demonstrated that insertion of HSV-2 sequences at the terminus of the HSV-1 U_L sequence resulted in an avirulent virus (12, 13). Virulence was with the insertion of the homologous HSV-1 fragment. Taha et al. (14–16) described spontaneous HSV-2 deletion mutants, lacking less than 1.5 kbp at the termini of the L component, which were less virulent than a recombinant obtained by marker rescue.

To determine the ability to grow in the central nervous system (CNS), one hallmark of neurovirulence, a series of mutant viruses were constructed (Fig. 1) and tested which of these viruses map within the coding sequences of γ_1 34.5. For mutant R3616, 1 kbp was deleted in both copies of the coding sequences of the gene. To insure that the phenotype of the mutant reflected the deletions only, the deleted sequences were restored in both copies of the gene by marker rescue, [HSV-1 (F)R]. To control for the possibility that the deletions cause gross perturbations of the viral genome, a stop codon was inserted into the open reading frame of both copies of the γ_1 34.5 gene. This mutant was designated as R4009. In studies published elsewhere, it was shown that the mortality resulting from direct inoculation into the brain of mutant viruses R3616 and R4009 was reduced by a factor of over six logs as compared to either the parent, HSV-1 (F), or the restored, HSV-1 (F)R, viruses (17). Although the γ_1 34.5 gene appears to be nonessential for viral replication in cell culture, the product of the gene appeared to be essential for the ability of the virus to replicate in the CNS and produce mortality in the mouse (17).

Address correspondence to Dr. Richard J. Whitley, Suite 616, ACC, 1600 Seventh Avenue South, Birmingham, AL 35294.

Received for publication 15 April 1992 and in revised form 5 January 1993.

1. Abbreviations used in this paper: BSC-1, basic cell culture number 1; HFF, human foreskin fibroblast cells; HSV-1, herpes simplex virus, type-1; MEF, primary mouse embryo fibroblasts; pfu, plaque-forming units; RK, primary rabbit kidney cells.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/93/06/2837/07 \$2.00

Volume 91, June 1993, 2837–2843

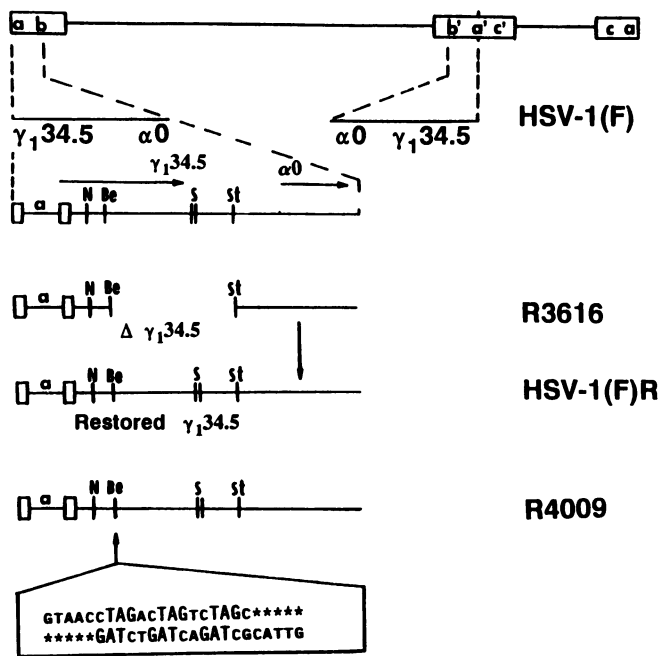


Figure 1. Schematic representation of sequence and gene arrangements of R3616, and R4009, HSV-1(F) and HSV-1(F)R viruses. The boxes on top line represent the inverted repeat sequence flanking the long and short components of HSV DNA represented by thin lines. The expanded domains of the repeat regions show the location of the $\gamma_134.5$ and $\alpha 0$ genes. The lines with arrows show the transcribed sequences and the direction of transcription. The diagram shows the sequences deleted from R3616, and the sequence and location of the stop codon inserted into both copies of $\gamma_134.5$ gene in the recombinant virus R4009. The procedures for the generation of the mutants and for the restoration of the deleted sequences were as described in reference 5. The abbreviations are N-, Be-, -, S-, and St- for NcoI, BstEII, SacI, and StuI restriction endonucleases.

We report for the first time, to our knowledge, studies which characterize the contribution of the $\gamma_134.5$ to the biologic properties of HSV-1 and, in particular, define the ability of these mutants to replicate in peripheral tissues of mice, to establish latency in the trigeminal and dorsal root ganglia, and to be reactivated from explanted sensory ganglia. We believe that these results are directly applicable to the development of live, attenuated, genetically engineered HSV vaccines.

