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High-Dose Ocular Infection with a Herpes Simplex Virus Type 1 ICP34.5 Deletion Mutant Produces No Corneal Disease or Neurovirulence yet Results in Wild-Type Levels of Spontaneous Reactivation

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We report here that in the rabbit ocular model of herpes simplex virus type 1 (HSV-1) latency, spontaneous reactivation of the HSV-1 ICP34.5 deletion mutant *d*34.5 increased significantly in response to increasing infectious doses. At the highest infectious dose of *d*34.5, the spontaneous reactivation rate was indistinguishable from that of wild-type virus (average spontaneous reactivation rates for *d*34.5, 0.3 to 1.4% at 2×10^5 PFU per eye, 3.4% at 2×10^6 PFU per eye, and 6.3 to 11.5% at 1×10^8 PFU per eye; average spontaneous reactivation rates for marker-rescued virus, 7.7 to 19.6% at 2×10^5 PFU per eye). The percentage of latency-associated transcript (LAT) RNA-positive neurons in sections from trigeminal ganglia (TG) of rabbits latently infected with *d*34.5 demonstrated a similar dose-response effect as estimated by in situ hybridization (0.05% LAT RNA-positive neurons at 2×10^5 PFU per eye and 0.1% LAT RNA-positive neurons at 1×10^8 PFU per eye; $P = 0.002$). In contrast, even at the highest infectious dose (1×10^8 PFU per eye), *d*34.5 was less virulent (23 of 23 survivors) than the normal infectious dose (2×10^5 PFU per eye) of marker-rescued virus (14 of 27 survivors; $P < 0.0001$). In addition, at 1×10^8 PFU per eye, *d*34.5 produced virtually no corneal disease, compared with the production of severe corneal disease by 2×10^5 PFU of marker-rescued virus per eye ($P < 0.0001$). Thus, at increasing infectious doses of *d*34.5, both spontaneous reactivation and the percentage of neurons expressing LAT appeared to increase, without a corresponding increase in virulence. These results strongly suggest that (i) the phenotypes of neurovirulence and spontaneous reactivation are separable, (ii) the phenotypes of corneal disease and spontaneous reactivation are separable, and (iii) the decreased rate of spontaneous reactivation previously reported for *d*34.5 (G. C. Perng, R. L. Thompson, N. M. Sawtell, W. E. Taylor, S. M. Slanina, H. Ghiasi, R. Kaiwar, A. B. Nesburn, and S. L. Wechsler, J. Virol. 69:3033–3041, 1995) is at least partially due to a reduced rate of establishing latency.

One of the hallmarks of herpes simplex virus type 1 (HSV-1) infection is the propensity of the virus to establish a lifelong latent infection which at various times throughout the life of the latently infected individual can reactivate and cause recurrent infection. Recurrent HSV-1 corneal infection can result in corneal scarring. This is the major cause of infectious blindness in developed countries (8).

Studies with ICP34.5 mutants have shown that in mice the ICP34.5 gene is a neurovirulence gene (1, 2, 7, 18, 21). ICP34.5 mutants have also been shown to reactivate less efficiently than wild-type virus, as measured by explant in vitro reactivation from mouse ganglia (1, 15, 21). This suggests that in mice ICP34.5 may play a role in the HSV-1 latency-reactivation cycle. Recently, we and others extended these findings to include spontaneous reactivation and virulence in rabbits. We constructed an ICP34.5 deletion mutant (*d*34.5) in the McKrae strain of HSV-1 (14). We chose McKrae as the parental HSV-1 strain because its high in vivo spontaneous reactivation rate in rabbits makes it possible to analyze the effect of mutants on spontaneous reactivation (13). Both neurovirulence in rabbits and spontaneous reactivation were greatly reduced by deletion of the ICP34.5 gene. Although we found that spontaneous reactivation was decreased by more than 10-fold in *d*34.5 compared with that of the *d*34.5R marker-rescued virus, it was still readily detectable. Thus, although ICP34.5 clearly was somehow involved in efficient spontaneous reactivation, it was not absolutely essential in the rabbit ocular model of HSV-1. Since the rabbit ocular model closely mimics clinical HSV-1 infection, latency, and reactivation, it is likely that ICP34.5 is also not essential for spontaneous reactivation in humans.

