An Avirulent ICP34.5 Deletion Mutant of Herpes Simplex Virus Type 1 Is Capable of In Vivo Spontaneous Reactivation

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The herpes simplex virus type 1 (HSV-1) ICP34.5 gene is a neurovirulence gene in mice. In addition, some ICP34.5 mutants have been reported to have a reduced efficiency of induced reactivation as measured by in vitro explantation of latently infected mouse ganglia. However, since spontaneous reactivation is almost nonexistent in mice, nothing has been reported on the effect of ICP34.5 mutants on spontaneous reactivation in vivo. To examine this, we have deleted both copies of the ICP34.5 neurovirulence gene from a strain of HSV-1 (McKrae) that has a high spontaneous reactivation rate in rabbits and used this mutant to infect rabbit eyes. All rabbits infected with the ICP34.5 mutant virus (*d34.5*) survived, even at challenge doses greater than 4×10^7 PFU per eye. In contrast, a 200-fold-lower challenge dose of 2×10^5 PFU per eye was lethal for approximately 50% of rabbits infected with either the wild-type McKrae parental virus or a rescued ICP34.5 mutant in which both copies of the ICP34.5 gene were restored. In mice, the 50% lethal dose of the ICP34.5 mutant was over 10^6 PFU, compared with a value of less than 10 PFU for the rescued virus. The ICP34.5 mutant was restricted for replication in rabbit and mouse eyes and mouse trigeminal ganglia in vivo. The spontaneous reactivation rate in rabbits for the mutant was 1.4% as determined by culturing tear films for the presence of reactivated virus. This was more than 10-fold lower than the spontaneous reactivation rate determined for the rescued virus (19.6%) and was highly significant ($P < 0.0001$, Fisher exact test). Southern analysis confirmed that the reactivated virus retained both copies of the ICP34.5 deletion. Thus, this report demonstrates that (i) the ICP34.5 gene, known to be a neurovirulence gene in mice, is also important for virulence in rabbits and (ii) in vivo spontaneous reactivation of HSV-1 in the rabbit ocular model, although reduced, can occur in the absence of the ICP34.5 gene.

In 1983, Thompson and Stevens showed that mutations in the region now known to contain the ICP34.5 gene reduced neurovirulence in mice (19). More recently, it has been shown that deletion mutants, translation termination mutants, and frameshift mutants in the ICP34.5 open reading frame also decrease neurovirulence in mice (2, 10, 21). In addition, some (2, 15, 21), but not all (10, 11), ICP34.5 mutants have been reported to reactivate less efficiently than wild-type virus, as measured by explant in vitro reactivation from mouse ganglia.

While spontaneous reactivation in the mouse has been reported, it is an extremely rare event (5, 18). In contrast, in rabbits, some herpes simplex virus type 1 (HSV-1) strains, such as McKrae, have spontaneous reactivation rates similar to those seen in humans with recurrent ocular herpes infection. To date, all reactivation studies of ICP34.5 mutants have used mouse models, except for one using guinea pigs (21). Unfortunately, since the parental F strain did not reactivate spontaneously in the guinea pig, no information regarding spontaneous reactivation was obtained.

We report here the construction and analysis in rabbits of an ICP34.5 deletion mutant in McKrae. This HSV-1 strain was chosen because it has a much higher in vivo spontaneous re-

activation rate in rabbits than most other strains of HSV-1 (6). The ICP34.5 mutant, designated *d34.5*, was compared with its parental virus and with a rescued virus, designated *d34.5R*, in which both copies of the ICP34.5 gene were completely restored. *d34.5* replicated with normal kinetics and to normal titers in RS (rabbit skin) cells in tissue culture but grew poorly in CV-1 cells. As with other ICP34.5 mutants, neurovirulence in mice was greatly reduced, as was replication in eyes and trigeminal ganglia (TG). The virulence of *d34.5* was also greatly reduced in rabbits. This is the first report to show that ICP34.5 is involved in virulence in an animal other than mice.

We also report here for the first time on the effect of deletion of the ICP34.5 gene on HSV-1 spontaneous reactivation. We found that spontaneous reactivation was decreased by over 10-fold in *d34.5* compared with the *d34.5R* rescued virus. Despite the large reduction in the frequency of spontaneous reactivation, it was still readily detectable. Thus, ICP34.5 was not essential for spontaneous reactivation in the rabbit ocular model of HSV-1. If, as expected, the rabbit model accurately reflects the mechanisms involved in clinical HSV-1 infection, latency, and reactivation, our results would suggest that ICP34.5 is not essential for spontaneous reactivation in humans. This possibility may have implications for the feasibility of using ICP34.5 deletion mutants as genetically engineered HSV vaccines (15, 21).

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MATERIALS AND METHODS

Virus and cells. All mutants were derived from HSV-1 strain McKrae. The parental McKrae virus and all mutants were triple plaque purified and passaged a maximum of two times prior to use. Rabbit tear films were cultured on primary rabbit kidney cell monolayers to look for the presence of reactivated HSV-1. RS cells were used for all other experiments unless otherwise indicated. All cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum.