Methods

Viruses and cells. The isolation and properties of HSV-1 (F) have been previously described (18). The construction of R3616, R4009, and the restored HSV-1(F)R have been described elsewhere (17). These viruses were passaged only once prior to freezing for future studies. Virus stocks were prepared in Vero cells or primary rabbit kidney (RK) cells and titered in BSC-1, primary mouse embryo fibroblasts (MEF) or RK cells. Additional cell lines utilized for these studies included Vero, the 143TK-, African green monkey kidney cell line BS-C-1, and human foreskin fibroblast (HFF) cells. The continuous cell lines are maintained routinely in our laboratories.

Viral assays. Monolayers of BSC-1, RK, or Vero cells in 24-well plates were infected with serial 10-fold dilutions of samples obtained from the vaginal tract or homogenized, clarified (600g for 20 min) brain, eye, or ganglionic tissues. These tissues were weighed and prepared as 10% (wt/vol) homogenates. Cultures were incubated at 37°

for 48–72 h. The pfu per milliliter or gram of tissue (pfu/ml or pfu/g) were calculated after the cells were stained with May-Grunwald-Giemsa, or neutral red.

Animals. Female Balb/C mice (3 wks old) or Swiss Webster (3 or 6 wks of age), obtained from Charles River Breeding Laboratories (Kingston, NY) were used in these experiments. Hartley strain female guinea pigs (250–350 g) were purchased from Charles River Breeding Laboratories, and housed for 1 wk prior to virus inoculation.

Experimental viral infections. Three model infections were utilized to assess the ability of these viruses to replicate and cause pathology in peripheral and CNS tissues of mice. The first model evaluated accessions and replication of these viruses in the CNS and trigeminal ganglia following intranasal inoculation of 10^5 to 10^6 pfu per 0.03 ml directly into the nares of 3-wk-old Swiss Webster mice (19). Animals (five mice per group) were killed on days 1, 3, 5, and 7. Trigeminal ganglia and brain tissue were harvested, homogenized (10% wt/vol), and assayed on RK cells to determine the quantity of infectious virus. Trigeminal ganglia were removed from parallel groups of animals on day 28 for quantitation of latent virus. The second model evaluated the ability of these viruses to replicate in the eye, CNS, and trigeminal ganglia following inoculation by the ocular route (20). Suspensions of virus preparations at a concentration of $10^{3.8}$ to $10^{5.2}$ were inoculated on the surface of scarified cornea (20 μ l/eye) of Balb/C mice bilaterally. The eyes were examined sequentially for evidence of ocular disease. These examinations consisted of visual assessment of conjunctival edema and erythema, corneal lesions, and corneal opacification. Mice (five mice per group) were killed on days 1, 2, 3, 4, 5, and 7, and both eyes from each animal were harvested for quantitation of infectious virus. Tissue homogenization and inoculation were performed as noted above. Additionally, 10 mice were infected at day 0, killed at day 30, and the trigeminal ganglia processed, as noted below, for evaluation of latent virus. The third model evaluated the ability of these viruses to replicate in the vaginal tract, invade into local peripheral nerves, and establish latency in the sensory ganglia of both Balb/C and Swiss Webster mice after intravaginal inoculation (20). 20 Swiss Webster and 25 Balb/c mice per virus were inoculated intravaginally with 10^4 or 10^6 pfu in 50 μ l with cotton-tipped applicators. Vaginal swabs were collected on days 1, 3, 5, and 7 for quantitation of infectious virus. Sacral-dorsal ganglia were removed from the Swiss Webster mice (20 mice per group) on days 1, 3, 5, and 8 postinfection, homogenized, and assayed for the presence of infectious virus. In addition, 10 Balb/C and 5 Swiss Webster mice per virus were killed on day ~ 30, and sacral ganglia were collected for reactivation of latent virus.

Finally, groups of 10 guinea pigs were inoculated intravaginally with 10^6 pfu's of each of the four viruses (21–23). Infection was accomplished by use of virus-soaked cotton tip applicators. On days 1, 3, 5, 7, and 10 postinfection, vaginal swabs were collected, added to 2.0 ml of medium, and frozen until titrated for infectious virus. On days 2, 5, and 8 postinoculation, three animals from each virus group were killed, sacral-dorsal root ganglia were removed, homogenized in 1.0 ml of medium, clarified at 600 g, and serial tenfold dilutions were assayed on RK cells for infectious virus.

Determination of latent virus infections. The frequency of establishment of latency and the quantity of latent virus recovered per ganglion were determined in groups of five animals per virus, following either ocular, intranasal, or genital inoculation. In representative experiments, bilateral trigeminal and sacral-dorsal ganglia were removed from surviving animals. The procedures for virus reactivation and for measurement of latent virus have been previously described (22). Briefly, ganglia were incubated in culture tubes with 1 ml of growth medium for 5–7 d in a CO₂ incubator to reactivate latent virus. Ganglia were then homogenized and titered in BSC-1 or RK cells. Representative ganglia were stored at -95°C for DNA extraction.