Because of the possible implications that the low levels of spontaneous reactivation in our ICP34.5 deletion mutant posed for use of ICP34.5 deletion mutants as HSV vaccines (15, 21), we were interested in determining what would happen to the spontaneous reactivation rate at higher infectious doses of *d*34.5. We report here that regardless of the input infectious dose of *d*34.5 in rabbit eyes, all rabbits survived the infection, and the severity of corneal disease remained at trace levels. In sharp contrast, there was an obvious dose-response effect on spontaneous reactivation. As the input infectious dose of *d*34.5 rose from 2×10^5 to 2×10^6 to 1×10^8 PFU per eye, the average rate of spontaneous reactivation increased from $<1.5\%$ to 3.4% to $>10\%$. At the highest dose of *d*34.5, the level of spontaneous reactivation was indistinguishable from that of wild-type virus. In addition, there was also a dose-response effect on the percentage of neurons containing latency-associated transcript (LAT) RNA, as judged by in situ hy-

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bridization. These results strongly suggest that the decreased spontaneous reactivation seen in rabbits infected with the standard dose of *d*34.5 was due to a decrease in the establishment of latency.

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. *d*34.5 contains a complete deletion of both copies of the ICP34.5 gene (one in each long repeat), as we previously described (14). We have also previously described the marker-rescued virus, *d*34.5R, in which both copies of ICP34.5 have been completely restored, and showed that it behaves like the original wild-type McKrae parental virus for all parameters examined, including tissue culture replication, replication in rabbit and mouse eyes and trigeminal ganglia (TG), virulence in rabbits and mice, and spontaneous reactivation in rabbits (14). Rabbit tear films were cultured on primary rabbit kidney cell monolayers to look for the presence of reactivated HSV-1. Rabbit skin cells were used for all other experiments. All cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum.

Rabbits. Ten-week-old New Zealand White female rabbits were used for all rabbit experiments. Rabbits 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. With the McKrae strain of HSV-1, rabbits are routinely bilaterally infected without scarification or anesthesia by placing 2×10^5 PFU of HSV-1 per eye into the conjunctival cul-de-sac, closing the eye, and rubbing the lid gently against the eye for 30 s (16). This is our standard infectious dose. It produces a bilateral latent HSV-1 infection in the TG of virtually all surviving rabbits, which in turn results 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 cultures of tear film specimens collected on days 3 and 4 postinfection. For the high-dose infection (1×10^8 PFU per eye), eyes were infected as described above with 4×10^7 PFU per eye three times at 5-min intervals. For the high-dose infection with scarification (1×10^8 PFU per eye), the rabbits were anesthetized and each eye was scarified with a 26-gauge needle as previously described (14) and then infected three times with *d*34.5 as described above.

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 (10), with 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-contrast 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 *d*34.5 and analyzed by restriction enzyme digestion and Southern blotting to confirm that the reactivated virus retained both copies of the ICP34.5 deletion. In both high- and low-dose infections, all of the *d*34.5 spontaneously reactivated virus was indistinguishable from the input *d*34.5 virus, as we previously described for the low-dose *d*34.5 infection (14).

Virus replication in rabbit eyes during the acute phase of infection. Tear film specimens were collected as described above on various days postinfection. The amount of virus in each tear film was determined by standard plaque assays with rabbit skin cells.

Acute eye disease. Acute eye disease was determined by examining eyes for herpes simplex dendritic and geographical ulceration (epithelial disease) by slit lamp biomicroscopy using 1% Fluorescein (11, 12). Iritis, conjunctivitis, and stromal clouding were determined by direct visual observation. Eye readings were done in a blind fashion by an observer unaware of the history of the rabbit. Each eye disease parameter was scored on a scale of 0 to 4 (0 = no disease, 4 = maximum disease) as previously described (9).

Purification of DNA from TG of latently infected rabbits. Extraction of DNA from latently infected rabbit TG was done as we previously described (13, 14). Briefly, individual TG were removed from euthanized rabbits, washed in 100% ethanol at room temperature, 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 TG were incubated at 55°C for 16 h. The treated mixture was extracted once with water-saturated phenol and then with chloroform-isoamyl alcohol (24:1). The final supernatant was precipitated with 2.5 volumes of 95% ethanol and vacuum dried.