Construction of the ICP34.5 deletion mutant *d34.5*. Plasmid PEV-vrf3 (14) containing the HSV-1 McKrae DNA restriction fragment *EcoRV* to *EcoRI* (118,640 to 131,534; Fig. 1) was digested with *SstI* to remove a 4-kb *SstI*-to-*SstI* fragment. The plasmid was then self-ligated and used to transform *Escherichia coli* as previously described (14). A plasmid containing the *EcoRV*-*EcoRI* fragment with a 4-kb deletion was isolated and designated pd34.5 (plasmid with a deletion of ICP34.5) (Fig. 1). The deletion in pd34.5 encompasses the entire ICP34.5 gene and also includes the essential gene, ICP4. This plasmid was cotransfected with infectious McKrae DNA as previously described (13). Virus plaques were picked and screened by Southern analysis. No viruses containing the entire deletion were found. Instead, several viruses that contained identical deletions of approximately 0.9 kb in both viral long repeats were found. Sequence analysis showed that the left end of the deletion is the left *SstI* site in the original pd34.5 plasmid (viral nucleotide position in the internal long repeat, 125,062), while the right end of the deletion is at viral nucleotide position 125,979 (Fig. 1). The restoration of the right side of the original deletion was expected, since without ICP4 the virus would not have replicated. The restoration of the ICP4 region is assumed to be the result of recombination with the terminal short repeat of the virus. One of the viruses was arbitrarily chosen and designated *d34.5*. A rescuant of *d34.5* was made by cotransfection of *d34.5* DNA with the original *EcoRV*-to-*EcoRI* restriction fragment that still contained the area deleted from *d34.5*. Using the same techniques used to make *d34.5*, a virus in which both copies of the deletion were restored was isolated, triple plaque purified, and designated *d34.5R*.

Replication of virus in tissue culture. Monolayers of RS or CV-1 cells at 75 to 80% confluency were infected with McKrae, *d34.5*, or *d34.5R* virus at various multiplicities as indicated in the text. All monolayers were refed with exactly the same amount of minimal essential medium containing 10% fetal calf serum. At indicated times, virus was harvested for titration by two cycles of freeze-thawing the monolayers plus media (-80°C to room temperature). Titers (PFU per milliliter) were determined by standard plaque assays on RS cells.

Rabbits and mice. Eight- to ten-week-old New Zealand White female rabbits (Irish Farms) and 4- to 5-week-old outbred Swiss Webster mice (Harland) were used for all rabbit and mouse experiments. Animals were treated in accordance with Association for Research in Vision and Ophthalmology, American Association for Laboratory Animal Care, and National Institutes of Health guidelines.

Rabbit model of ocular HSV-1 infection, latency, and spontaneous reactivation. Rabbits were bilaterally infected without scarification or anesthesia by placing 2×10^5 PFU of HSV-1 into the conjunctival cul-de-sac of each eye, closing the eye, and rubbing the lid gently against the eye for 30 s (16). At this dose of virus, virtually all of the surviving rabbits harbor a bilateral latent HSV-1 infection in both TG, resulting in a high group rate of spontaneous reactivation with the McKrae strain of HSV-1. Latency is assumed to have been established by 28 days postinfection. Acute ocular infection of all eyes was confirmed by HSV-1-positive tear film cultures collected on days 3 and 4 postinfection. For the high-dose infection with *d34.5*, each eye was scarified with a 26-gauge needle and then infected as described above with 4×10^7 PFU per eye, and this was repeated two additional times at 5-min intervals.

Virus replication in mouse eyes and trigeminal ganglia. Virus replication following eye inoculation was assayed as previously described (2, 19). Briefly, mice were anesthetized with sodium pentobarbital and inoculated bilaterally with 10^7 PFU in 30 μl following corneal scarification with a 27-gauge needle. Mice were sacrificed at various times postinfection (three mice per time point), and eyes and TG were collected, stored at -80°C , and assayed for virus content by plaque assay.

Neurovirulence assays in mice. Mice (five per dilution) were inoculated intracranially in the left brain hemisphere (19) with serial 10-fold dilutions ranging from 10^7 to 10^0 PFU of virus in 30 μl of minimal essential medium. Mice were maintained for 21 days and scored for death by encephalitis. The PFU/50% lethal dose (LD_{50}) ratios were calculated by the method of Karber (9).

Ocular shedding. To test for the presence of spontaneously reactivated virus in rabbit eyes, beginning on day 30 postinfection, tear film specimens were collected daily from each eye as previously described (12), using a nylon-tipped swab. The swab was then placed in 0.5 ml of tissue culture medium and squeezed, and the inoculated medium was used to infect primary rabbit kidney cell monolayers. These cell monolayers were observed in a masked fashion by phase light microscopy for up to 30 days for HSV-1 cytopathic effects. All positive monolayers were blind passaged onto fresh cells to confirm the presence of virus. DNA was purified from all positive cultures derived from rabbits latently infected with *d34.5* and analyzed by restriction enzyme digestion and Southern blotting to confirm that the reactivated virus retained both copies of the ICP34.5 deletion. In all cases, the spontaneously reactivated virus was indistinguishable from the input *d34.5*.

Virus replication in rabbit eyes. Tear films were collected as described above on various days postinfection. The amount of virus in each tear film was determined by standard plaque assays on RS cells.

Purification of DNA from TG of latently infected rabbits. Individual TG were removed from euthanized rabbits and stored at -80°C until use. To remove debris, individual TG were transferred to sterile Eppendorf tubes containing 1.0 ml of 100% ethanol at room temperature. Each tube was vortexed for 1 min, and the TG was pelleted in a microcentrifuge for 2 min and resuspended in 100% ethanol; the procedure was repeated twice. The final pelleted TG was vacuum dried and suspended in 500 μl of Tris-EDTA containing 0.1% sodium dodecyl sulfate and 100 μg of proteinase K per ml. The mixture was incubated at 55°C for 16 h. The treated mixture was extracted once with water-saturated phenol and then extracted with chloroform-isoamyl alcohol (24:1). The final supernatant was precipitated with 2.5 volumes of 95% ethanol and vacuum dried.