Isolation of DNA from ganglia and analysis on ganglionic DNA for viral DNA sequences by polymerase chain reaction. Isolation and extraction of DNA from mouse ganglia have been described (24). The primers, probes, and the reaction conditions used in the PCR reaction and the Southern blot analysis of the PCR products were identical to

those as described (24), except that washing procedures of the Southern blots were carried out at 46°C rather than 50°C as reported.

Results

Replication of mutants in vitro

Replication competence of HSV-1(F), R3616, R4009, and HSV-1(F)R was determined in MEF cells. The moi was 0.01. For HSV-1(F) and HSV-1(F)R, peak viral titers of 10⁶ TCID₅₀/ml of sample were achieved 48–72 h after inoculation. With R3616 and R4009, peak titers were attained later at 72–96 h after infection and at slightly lower quantities of 10⁴ TCID₅₀/ml of sample.

Pathogenesis of acute infection

Intranasal infection. After intranasal inoculation of either HSV-1(F) or HSV-1(F)R in Swiss Webster mice, virus was readily detected in both the trigeminal ganglia and brain within three days of infection, as shown in Table I. The quantity of virus peaked ~ 5 d after infection with both viruses. In contrast, R3616 and R4009 were not detected either in the ganglia or brain with the exception of one animal. Furthermore, the quantity of virus detected in the brain of this mouse was significantly lower than that following infection with HSV-1(F) or HSV-1(F)R. We repeated these experiments in Balb/C mice and obtained virtually identical results.

Ocular infection. As shown in Table II, HSV-1(F) and HSV-1(F)R also replicated well in corneal tissue of Balb/C mice. All animals became infected and the quantity of virus recovered was similar for both viruses. In contrast, R3616 and

Table II. Acute Replication of HSV-1, (F) and Mutant Strains in the Eye of Balb/C Mice

Virus (Inoculum)*	Day post- inoculation	No. positive/ No. tested	Mean peak [‡] ocular yield [‡]	Mean latent virus titer [§]
				No. positive/ No. tested
				<i>PFU/ml</i>
HSV-1-(F) (7.1)	1	5/5	5.2	
	2	5/5	4.7	
	3	5/5	4.0	5.8
	4	5/5	3.8	(10/10)
	5	5/5	4.0	
R3616 (7.1)	1	0/5	< 1.0	
	2	0/5	< 1.0	
	3	0/5	< 1.0	< 1.0
	4	0/5	< 1.0	(0/10)
	5	0/5	< 1.0	
R4009 (7.1)	1	0/5	< 1.0	
	2	0/5	< 1.0	
	3	0/5	< 1.0	1.8
	4	0/5	< 1.0	(1/10)
	5	1/5	1.0	
HSV-1-(F)R (7.2)	1	5/5	3.6	
	2	5/5	3.6	
	3	5/5	3.5	4.8
	4	5/5	3.3	(10/1)
	5	5/5	3.0	
	7	5/5	3.2	

* Log₁₀ titer.

[‡] Log mean of virus titers from 5 animals. The titers are expressed as log₁₀ pfu/g tissue.

[§] Ganglia were harvested 30 d postinoculation. The titer is expressed as log₁₀ mean.

Table I. Replication of HSV-1 F and Mutant Viruses After Intranasal Inoculation of Swiss Webster Mice

Virus	Day post- inoculation	No. positive/No. tested		Virus titers*		Latent virus titers
		Ganglia [‡]	Brain	Ganglia [‡]	Brain	Ganglia [§]
F	1	3/5	1/5	1.4	0.3	7.0 (5/5)
	3	5/5	3/5	3.7	1.6	
	5	5/5	5/5	3.9	4.1	
	7	5/5	5/5	2.7	3.1	
R3616	1	0/5	0/5	< 1.0	< 1.0	0.5 (1/5)
	3	0/5	0/5	< 1.0	< 1.0	
	5	0/5	0/5	< 1.0	< 1.0	
	7	0/5	0/5	< 1.0	< 1.0	
R4009	1	0/5	0/5	< 1.0	< 1.0	2.5 (2/5)
	3	0/5	1/5	< 1.0	0.4	
	5	0/5	0/5	< 1.0	< 1.0	
	7	0/5	0/5	< 1.0	< 1.0	
F (R)	1	4/5	4/5	1.5	1.5	6.9 (5/5)
	3	5/5	5/5	4.2	2.8	
	5	5/5	5/5	3.2	3.9	
	7	3/5	5/5	1.3	3.6	

* Average of virus titers from 5 animals expressed as log₁₀ pfu per gram of tissue.

[‡] Trigeminal ganglia.

[§] Trigeminal ganglia removed 28 d postinfection. Average virus titer from 5 mice expressed as log₁₀ pfu per gram of tissue (No. positive/No. tested).

R4009 replicated poorly in corneal tissue. Overall, only one mouse inoculated with the R3616 and one with R4009 yielded detectable virus on days 7 and 5, respectively. When these experiments were repeated in Swiss Webster mice, HSV-1(F) and HSV-1(F)R behaved in an identical fashion as that following inoculation of Balb/C mice. For R3616 and R4009, we could not detect evidence of viral replication.