Quantitation of latent HSV-1 DNA by competitive PCR. The primer pair used for the competitive PCR analyses amplifies a 192-bp region of the HSV-1 gB gene as previously reported (12a). The competitor DNA corresponds to the same 192-bp region of gB but is missing 35 internal nucleotides. To construct the competitor DNA, the 192-bp wild-type region was amplified by PCR using the above primers. The PCR product was digested with *Bsa*JI and religated to delete 35 internal nucleotides. The resulting 157-bp product was cloned into pGEM-T (Promega Corporation, Madison, Wis.) and transformed into *Escherichia coli* RRI*λc*I857. The resulting plasmid was designated PT157. When the PT157 plasmid was subjected to PCR using the above gB primers, a 157-bp product was generated. This was separated from the 192-bp McKrae viral DNA gB PCR product by electrophoresis on 4% NuSieve GTG agarose (FMC Bioproducts, Rockland, Maine) in 1× Tris-borate-EDTA .

Competitive PCR assays were done as described previously (12a), with minor modifications. The DNA extracted from each TG was resuspended in 100 μl of double-distilled water, and 5-μl aliquots were placed in PCR tubes containing 10-fold serial dilutions of a known amount of competitor DNA (corresponding to 6×10^3 to 6×10^7 copies of HSV-1 DNA per TG). The competitive PCRs employed an excess of both primers and *Taq* polymerase. Each PCR was carried out in a final volume of 100 μl containing 10 μl of 10× PCR buffer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 1 μl of deoxynucleoside triphosphates (100 mM each dCTP, dGTP, dATP, and dTTP) (New England Biolab), 1 μl of the primer pair, and 0.5 μl (2.5 U) of *Taq* polymerase (Boehringer Mannheim Biochemicals). PCR amplification was carried out in a thermal cycler (Appligene, Pleasanton, Calif.) as previously described (14), with modifications. Briefly, one cycle was done at 95°C for 3 min and 30 cycles were done at 95°C for 15 s, 54°C for 15 s, and 71°C for 1.5 min with one subsequent extension at 72°C for 10 min. The PCR products were separated by electrophoresis on 4% NuSieve GTG agarose as described above. The DNA gel was denatured in 1.5 M NaCl–0.5 M NaOH for 20 min, and the DNA was transferred to a Hybond-N membrane (Amersham Life Science, Arlington, Ill.) and probed with a [^{32}P]dCTP-labeled cloned fragment corresponding to the 192-bp gB region. The amount of radioactivity in each band was determined with a radioanalytic imaging detector (AMBIS Inc., San Diego, Calif.).

For each experimental sample, the number of HSV-1 DNA copies in the original TG was determined from the dilution of the competitor at which the competitor and viral PCR products were present in equal amounts. If the intensity of the competitor and latent DNA PCR products did not exactly coincide at any of the competitor DNA dilutions, the competitor DNA dilution at which the PCR products would have been identical was estimated from a linear regression plot of the two PCR products made with the computer program Graph Pad. The point at which the plots cross indicates the point of equivalence of the competitor and viral PCR products.

In situ hybridization. In situ hybridization of TG sections from latently infected rabbits was done with a ^{32}P-labeled oligonucleotide probe specific for the 2-kb LAT RNA as previously described (20). The slides were examined in a blind fashion. The sections from each TG represent a sampling from throughout the TG, so that each section contains different neurons.

Statistical analyses. Statistical analyses were performed with Instat, a personal computer software program. For analyses using either the Student *t* test, the Mann-Whitney rank sum test, the chi-square test, or the Fisher exact test, results were considered statistically significant when P was <0.05.

RESULTS

Survival of rabbits ocularly infected with *d*34.5. *d*34.5 and its marker-rescued virus *d*34.5R are described in Materials and Methods. Rabbits were infected, and survival was determined 21 days postinfection (Fig. 1). All rabbits infected with *d*34.5 survived, regardless of challenge dose, including infection at approximately 10^8 PFU per eye following corneal scarification. Since corneal scarification enhances the apparent infectious dose severalfold, the effective relative challenge dose in the scarified group (infected with a total of 1.2×10^8 PFU per eye) was at least 1,000 times that of the standard dose without scarification (2×10^5 PFU per eye). In contrast, ocular challenge with 2×10^5 PFU of the marker-rescued virus, *d*34.5R, per eye resulted in a 48% death rate (13 of 27). This was significantly different from the results with the largest challenge dose of *d*34.5 ($P = 0.0005$, Fisher exact test; Fig. 1). These results confirm and extend those of our previous study (14).