Semiquantitation of latent HSV-1 DNA by PCR. PCR was performed as described by Coen et al. (4, 7), with minor modifications as we previously described (13). The DNA extracted from each TG was resuspended in 100 μl of double-distilled water. One half (50 μl) of the DNA was used for amplification of HSV-1 DNA; the other half was used for amplification of rabbit actin DNA. We split the sample and ran the primer sets in parallel because our experience is that running the primer sets together often produces anomalies. The primer set used for virus DNA amplification was from the thymidine kinase (TK) gene of HSV-1 and produces a PCR product of 110 bp as previously reported (19). The internal probe used to detect this product is from the same report. The actin primer set used for amplification of cellular actin and the internal probe used to detect the 124-bp PCR product was as previously reported (1).

Thirty cycles of amplification were done with Boehringer Mannheim (Indianapolis, Ind.) *Taq* polymerase and the supplied buffer. Cycling reactions were performed with an Appligene (Pleasanton, Calif.) thermal cycler. Cycles were as follows: (i) denaturation at 94°C for 1 min, (ii) annealing at 5°C below the melting temperature of the primers for 1.5 min, and (iii) extension for 2.5 min at 74°C . The final cycle was terminated with a 10-min extension period at 74°C . The amplified products were fractionated on a 1.8% SeaKem agarose gel running in Tris-acetate-EDTA buffer. Alternate lanes contained the TK PCR product and the actin PCR product from the same TG.

The specificities of the amplified DNA products were confirmed by Southern blot hybridization of the PCR products with ^{32}P -end-labeled internal probes. The amount of radioactivity in each band was determined with a radioanalytic imaging detector (AMBIS, Inc., San Diego, Calif.). The relative amount of HSV-1 DNA was expressed as the ratio of the counts per minute of the TK PCR band divided by the counts per minute of the actin PCR band. To produce a standard dilution curve from which the number of HSV-1 DNA genomes could be estimated, twofold serial dilutions of a known amount of viral DNA were mixed with carrier DNA equivalent to the total DNA in an average rabbit TG. The dilutions were subjected to PCR in parallel under the identical conditions used for the experimental samples.

Statistical analyses. Statistical analyses were performed by using Instat, a personal computer software program. Results were considered statistically significant when the *P* value was <0.05 .

RESULTS

Description of the ICP34.5 deletion mutant *d34.5*. Figure 1A shows a schematic representation of the construction and final structure of the *d34.5* mutant. Additional details of the construction can be found in Materials and Methods. In the internal long repeat, the deletion extends from nucleotide position 125,062 (an *SstI* restriction enzyme site) at the left to nucleotide position 125,979 on the right (Fig. 1), as determined by sequence analysis. The corresponding position of the deletion in the terminal long repeat is nucleotides 1,312 to 2,229. The ICP34.5 open reading frame extends from nucleotides 125,110 to 125,904 in the internal long repeat (Fig. 1A) and nucleotides 1,237 to 2,181 in the terminal repeat. The ICP34.5 open reading frame is thus completely within the 917-nucleotide deletion and has therefore been unequivocally removed. The ICP34.5 deletion mutant, *d34.5*, is therefore incapable of making any ICP34.5 protein.

A rescued virus, *d34.5R*, in which both copies of ICP34.5 have been restored was made as described in Materials and Methods. Southern blot hybridization analyses of *d34.5* and *d34.5R* are shown in Fig. 1B. Parental McKrae, *d34.5R*, and *d34.5* DNAs were digested with *Bam*HI and subjected to Southern analysis using a probe that spans the ICP34.5 gene and is completely within the viral long repeat. For wild-type virus with a single copy of the "a" repeat in each viral long

FIG. 1. Construction and structure of the ICP34.5 deletion mutant *d34.5*. (A) The top of the schematic shows the HSV-1 McKrae genome in the prototypic orientation. The rectangles labeled TR_L and IR_L represent the terminal and internal (or inverted) long repeats, while the rectangles labeled TR_S and IR_S represent the terminal and internal (or inverted) short repeats. U_L and U_S represent the long and short unique regions, respectively. The expanded presentation of part of the internal long and short repeats indicates the location and direction of transcription of the ICP34.5 open reading frame. The locations of ICP0, ICP4, and the 8.3- and 2-kb LATs are shown for reference. Purified, infectious McKrae DNA was mixed with (+) plasmid pd34.5, which contains an *SstI-SstI* deletion (open rectangle), resulting in removal of the entire ICP34.5 gene, the viral junction, and part of ICP4, and the mixture was then cotransfected into RS cells (arrow) as described in Materials and Methods. Resulting virus plaques were screened by Southern analysis for deletion of ICP34.5. No viruses containing the entire plasmid deletion were detected. However, numerous, apparently identical viruses containing a 0.9-kb deletion from *SstI* (nucleotide 125,062) to nucleotide 125,979 as indicated at the bottom were isolated (open box). One such plaque was chosen arbitrarily, triple plaque purified, and used in the studies described here. The final deletion encompasses the entire ICP34.5 open reading frame. The locations of the "a" viral sequences containing the ICP34.5 promoter are shown by the double-headed arrow. An identical corresponding deletion is located in the terminal long repeat. (B) Purified McKrae (lane M), *d34.5* (lane D), and *d34.5R* (lane R) DNAs were individually digested with *Bam*HI and subjected to Southern analysis using a 32 P-end-labeled probe that was derived from the long repeat and that spans the entire ICP34.5 gene. Solid arrows 1 and 2 (lanes M and R) indicate the locations of the 5.9- and 2.9-kb bands expected if the virus has a single copy of the "a" region. Open arrows 1' and 2' (lane D) show the locations of the corresponding 5.0- and 2.0-kb bands due to the deletion in *d34.5*. The arrows labeled 1a, 2a, 1a', and 2a' correspond to the locations of 1, 2, 1', and 2' if there are two copies of the "a" region.