Ocular disease was significantly greater in mice inoculated with HSV-1(F) and HSV-1(F)R. By day 3 following inoculation with one of these two viruses, all mice exhibited ocular disease, including erythema, injection, corneal edema, and opacification of the cornea. In contrast, animals inoculated with R3616 and R4009 had virtually no ocular disease (only conjunctival erythema in two animals in each virus group) which appeared on day four and resolved over the next 4 d. No Swiss Webster mouse which was inoculated with R3616 or R4009 developed ocular disease.

Mouse vaginal infection. Inbred (Balb/C) and outbred (Swiss Webster) mice were selected for evaluation of the ability of these viruses to replicate in the genital tract in order to exclude differences in strains. As a preliminary experiment, we proved clearance of input virus by sampling vaginal secretions

Table III. Acute Replication of HSV-1(F) and Mutant Strains in the Vagina of Balb/C Mice

Virus (Inoculum)*	Day post-infection	No. infected/ No. tested	Mean peak vaginal yield [‡]
HSV-1(F) (7.1)	0.6 h	5/5	2.3
	1	5/5	4.3
	3	5/5	6.8
	5	5/5	4.8
	7	5/5	5.0
R3616 (7.1)	0.6 h	5/5	3.0
	1	5/5	3.1
	3	5/5	3.0
	5	1/5	0.3
	7	0/5	< 1.0
R4009 (7.1)	0.6 h	5/5	4.0
	1	5/5	4.6
	3	5/5	3.8
	5	5/5	2.4
	7	3/5	1.2
HSV-1(F)R (7.2)	0.6 h	5/5	4.1
	1	5/5	4.5
	3	5/5	5.2
	5	5/5	4.9
	7	5/5	4.3

* Log₁₀ titer.

[‡] Log mean of virus titers from five animals. The titers are expressed as log₁₀ pfu/ml inoculum.

at 3, 6, 9, 12, and 24 h after inoculation. Virus was cleared from all animals by 9 h after infection. As shown in Table III, 3 d after intravaginal inoculation of Balb/c mice, peak quantities of HSV-1(F) and HSV-1(F)R could be detected in vaginal secretions. All animals became infected and remained so throughout the 7-d study. In contrast, R4009 and R3616 vi-

ruses were readily detected during the first 3 days; however, peak titers were lower and these viruses were cleared faster. Furthermore, R4009 was detectable in the genital tract of the Balb/C mice on day 7, whereas R3616 was not.

Manifestations of disease was greatest with HSV-1(F) and HSV-1(F)R, consisting of edema, intense erythema, and numerous ulcerative lesions. In contrast, clinical disease was less evident in mice infected with R4009 or R3616. Among mice infected with these viruses, erythema was present in all animals but only on days 1 and 3. Whereas no Balb/C mouse developed hind limb paralysis or death following vaginal inoculation with R4009 and R3616, ~ 20% of animals infected with HSV-1(F) and HSV-1(F)R developed hind limb paralysis.

These studies were repeated and extended in Swiss Webster mice. The results of two separate experiments investigating the differences in the ability of these four viruses to replicate in the genital tract and spinal ganglia, cause diseases, and become reactivated on explantation of sacral ganglia, are illustrated in Table IV and Fig. 2 A. Several conclusions are relevant: (a) The peak vaginal virus titer of HSV-1(F) and HSV-1(F)R from day 1 to day 8 was, on the average, over 100-fold greater than that of R3616 or R4009. (b) In these experiments, we detected larger quantities of both R3616 and R4009 than those performed in the Balb/c mice but still significantly less than that found in the mice infected with HSV-1(F) or HSV-1(F)R. Virus replication of the mutants in the vaginal tract was of shorter duration compared to the parental and restored viruses. (c) There was no mortality in mice infected with either R3616 or R4009 whereas the mortality exceeded 50% for both HSV-1(F) and HSV-1(F)R (Table IV). (d) The mice infected with HSV-1(F) or HSV-1(F)R exhibited considerable genital disease consisting of erythema, edema, ulcerations of perianal and perivaginal skin, and paralysis. Essentially, no disease was observed in mice inoculated with R3616 or R4009.

Guinea pig vaginal infection. To determine if species differences existed following intravaginal inoculation, we repeated the vaginal inoculations in female Hartley guinea pigs. First, we documented clearance of input virus by obtaining vaginal

Table IV. Replication and Latency of HSV-1, F and Mutant Viruses After Intravaginal Inoculation in Swiss/Webster Mice

Virus	No. infected/ No. inoculated	Mortality*	Peak vaginal virus titers [‡]	Peak disease scores [§]	Virus titers	
					Acute ganglia [¶]	Latent ganglia ^{**}
F	20/20	13/20	5.6	2.3	4.6 (8/20)	6.8 (3/5)
	10/10	9/10	6.0			
R3616	15/20	0/15	2.7	0.1	2.8 (2/20)	2.6 (2/5)
	10/10	0/10	3.5			
R4009	16/20	0/16	3.9	0.1	ND ^{**} (0/20)	4.6 (3/5)
	10/10	0/10	4.5			
F(R)	19/10	11/19	5.2	2.1	4.3 (11/20)	6.9 (4/4)
	10/10	6/10	5.6			

* Mortality expressed as No. dead/No. infected. Infected animals were identified by positive vaginal virus culture.