Ocular replication of *d*34.5 following high-dose infection of rabbit eyes. Rabbits were infected ocularly with 2×10^5 PFU of either *d*34.5 or *d*34.5R per eye without scarification or with 1×10^8 PFU of *d*34.5 per eye with scarification as described in Materials and Methods. At each of the times postinfection for which results are shown, tear film specimens were collected from six eyes from each group, individual titers were determined, and the mean titers for each day were plotted (Fig. 2). The *d*34.5R rescued virus had a peak average titer of approximately 6×10^5 PFU per eye on day 5. In contrast, at the same infectious dose of 2×10^5 PFU per eye, *d*34.5 had a peak titer

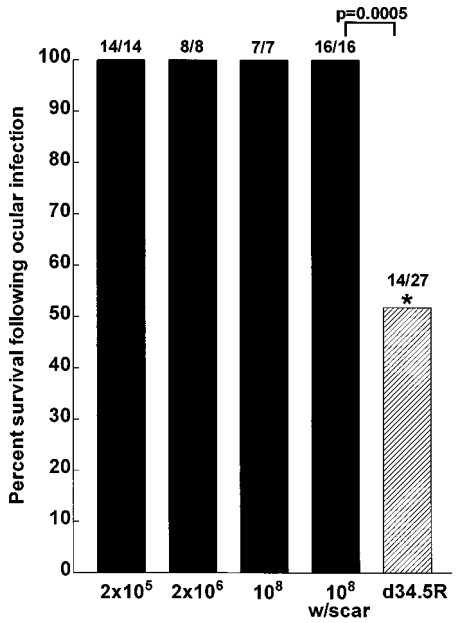

FIG. 1. Virulence of *d*34.5 in rabbits. Rabbits were infected ocularly as described in Materials and Methods. Survival was determined at day 21 postinfection. The infectious doses for *d*34.5 (in PFU per eye) are indicated below the graph. Control rabbits were infected with 2×10^5 PFU of the marker-rescued wild-type virus, *d*34.5R, per eye. The numbers at the top of each column indicate the number of surviving rabbits/number of rabbits infected. The *d*34.5R result is significantly different from each of the other results (*) ($P < 0.05$; Mann-Whitney rank sum test). The results for *d*34.5R (hatched bar) were significantly different (bracket) from the results for the high dose of *d*34.5 with scarification (w/scar). The results shown for *d*34.5 at 2×10^5 PFU per eye and *d*34.5R were done in parallel and at the same time as the higher-dose *d*34.5 infections. They were previously published as part of a different report (12a) and are included here for reference and as controls.

of <100 (day 3). Even at the extremely high infectious dose, the maximum titer of *d*34.5 (with scarification) reached only approximately 3×10^3 PFU per eye (Fig. 2). Thus, increasing the effective input dose of *d*34.5 by over 1,000-fold resulted in an apparent 10- to 100-fold increase of virus in the tears. However, this increased virus level was still approximately 100-fold less than that obtained with the standard dose of wild-type virus.