FIG. 2. Kinetics of *d34.5* replication in tissue culture. Subconfluent RS or CV-1 cell monolayers were infected with *d34.5*, McKrae, or *d34.5R* as described in Materials and Methods. Total virus was harvested at the indicated times postinfection by two cycles of freeze-thawing. The amount of virus at each time for each virus was determined by standard plaque assays on RS cells. (A) RS cells infected with a multiplicity of infection (MOI) of 0.05 PFU per cell; (B) RS cells infected with 0.001 PFU per cell; (C) CV-1 cells infected with 0.01 PFU per cell; (D) CV-1 cells infected with 10 PFU per cell.

repeat, the probe would be expected to hybridize to restriction fragments of 5.9 and 2.9 kb (Fig. 1B, lane M, arrows 1 and 2). The 5.9-kb fragment is bounded by a *Bam*HI site in the internal long repeat at nucleotide 123,459 and a *Bam*HI site in the internal short repeat at nucleotide 129,403. The 2.9-kb restriction fragment is derived from the terminal long repeat and is bounded by the start of the genome at nucleotide 1 and a *Bam*HI site at nucleotide 2907. Band 1a, which is approximately 400 nucleotides larger than band 1, and band 2a, which is approximately 400 nucleotides larger than band 2, are apparently derived from viral long repeats containing two copies of the "a" repeats. In addition, increased exposure time of the autoradiogram reveals additional bands approximately 400 nucleotides larger than the 1a and 2a bands. These bands presumably represent long repeats with three copies of the "a" repeat. Similar variation in the number of "a" repeats has been reported previously (20).

As estimated from electrophoretic mobilities, the *d34.5*-derived bands (1', 1a', 2', and 2a') each appear to be approximately 0.9 kb smaller than the corresponding (1, 1a, 2, and 2a) McKrae bands (Fig. 1b; compare lane D bands, open arrows, with the lane M bands, solid arrows). This size difference is consistent with the 917-nucleotide deletion in *d34.5* determined above by sequence analysis and confirms that the deletion is present in both viral long repeats. Southern analysis of *d34.5R* DNA (Fig. 1B, lane R) produced bands that were the same size as the McKrae bands. This confirmed that the deletion had been restored in both viral long repeats.

Tissue culture replication. In plaque assays on RS cells, *d34.5* plaques were not distinguishable in size or morphology from wild-type HSV-1 plaques (not shown). To assess the replication kinetics of *d34.5*, RS cell monolayers were infected in triplicate with either 0.05 or 0.001 PFU of *d34.5* or the parental virus McKrae per cell. There were no statistically

FIG. 3. Survival of rabbits infected with the ICP34.5 null mutant *d34.5*. Rabbits were ocularly infected with either 2×10^5 PFU of the *d34.5R* rescued virus (left bar), 2×10^5 PFU of *d34.5* (center bar), or $>4 \times 10^7$ PFU of *d34.5* (right bar) per eye as described in Materials and Methods. Survival was determined 21 days postinfection. For the $>4 \times 10^7$ -PFU-per-eye group, eyes were scarified and then infected three successive times at 5-min intervals with 4×10^7 PFU per eye as described in Materials and Methods. The *P* value above the two rightmost bars was determined by using the Fisher exact test to compare survival in the *d34.5R*-infected rabbits with the pooled results of both *d34.5*-infected rabbit groups.

significant differences between the virus growth curves at either input multiplicity of infection (Fig. 2A and B; *P* > 0.05 for all time points, Student *t* test). Thus, deletion of the ICP34.5 gene had no effect on viral replication kinetics in RS cells in tissue culture.

In contrast, *d34.5* produced no plaques on CV-1 cells. The plating efficiency of *d34.5* on CV-1 cells compared with the plating efficiency of *d34.5* on RS cells was less than 10^{-7} (data not shown). To assess the replication kinetics of *d34.5* in CV-1 cells, CV-1 cell monolayers were infected with 0.01 or 10 PFU of either *d34.5* or the rescued virus *d34.5R* per cell. At various times, the monolayers were harvested by two cycles of freeze-thawing, and the amount of virus was measured by plaque assays on RS cells. At a multiplicity of infection of 0.01 PFU per CV-1 cell, the maximum yield of *d34.5* virus was over 200,000-fold lower than the yield of *d34.5R* virus (Fig. 2C; 500 versus 1.2×10^8 PFU/ml). Even at a high multiplicity of infection (10 PFU per CV-1 cell), the mutant yield at 72 h postinfection was over 300-fold lower than that of the rescued virus

FIG. 4. Replication of *d34.5* in rabbit eyes. Rabbits were ocularly infected with 2×10^5 PFU of each virus per eye as described in Materials and Methods. Tear films were collected on the days indicated, and virus titers were determined by standard plaque assays. Each point represents the mean of the titers from six eyes.