[‡] Mean peak virus titer expressed as TCID₅₀ log₁₀ per ml of sample.

[§] Mean peak disease score.

^{||} Average virus titers of positive mice, expressed as log₁₀ pfu per g of tissue.

[¶] Sacral ganglia harvested on days 1, 3, 5, and 8 postinoculation (No. positive/No. tested).

^{**} Sacral ganglia harvested on day 32 postinoculation (No. positive/No. tested).

^{††} Not detected.

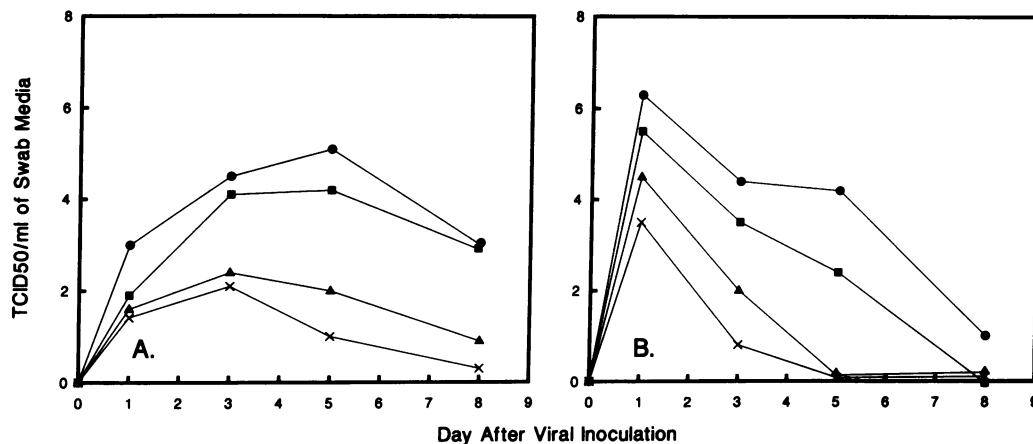


Figure 2. The quantitative evaluation of the replication of HSV-1(F) [●], HSV-1(F)R [■], R4009 [△], and R3616 [×] in the vaginal tract of mice (A) and guinea pigs (B). Each point represents a mean of 10 animals.

swabs at 3, 6, 9, 12, and 24 h after infection. All animals cleared the input inoculum by 12 h. As shown in Fig. 2 B and Table V, the peak virus titers in vaginal swabs from guinea pigs infected with R3616 and R4009 was lower by a factor of 10- to 100-fold compared to HSV-1(F) and HSV-1(F)R. Larger quantities of both HSV-1(F) and HSV-1(F)R for longer periods of time were detected compared to R3616 and R4009.

Guinea pigs inoculated with HSV-1(F) developed external lesions on the genital skin with peak scores of 1.5. In contrast, animals inoculated with HSV-1(F)R, R3616, and R4009 rarely developed a genital lesion.

We were unable to detect infectious or latent virus in the ganglia of guinea pigs infected with R3616 or R4009. However, only 2 of 18 animals which received wild-type or restored virus had virus detectable in the ganglia during either acute or latent infection.

Reactivation of latent virus from murine ganglia. One objective of these studies was to determine whether the mutants incapable of expressing $\gamma_1 34.5$ established latency and could be reactivated. Bilateral trigeminal and sacral-dorsal ganglia of infected mice were tested for the presence of reactivable virus. HSV-1(F) and HSV-1(F)R were recovered uniformly from both trigeminal (Table I and Table II) and sacral ganglia (Tables III and IV). The quantities of virus present in these infected ganglia were 10^7 pfu from trigeminal ganglia of Swiss

Webster mice inoculated intranasally and 7.0×10^5 and 7.0×10^4 pfu from the trigeminal ganglia of Balb/C mice inoculated by the ocular route. Approximately 10^7 pfu (both viruses) from the sacral ganglia of Swiss Webster mice inoculated intravaginally were recovered (Table IV). In contrast, R3616 could be detected in only one of the 30 trigeminal ganglia tested in these studies and R4009 was found in only three trigeminal ganglia.

Analyses of murine trigeminal ganglia for the presence of latent virus by polymerase chain reaction. Trigeminal ganglia from mice infected with HSV-1(F), R4009, R3616, and mock infected mice were tested 30 d after infection with these viruses. As shown in Fig. 3, viral DNA was readily detected in mice infected with HSV-1(F). The average quantity of virus recovered was ~ 0.1 viral DNA equivalent/ganglionic cell DNA equivalent. In contrast, the amounts of R3616 and R4009 were much lower; in the few ganglia that were positive, only between 0.001 and 0.01 viral DNA equivalents per ganglionic cell DNA equivalent were obtained.

Discussion

The studies described in this report extend the previous observations that HSV-1 mutants lacking a functional $\gamma_1 34.5$ gene were incapable of replicating in and destroying the CNS of

Table V. Replication and Latency of HSV-1, F and Mutant Viruses After Intravaginal Inoculation in Guinea Pigs

Virus	No. infected/ No. inoculated	Vaginal virus titers		Lesion scores		Virus titers ¹	
		AUC*	Peak [‡]	AUC [§]	Peak	Acute ganglia**	Latent ganglia**
F	11/11	30.5	6.6	7.7	1.5	0.2 (1/9)	0.0 (0/)
R3616	12/12	7.2	3.5	0.1	0.1	0.0 (0/9)	0.0 (0/5)
R4009	12/12	10.9	4.5	0.3	0.1	0.0 (0/9)	0.0 (0/5)
F(R)	12/12	20.7	5.4	0.2	0.2	0.3 (1/9)	0.0 (0/5)

* AUC, Area under the vaginal virus titer-day curve.

[‡] Mean peak virus titer expressed as TCID₅₀ per ml of sample.

[§] AUC, Area under the lesion score-day curve.

^{||} Mean peak lesion score, based on a 0-4+ scale in 0.5 increments.

¹ Average virus titers, expressed as log₁₀ pfu per g of tissue (number positive out of number tested).

** Sacral-dorsal ganglia harvested on days 2, 5, and 8 postinoculation.

** Sacral-dorsal ganglia harvested on day 31 postinoculation.

^{|||} Not detectable.

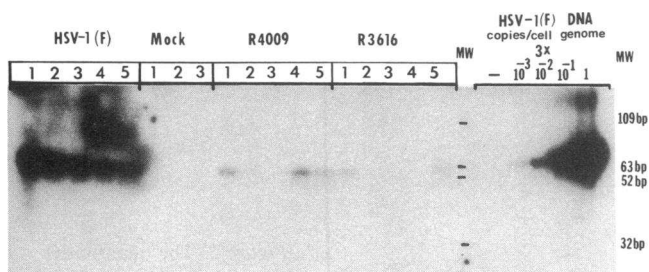


Figure 3. Autoradiographic images of the electrophoretically separated DNA fragments amplified by PCR, transferred to nitrocellulose sheet and hybridized with HSV-1 DNA probe as described in Methods. The PCR analyses were done on DNA extracted from trigeminal ganglia of mice inoculated by ocular route with HSV-1 (F), R4009, and R3616, or with tissue culture medium. Only the left ganglia from those animals were used. For quantitation, per cell genome equivalent was spiked into the mock ganglia No. 2 and used as standards as indicated in the rightmost lane. HSV-1(F) DNA was then serially 10-fold diluted for the PCR reaction to indicate 10-fold reduction in copy number in the ganglia. DNA molecular weight standards shown in the far right were run to indicate the correct PCR size products as expected.

mice following direct intracerebral inoculation (17). The salient features of the results presented in this report are that viruses lacking $\gamma_134.5$ (*a*) failed to multiply in the cornea or cause significant eye pathology following eye inoculation; (*b*) had a significantly reduced capacity to spread into the CNS following intranasal inoculation; (*c*) replicated, but to a reduced level in the vaginal tract of two strains of mice and in guinea pigs; (*d*) could be reactivated from trigeminal ganglia of mice inoculated by the ocular or intranasal routes only at very low levels, and analyses of the sensory ganglia by PCR indicated that the amounts of viral DNA were 10- to 100-fold lower than those present in ganglia of mice infected with the wild-type parent; (*e*) was reactivated from about 50% of sacral ganglia of mice infected by the intravaginal route. Thus, this gene exerts its function(s) in tissues other than just the CNS.

The significance of these studies may be summarized as follows:

(*a*) A striking feature of $\gamma_134.5$ virus to emerge from these studies is that the requirement for the gene product for HSV replication is not uniform in all tissues. Thus, the deletion mutants replicated in the vaginal tract but not in the cornea or the CNS. In the absence of additional information regarding this gene and the activity of its expressed protein product, we must conclude that vaginal mucosal cells or other localized host cells of mice express a function which complements the virus missing this gene, whereas corneal or CNS cells do not complement the missing gene. We do note that these deletion mutant viruses do not replicate as well as parental or restored strains in primary MEF cells. This finding is not unexpected, as a large number of deletion mutants tested to date exhibit at least some deficiency in the capacity to replicate both *in vitro* and *in vivo*. Some of the deletion mutants showed a reduced capacity to replicate in rodent cells (25).