(Fig. 2D; 5.4×10^4 versus 1.7×10^7 PFU/ml). CV-1 is an African green monkey kidney cell-derived continuous cell line that is commonly used for growing a variety of viruses, including HSV-1. McKrae's efficiencies of plating and replication are similar on CV-1 cells and RS cells. Rescue of the deletion in *d34.5* fully restored the ability of the virus to grow in CV-1 cells.

Restricted replication of an ICP34.5 mutant has been previously reported in confluent primary mouse embryo cell cultures (2). The mutant grew with much greater efficiency in subconfluent mouse embryo cells, suggesting that the physiological state of the cell might be important for efficient replication. Replication of another ICP34.5 mutant was reported to be restricted in a human neuroblastoma cell line but not in cells of nonneuronal origin (3). CV-1 cells are not of neuronal origin. In addition, the restriction of *d34.5* replication occurred in subconfluent as well as confluent CV-1 cell monolayers. The reason for the restricted replication of *d34.5* in CV-1 cells remains to be determined.

Virulence in rabbits. Rabbits were ocularly infected with 2×10^5 PFU of the *d34.5R* rescued virus per eye as described in Materials and Methods. The rescued virus produced a lethal encephalitis in approximately half of the infected rabbits, resulting in a 53% survival rate (9 of 17) (Fig. 3, *d34.5R*). This was similar to all our previous experience with the parental McKrae virus. In contrast, all of the eight rabbits infected with the same amount of *d34.5* survived (Fig. 3, center bar). This was significantly more survival, and hence less virulence, than seen with the rescued virus (*P* = 0.026, Fisher exact test). In addition, all of eight rabbits infected with greater than a 200-fold-higher input dose of *d34.5* ($>4 \times 10^7$ PFU per eye; see Materials and Methods) also survived (Fig. 3, right bar). Comparison of the combined results of both doses of *d34.5* (16 of 16 survived) and *d34.5R* (9 of 17 survived) was highly significant (*P* < 0.01). Thus, deletion of ICP34.5 produced a profound decrease (>200 -fold) in the virulence of HSV-1 in ocularly infected rabbits.

TABLE 1. Survival following intracranial injection of mice with the ICP34.5 null mutant *d34.5*^a

Virus	PFU of input virus	No. of survivors	LD ₅₀ (PFU)
McKrae	1.5×10^2	0	7.5 ± 5.0
	1.5×10^1	1	
	1.5×10^0	5	
<i>d34.5</i>	1.5×10^7	0	$1.9 \times 10^6 \pm 1.3 \times 10^6$
	1.5×10^6	5	

^a Mice (five per group) were inoculated intracranially with 10-fold serial dilutions of McKrae or *d34.5*, and LD₅₀s were calculated as described in Materials and Methods.

FIG. 5. Replication of *d34.5* in mouse eyes and TG. Mice were ocularly infected as described in Materials and Methods. Mice were euthanized on the indicated days. (A) Eyes were removed and ground up, and virus titers were determined as described in Materials and Methods. (B) TG were removed and ground up, and virus titers were determined.

Virulence in mice. For purposes of comparison with other ICP34.5 mutants made in other virus strains, groups of five mice were infected intracranially with 10-fold serial dilutions of either McKrae or *d34.5* as described in Materials and Methods (Table 1), and the LD₅₀ was calculated for each virus. Following direct injection of virus into the brain, McKrae had an LD₅₀ of 7.5 PFU, while *d34.5* had an LD₅₀ of 1.9×10^6 PFU. Thus, deletion of ICP34.5 produced over a 250,000-fold decrease in neurovirulence of HSV-1 as measured by survival following intracranial injection. Similar results were obtained in two additional determinations (not shown).

Replication in rabbit and mouse eyes. Rabbits were infected ocularly as described in Materials and Methods. At the times shown postinfection, tear films were collected from six eyes per virus, virus titers were determined individually, and the mean titers for each day were plotted (Fig. 4). McKrae and the rescued *d34.5R* virus had similar peak titers of approximately 6×10^5 to 1×10^6 PFU per eye. In contrast, *d34.5* had a peak titer of less than 100. This decrease was highly significant ($P < 0.0001$, Student *t* test). Thus, deletion of ICP34.5 resulted in a decrease in HSV-1 growth in the rabbit eye of approximately 6,000-fold.

Similar results were obtained in the mouse. Mice were ocularly infected with *d34.5* or *d34.5R* as described in Materials and Methods. The peak virus titer for the rescued virus was approximately 2.3×10^5 , while the peak titer for the *d34.5* mutant was only 300 (Fig. 5A), a more than 700-fold decrease. Thus, deletion of ICP34.5 resulted in large decreases in HSV-1 replication in both rabbit and mouse eyes.

Replication in TG. Following ocular infection, mice were euthanized at the times indicated (Fig. 5B), and the level of infectious virus in the TG was determined by plaque assay. *d34.5R* had a peak titer in the TG of 1.5×10^5 PFU on day 5. The peak titer for *d34.5* was also on day 5 but was only 50. This 3,000-fold difference indicated that *d34.5* was restricted in its ability to replicate in TG.