(*b*) It has been reported that a number of HSV-1 mutants, from which genes not essential for replication in cells in culture had been deleted, either failed to establish latency or could not be reactivated from the latent state (26, 27). These deletion mutants could only be reactivated at a very low frequency. One

explanation for this observation is that in the absence of replication in the cornea and nasopharynx, the amount of virus which reached the trigeminal neurons and CNS were considerably lower than that which reached the sacral ganglia from the vaginal tract—a site at which the virus did multiply. We should also note recovery of reactivated virus depends on its ability to multiply initially in the sensory neuron and subsequently in the cocultivated nonneuronal cells. Inasmuch as R3616 does not multiply efficiently in neuronal cells *in situ*, it is conceivable that the small amount of virus recovered from sensory ganglia may represent neuronal cells with a very high genome copy number.

(*c*) Our studies suggest that the $\gamma_134.5$ mutants exhibit properties desirable in an ideal attenuated HSV-1 vaccine. This conclusion is based on the recognition that, above all other manifestations of pathology induced by HSV, the most hazardous to health is the ability of this virus to access and replicate in the CNS of both newborn and adults. In consequence, elimination of the capacity of HSV to multiply in the CNS and, coincidentally in the eye (a major cause of blindness in developed societies), would be a major safety requirement of for a live HSV vaccine. We should note, however, that it is possible to over-attenuate a HSV mutant and, therefore, negate its utility as a vaccine (11).

(*d*) The observation that the $\gamma_134.5$ virus fails to replicate in the CNS could potentially be exploited to selectively destroy tumor cells without affecting CNS tissue, as has recently been reported (28, 29). Specifically, when R3616 is inoculated directly into glioma cell lines *in vitro*, viral replication progresses without impediment. Recently, we reported that the 34.5 gene functions to prevent virus mediated shut-off of protein synthesis in neuroblastoma cells (30). These observations may have relevance for adjunctive therapy of CNS tumors of nonneuronal origin.

Among the many remaining unresolved questions are the actual functions of $\gamma_134.5$ at the molecular level, its precise role in the pathogenesis of disease(s), and whether the immune response to the gene product is a determinant of the outcome of infection. Studies designed to resolve these issues are in progress.

Acknowledgments

We wish to thank Samita Sarkar, Peggy Vogt, and Joyce Palmer for technical assistance.

These studies were aided by grants from the National Cancer Institute (CA47451) and the National Institute for Allergy and Infectious Diseases (AI124009, AI15098, and AI1588-11), the United States Public Health Service, and the State of Alabama.

References

1. Kieff, E. D., S. L. Bachenheimer, and B. Roizman. 1971. Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. *J. Virol.* 8:125-132.
2. McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor. 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.* 69:1531-1574.
3. Sheldrick, P., and N. Berthelot. 1975. Inverted repetitions in the chromosome of herpes simplex virus. *Cold Spring Harbor Symp. Quant. Biol.* 39:667-678.
4. Wadsworth, S., R. J. Jacob, and B. Roizman. 1975. Anatomy of herpes simplex virus DNA. II. Size, composition, and arrangements of inverted terminal repetitions. *J. Virol.* 15:1487-1497.

5. Hayward, G. S., R. Jacobs, S. Wadsworth, and B. Roizman. 1975. Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short segments. *Proc. Natl. Acad. Sci. USA.* 72:4243-4247.
6. Mocarski, E. S., and B. Roizman. 1982. The structure and role of the herpes simplex virus DNA termini in inversion, circularization, and generation of virion DNA. *Cell.* 31:89-97.
7. Chou, J., and B. Roizman. 1986. The terminal *a* sequence of the herpes simplex virus genome contains the promoter of a gene located in repeat sequences of the L component. *J. Virol.* 57:629-637.
8. Ackermann, M., J. Chou, M. Sarmiento, R. A. Lerner, and B. Roizman. 1986. Identification by antibody to a synthetic peptide of a protein specified by a diploid gene located in the terminal repeats of the L component of herpes simplex virus genome. *J. Virol.* 58:843-850.
9. Chou, J., and B. Roizman. 1990. The herpes simplex virus 1 gene 34.5 mapping in inverted repeats is conserved in several limited passage isolates but not in strain 17 syn+. *J. Virol.* 64:1014-1020.
10. Centifanto-Fitzgerald, Y., T. Yamakuchi, H. Kaufman, M. Tognon, and B. Roizman. 1982. Ocular disease pattern induced by herpes simplex virus is genetically determined by a specific region of viral DNA. *J. Exp. Med.* 155:475-489.
11. Meignier, B., R. Longnecker, and B. Roizman. 1988. In vivo behavior of genetically engineered herpes simplex viruses R7017 and R7020: construction and evaluation in rodents. *J. Infect. Dis.* 158:602-614.
12. Javier, R. T., R. L. Thompson, and J. G. Stevens. 1987. Genetic and biological analyses of a herpes simplex virus intertypic recombinant reduced specifically for neurovirulence. *J. Virol.* 65:1978-1984.
13. Thompson, R. L., S. K. Rogers, and M. A. Zerhusen. 1989. Herpes simplex virus neurovirulence and productive infection of neural cells is associated with a function which maps between 0.82 and 0.832 map units on the HSV genome. *Virology.* 172:435-450.
14. Taha, M. Y., G. B. Clements, and S. M. Brown. 1989. A variant of herpes simplex virus type 2 strain HG52 with a 1.5 kb deletion in RL between 0 to 0.02 and 0.81 to 0.83 map units is non-neurovirulent for mice. *J. Gen. Virol.* 70:705-716.
15. Taha, M. Y., G. B. Clements, and S. M. Brown. 1989. The herpes simplex virus type 2 (HG52) variant JH2604 has a 1488 bp deletion which eliminates neurovirulence in mice. *J. Gen. Virol.* 70:3073-3078.
16. Taha, M. Y., S. M. Brown, G. B. Clements, and D. I. Graham. 1990. The JH2604 deletion variant of herpes simplex virus type 2 (HG52) fails to produce necrotizing encephalitis following intracranial inoculation of mice. *J. Gen. Virol.* 71:1597-1601.
17. Chou, J., E. R. Kern, R. J. Whitley, and B. Roizman. 1990. Mapping of herpes simplex virus -1 neurovirulence to γ 1 34.5, a gene nonessential for growth in culture. *Science (Wash. DC).* 250:1262-1266.
18. Ejercito, P. M., E. D. Kieff, and B. Roizman. 1968. Characterization of herpes simplex virus strains differing in their effect on social behavior of infected cells. *J. Gen. Virol.* 2:357-364.
19. Kern, E. R., J. T. Richards, and J. C. Overall Jr. 1986. Acyclovir treatment of disseminated herpes simplex virus type 2 infection of mice: Alteration of mortality and pathogenesis. *Antiviral Res.* 6:189-195.
20. Kern, E. R., J. T. Richards, J. C. Overall Jr., and L. A. Glasgow. 1983. Acyclovir treatment of experimental genital herpes simplex virus infections. I. Topical therapy of type 2 and type 1 infections of mice. *Antiviral Res.* 3:253-267.
21. Berman, P. W., P. E. Vogt, T. Gregory, L. A. Lasky, and E. R. Kern. 1988. Efficacy of recombinant glycoprotein D subunit vaccines on the development of primary, recurrent, and latent genital infections with herpes simplex virus type 2 in guinea pigs. *J. Infect. Dis.* 157:897-902.
22. Stanberry, L. R., E. R. Kern, J. T. Richards, and J. C. Overall Jr. 1985. Recurrent genital herpes simplex virus infection in guinea pigs. *Intervirology.* 24:226-231.
23. Stanberry, L. R., E. R. Kern, J. T. Richards, T. M. Abbott, and J. C. Overall, Jr. 1982. Genital herpes in guinea pigs: Pathogenesis of the primary infection and description of recurrent disease. *J. Infect. Dis.* 146:397-404.
24. Katz, J. P., E. Bodin, and D. Coen. 1990. Quantitative polymerase chain reaction analysis of herpes simplex virus DNA in ganglia of mice infected with replication-incompetent mutants. *J. Virol.* 64:4288-4295.
25. Sears, A. E., I. W. Halliburton, B. Meignier, S. Silver, and B. Roizman. 1985. Herpes simplex virus mutant deleted in the *alpha* 22 gene: growth and gene expression in permissive and restrictive cells, and establishment of latency in mice. *J. Virol.* 55:338-346.
26. Coen, D. M., M. Kosz-Vnenchak, J. G. Jacobson, D. A. Leib, C. L. Bogard, P. A. Schaffer, K. L. Tyler, and D. M. Knipe. 1989. Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. *Proc. Natl. Acad. Sci. USA.* 86:4736-4740.
27. Jacobson, J. G., D. A. Leib, D. J. Goldstein, D. L. Bogard, P. A. Schaffer, S. K. Weller, and D. M. Coen. 1989. A herpes simplex virus ribonucleotide reductase deletion mutant is defective for productive acute and reactivate latent infections of mice and for replication in mouse cells. *Virology.* 173:276-283.
28. Martuza, R. L., A. Malick, J. M. Markert, K. L. Ruffner, and D. M. Coen. 1991. Experimental therapy of human glioma in nude mice by means of a genetically engineered virus mutant. *Science (Wash. DC).* 252:854.
29. Markert, J. M., A. Malick, D. M. Coen, and R. L. Martuza. 1993. Reduction and elimination of encephalitis in an experimental glioma therapy model with attenuated herpes simplex mutants that retain susceptibility to acyclovir. *Neurosurgery.* 32:597-603.
30. Chou, J., and B. Roizman. 1992. The gamma-1 34.5 gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. *Proc. Natl. Acad. Sci. USA.* 89:3266-3270.