Spontaneous reactivation of the *d34.5* mutant. Rabbit eyes were infected with *d34.5* or *d34.5R*. Beginning 30 days postinfection (at which time latency had already been established), all eyes were swabbed once a day for 26 days to collect tear films for analysis of reactivated virus as described in Materials

and Methods. The cumulative number of virus-positive tear film cultures is shown in Fig. 6. Because of the different numbers of rabbits (and eyes) in the ICP34.5-positive and -negative virus groups, the data were standardized to represent cumulative positive cultures per eye. The cumulative spontaneous reactivation rate in rabbits latently infected with the ICP34.5 deletion mutant appeared to be much lower than that in the rescued virus-infected rabbits (Fig. 6). The cumulative data for positive (spontaneously reactivated) cultures versus negative cultures indicated that 80 of 408 (19.6%) of the tear film cultures from rabbits latently infected with *d34.5R* virus contained spontaneously reactivated virus. In contrast, only 6 of 416 (1.4%) of the tear films from eyes infected with *d34.5* were positive for reactivated virus (Table 2). This difference was highly significant ($P < 0.0001$).

Because the foregoing analyses do not take into account the

FIG. 6. In vivo spontaneous reactivation of *d34.5* in rabbits. Rabbits were infected with *d34.5* or *d34.5R*. Beginning 30 days postinfection, tear films were collected daily and the presence or absence of spontaneously reactivated virus was determined by plating on primary rabbit kidney cells as described in Materials and Methods. The average cumulative number of virus-positive tear film cultures per eye is plotted for each virus-infected group. Statistical analyses of the data are shown in Table 2.

TABLE 2. Spontaneous reactivation in rabbits latently infected with the ICP34.5 null deletion mutant *d34.5*

Virus	No. of HIV-1-positive tear film cultures/total (%) ^a	Fraction of positive cultures/eye ^b (no. of eyes)	No. of eyes that spontaneously reactivated at least once/total (%)	No. of rabbits that spontaneously reactivated at least once/total (%)	No. of reactivation episodes/eye ^c
<i>d34.5</i> virus	6/416 (1.4)	0.01 (16)	4/16 (25)	4/8 (50)	0.31
<i>d34.5R</i>	80/408 (19.6)	0.19 (18)	17/18 (94)	9/9 (100)	1.94
<i>P</i> , <i>d34.5</i> vs <i>d34.5R</i>	<0.0001 ^d	<0.0001 ^e	<0.0001 ^f	0.03 ^f	<0.001 ^e

^a Tears were collected daily from all eyes for 26 days and cultured on indicator cells for the presence of spontaneously reactivated HSV-1.

^b Average for all eyes of the total HSV-1-positive cultures for eye *x* per total cultures for eye *x*.

^c Total number of reactivations in each group, assuming that consecutive days of HSV-1-positive tear film cultures from a single eye are the result of a single reactivation event.

^d Chi square, two sided.

^e Mann-Whitney rank sum test, two sided.

^f Fisher exact test, two sided.

number of eyes in each of the groups, the data were analyzed by additional methods. The fraction of virus-positive cultures for each eye in each group (i.e., the fraction of time each eye was virus positive) was calculated, and these fractions were analyzed by the Mann-Whitney rank sum test (Table 2). The decreased spontaneous reactivation rate of the ICP34.5 deletion mutant compared with that of the rescued virus was highly significant ($P < 0.0001$).

The number of eyes in each group that had at least one spontaneous reactivation was also analyzed (Table 2). In the group of rabbits infected with the rescued virus, 17 of 18 eyes (94%) had at least one spontaneous reactivation. In the rabbits infected with *d34.5*, only 4 of 16 (25%) of the eyes reactivated spontaneously. Again, this difference was highly significant ($P < 0.0001$, Fisher exact test).

The number of rabbits in each group that had at least one spontaneous reactivation was also analyzed (Table 2). With the rescued virus, 9 of 9 rabbits (100%) had at least one spontaneous reactivation, compared with only 4 of 8 (50%) of the rabbits infected with *d34.5*. Again, this difference was significant ($P < 0.03$, Fisher exact test).

Another method of examining the effect of deleting ICP34.5 on spontaneous reactivation is to analyze the number of times spontaneous reactivation is detected in each eye, regardless of the length of time virus is present. This is equivalent to the number of episodes in which reactivated virus is detected in the tears, with consecutive days of positive cultures being treated as a single event. Thus, an eye that sheds virus for a single day and an eye that sheds virus for 5 consecutive days would both constitute one episode of spontaneous reactivation. The average number of spontaneous reactivations per eye in each group calculated in this manner is shown in Table 2. The number of spontaneous reactivations in *d34.5*-infected eyes averaged 0.31, compared with 1.94 for eyes infected with *d34.5R* ($P < 0.001$). All of these analyses strongly support the notion that ICP34.5 is involved in spontaneous reactivation in the rabbit eye model. However, they do not distinguish between a role for ICP34.5 in the establishment of latency, reactivation from latency, or both.

Rate of establishment of latency. Deletion of ICP34.5 might result in a reduced rate of establishing latency, and this could account for the apparent reduced spontaneous reactivation seen with *d34.5*. To address this, at the termination of the experiment shown in Fig. 6, rabbits were sacrificed and TG were removed. Total DNA was isolated, and the relative amounts of HSV-1-specific DNA were determined for each TG by semiquantitative PCR analysis as described in Materials and Methods. Two sets of PCR primers were used. One set was specific for a region of the HSV-1 TK gene. The other set was specific for a portion of cellular actin. This was used as an

internal control to standardize the recovery of DNA from the trigeminal ganglia and to standardize any differences in efficiency of DNA transfer during Southern blotting. The relative amount of each PCR product band following Southern analysis with internal probes was determined by direct counting of the blot as described in Materials and Methods. The ratio of each HSV-1-specific band and the corresponding actin band was calculated. This number represents the standardized relative amount of HSV-1 DNA.

To ensure that the PCR was linear over the range of HSV-1 DNA present in the samples, and also to produce a standard curve from which the amount of HSV-1 DNA in the latent trigeminal ganglia could be extrapolated, a control experiment was run in parallel. Twofold serial dilutions containing known amounts of HSV-1 genomic DNA in the range of approximately 10^3 to 10^7 copies were added to test tubes containing carrier DNA equivalent to one TG. The DNA was then subjected to PCR using the same procedures used for the DNA from the latently infected rabbits. Following Southern analysis with internal probes, the counts per minute of the HSV-1 PCR products of the serially diluted DNA was plotted on a log/log scale versus the known amount of input HSV-1 DNA (Fig. 7A). The result was a relatively straight line, indicating that in this range of HSV-1 DNA our PCR assay was semiquantitative.

An autoradiogram of a typical set of experimental PCRs is shown in Fig. 7B. Visually, the average intensity of the HSV-1 PCR bands from the *d34.5* mutant appeared to be slightly less than that of the *d34.5R* PCR bands (open arrow) relative to the intensity of their corresponding actin bands (solid arrow). The original blots of this and similar Southern analyses were counted, the amounts of viral DNA were standardized to the actin level, and the average copy number of HSV-1 DNA genomes was extrapolated from the standard curve. In experiment 1, PCRs were done on 16 individual TG from *d34.5* latently infected rabbits and 12 individual TG from *d34.5R* latently infected rabbits (Table 3, experiment 1). The estimated number of HSV-1 genomes was 6.8×10^5 per *d34.5* mutant-infected TG, compared with an average of 3.0×10^6 copies per *d34.5R*-virus TG. The apparent decrease in the *d34.5* establishment rate (23% of the *d34.5R* control rate) was not statistically significant ($P = 0.72$, Student *t* test). This was likely due to the large variation in HSV-1 DNA copy number seen from TG to TG in this experiment (3×10^2 to 7×10^8 copies per *d34.5*-infected TG; 1×10^3 to 7×10^8 copies per *d34.5R*-infected TG) and others (7, 13).

In a second analysis, the DNAs extracted from the TG of 12 *d34.5* and 10 *d34.5R* latently infected rabbits were pooled, and an aliquot of each mix corresponding to one TG was analyzed

FIG. 7. Quantitation of latent HSV-1 DNA by PCR. (A) A standard curve was generated in parallel with and under conditions identical to those used for the experimental PCR samples in panel B and Table 3. Twofold serial dilutions containing known amounts of HSV-1 genomic DNA in the range of approximately 10^3 to 10^7 copies were added to test tubes each containing carrier DNA approximately equivalent to the amount of TG DNA in each experimental sample. The DNA was then subjected to PCR and Southern analysis as described in Materials and Methods. The counts per minute for the HSV-1 PCR product from each DNA dilution was determined by radioanalytic imaging and plotted on a log/log scale versus the known amount of input HSV-1 DNA. The resulting relatively straight line indicated that in this range of HSV-1 DNA this PCR assay was semiquantitative. (B) DNA isolated from individual TG was divided, and the two aliquots were subjected to 30 cycles of PCR using primer pairs for either TK (HSV-1 DNA) or for cellular actin (standard) as described in Materials and Methods. The resulting PCR products from each TG were run on agarose gels in adjacent lanes. To improve separation of the TK and actin bands, the TK PCR products were loaded and the gel was run for 10 min. The current was then stopped, the actin PCR products were loaded, and the current was restored. The positions and quantities of the TK and actin PCR bands were determined by Southern analysis using labeled internal probes for TK and actin concurrently. Representative results for the DNA from five *d34.5* latently infected TG and four *d34.5R* latently infected TG are shown. All panels were run at the same time on the same or parallel gels, along with the DNA standards in panel A. To ensure consistency, all of the Southern blots were hybridized together in the same hybridization bag and then exposed to X-ray film for the same length of time on a single large sheet of film.

as described above (Table 3, experiment 2). The HSV-1 DNA copy number was estimated to be 1.2×10^5 for the *d34.5*-infected rabbits and 6.4×10^5 for the *d34.5R*-infected rabbits. No statistical analysis could be done because of the use of a single sample for each virus. Additional rabbits were infected for a third experiment, and three pools of three TG each were analyzed as described above (Table 3, experiment 3). The estimated HSV-1 genomic copy numbers per TG in this experiment were 1.6×10^6 for *d34.5* and 4.4×10^6 for *d34.5R* ($P = 0.17$). Thus, in three independent experiments, the HSV-1 genome copy numbers in *d34.5* latently infected TG appeared to be 23, 19, and 36% of that of the *d34.5R* control (Table 3). Although in each individual experiment the results could not be shown to be statistically significant, the consistency of the results suggest that ICP34.5 may be required for efficient establishment of latency in rabbit TG.

TABLE 3. Estimated number of HSV-1 genomes in TG of latently infected rabbits^a

Expt	Virus	Avg. no. of genomic copies/TG (<i>n</i>)	% Wild type	<i>P</i> ^b
1	<i>d34.5R</i>	3×10^6 (12 individual)	23	0.72
	<i>d34.5</i>	6.8×10^5 (16 individual)		
2	<i>d34.5R</i>	6.4×10^5 (10 pooled)	19	NA
	<i>d34.5</i>	1.2×10^5 (12 pooled)		
3	<i>d34.5R</i>	4.4×10^6 (9, 3 pools)	36	0.17
	<i>d34.5</i>	1.6×10^6 (9, 3 pools)		
Total/avg	<i>d34.5R</i>	2.7×10^6	30	
	<i>d34.5</i>	8.0×10^5		

^a In experiment 1, DNA was isolated individually from 16 TG from *d34.5* latently infected rabbits and 12 TG from *d34.5R* latently infected rabbits. PCRs were run on aliquots of each DNA sample, values were standardized to those for actin, and genomic copy numbers were estimated by extrapolation against a standard curve as described in Materials and Methods. In experiment 2, the DNA from 12 TG latently infected with *d34.5* were pooled and the DNAs from 10 TG latently infected with *d34.5R* were pooled. A sample of each pool (representing the same fraction of a TG) was analyzed by PCR as described above. In experiment 3, three sets of three TG from *d34.5* or *d34.5R* latently infected rabbits were analyzed as described above.

^b Student *t* test, double sided. NA, not applicable.

DISCUSSION

The results reported here strongly suggest that the HSV-1 ICP34.5 gene is a virulence gene in rabbits as well as mice. As has been reported for all other null mutations at this locus in mice (2, 15), in rabbits the McKrae *d34.5* mutant did not induce any signs of central nervous system disease following inoculation at the periphery. These findings for rabbits support the results of previous studies using mice and strengthen the notion that ICP34.5 may also be an important virulence or neurovirulence factor in humans. Since *d34.5* is defective for replication in the eye and other nonneuronal cells, the reduced virulence seen following ocular infection of rabbits may be partially or completely due to a defect in neuroinvasiveness or neurovirulence (or a combination of both). In this report, we have therefore described *d34.5* as having a defect in virulence, a term which can be applied to either neurovirulence or neuroinvasiveness.

Previous studies have demonstrated that ICP34.5 null mutants can reactivate from mouse TG following explant cocultivation (10, 11, 15, 21). However, many viral isolates that reactivate efficiently in explanted mouse ganglia (for example, strain KOS) fail to spontaneously reactivate and recur in the rabbit ocular model (6). It was therefore of interest to determine if deletion of ICP34.5 would affect spontaneous reactivation in the rabbit. Our results showed that deletion of the region of the genome encoding ICP34.5 reduced *in vivo* spontaneous reactivation by about 10-fold. Consistent with this reduced *in vivo* reactivation, an ICP34.5 null mutant (17TermA) (2) containing a subtle mutation abolishing the ICP34.5 reading frame has a hyperthermic stress-induced *in vivo* reactivation frequency (17) of about one-third of that of its parent strain 17syn+ (18a).

Our results, and those of others for mice (2, 21), indicate that ICP34.5 null mutants replicate poorly in eyes and TG. This probably accounts for the lower amount of latency detected with *d34.5*. Because of these same replication impairments, the ability to detect an appreciable amount of spontaneous reactivation with *d34.5* was unexpected. Following the molecular triggers that produce spontaneous reactivation of HSV-1 in neurons in the TG, the virus must presumably complete at least one replicative cycle in the neuron prior to traveling back to the eye. In addition, once it arrives at the eye, it

is assumed that the spontaneously reactivated virus must undergo further replicative amplification in order for easy detection in tear films. Nonetheless, spontaneous reactivation was readily detected, although the rate of detection was significantly reduced compared with that of McKrae. A thorough explanation for these findings awaits additional study.

In three separate experiments, the ICP34.5 deletion mutant appeared to establish (or maintain) latency with approximately 19 to 36% of the efficiency of the rescued virus, as judged by semiquantitative PCR analysis of viral DNA. Although the decreases in ICP34.5 DNA were similar in all three experiments, the differences were not statistically significant in any individual experiment. This was likely due to the large range of DNA values in TG within each group, a phenomenon known to occur with this type of analysis (7, 13). Nonetheless, there did appear to be a tendency for the ICP34.5 mutant to establish latency at a reduced level. The mutant's poor replication in eyes and TG and the resulting decreased amount of virus available for establishing latency probably account for at least some of the apparent reduction in the amount of latency with *d34.5*. Likewise, the approximately 3- to 5-fold reduced level of latency detected by PCR may account for the observed 10-fold reduction in spontaneous reactivation with *d34.5*. However, other factors may also contribute. (i) We recently showed that a latency-associated transcript (LAT) null mutant in McKrae (*dLAT2903*) significantly reduced spontaneous reactivation in latently infected rabbits without affecting the rate of establishment of latency (13). Since the 0.9-kb ICP34.5 deletion in *d34.5* also deleted 0.9 kb of the overlapping LAT gene (kb 6.2 to 7.1 of the 8.3-kb LAT; Fig. 1), it is possible that a portion of the reduced spontaneous reactivation of *d34.5* was due to alteration of the LAT gene. (ii) Similarly, it is formally possible that a portion of the reduced spontaneous reactivation is due to deletion of an open reading frame recently shown to be present on the opposite strand of ICP34.5 (8), i.e., within the 6.2- to 7.1-kb region of LAT. (iii) In addition to decreasing the establishment of latency, ICP34.5 may also be directly involved in the spontaneous reactivation process. (iv) Finally, the reduced ability of *d34.5* to replicate in TG and eyes may make it more difficult to detect reactivated virus in tear films.

In summary, we have found that deletion of the ICP34.5 open reading frame from McKrae, a highly virulent HSV-1 strain, results in an avirulent virus that is severely restricted for growth in eyes and TG and probably establishes latency at a reduced rate. Despite these impairments, this mutant was still capable of low levels of spontaneous reactivation in the rabbit. This may be an important consideration with respect to the use of ICP34.5 deletion mutants as genetically engineered HSV vaccines.

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