Eye disease induced by the ICP34.5 deletion mutant *d*34.5. Rabbit eyes were infected without scarification with 2×10^5 PFU of *d*34.5 per eye (12 eyes), 1×10^8 PFU of *d*34.5 per eye (14 eyes), 2×10^5 PFU of the marker-rescued virus *d*34.5R per eye (20 eyes), or, as a control, 2×10^5 PFU of parental wild-type McKrae per eye (20 eyes). An additional group of rabbits was infected with 10^8 PFU of *d*34.5 per eye following scarification (14 eyes) as described in Materials and Methods. The pattern of disease seen in eyes infected with the marker-rescued *d*34.5R virus (Fig. 3) was similar to that seen with wild-type McKrae virus. With these two viruses, the peak average score for epithelial disease was 1.4 to 1.8 on day 6 postinfection (Fig. 3A). The peak average score for conjunctivitis was 1.6 to 2 on day 4 (Fig. 3B). The peak average score for iritis was 0.4 to 0.5 on day 6 (Fig. 3C). The peak average score for clouding was 0.3 to 0.5 on day 6 (Fig. 3D). In contrast, eyes infected with 2×10^5 PFU of *d*34.5 per eye showed virtually no disease for any parameter (Fig. 3). Infection with 10^8 PFU of *d*34.5 per eye, with or without scarification, also produced no significant iritis or clouding (Fig. 3C and D). The high dose of *d*34.5, however, did produce a modest amount of conjunctivitis that cleared rapidly (scores of 1.5 and 0.6 with and without scarification, respectively, on day 2; Fig. 3B). In addition, with scarification, but not without scarification, 10^8 PFU of *d*34.5 per eye produced a modest amount of epithelial disease that cleared rapidly (approximately 0.5 on day 2; Fig. 3A).

The incidences of epithelial disease, conjunctivitis, iritis, and clouding were plotted as scattergrams for the day of peak disease severity (day 4 for conjunctivitis and day 6 for epithelial disease, iritis, and clouding) (Fig. 4). The incidence of disease for each of the parameters was less for all three of the *d*34.5 infections than for the *d*34.5R and McKrae infections ($P < 0.02$). This was consistent with the average disease severities in Fig. 3. Thus, even at very high infectious doses and even with scarification, *d*34.5 produced much less eye disease than the wild-type or marker-rescued virus.

Spontaneous reactivation following infection with the ICP34.5 deletion mutant occurs in a dose-response manner. Rabbit eyes were infected with 2×10^5 PFU of the marker-rescued virus *d*34.5R per eye or with various doses of the mutant *d*34.5. Beginning 30 days postinfection (at which time latency had already been established), all eyes were swabbed once a day to collect tear films for analysis of reactivated virus as described in Materials and Methods.

The cumulative numbers of virus-positive tear film cultures during 26 days are shown in Fig. 5 for two independent experiments. The data were standardized to represent cumulative positive cultures per eye. As we previously reported (14), the cumulative spontaneous reactivation rate in rabbits latently infected with 2×10^5 PFU of *d*34.5 per eye appeared greatly reduced compared with that in comparably *d*34.5R-infected rabbits (Fig. 5). The cumulative spontaneous reactivation in rabbits infected with a 10-fold-higher inoculum of *d*34.5 (2×10^6 PFU per eye) appeared to be slightly higher (Fig. 5A). In both experiments, cumulative spontaneous reactivation in rabbits infected with 10^8 PFU of *d*34.5 per eye without scarifica-

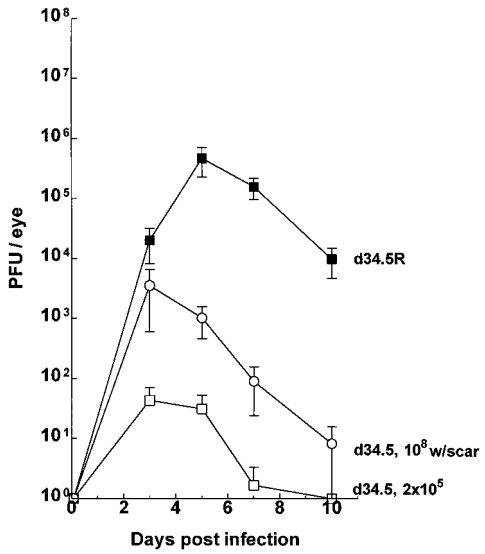

FIG. 2. Replication of *d*34.5 in rabbit eyes. Rabbits were infected with 2×10^5 PFU of *d*34.5 per eye, 10^8 PFU of *d*34.5 per eye with scarification (w/scar), or 2×10^5 PFU of *d*34.5R per eye as described in Materials and Methods. Tears were collected at the times indicated, and the amount of HSV-1 present was determined by plaque assay as described in Materials and Methods. Each time point represents the average virus titer from six eyes per group ± the standard error (error bars). Results for the low-dose *d*34.5 and *d*34.5R controls were previously published (12a).

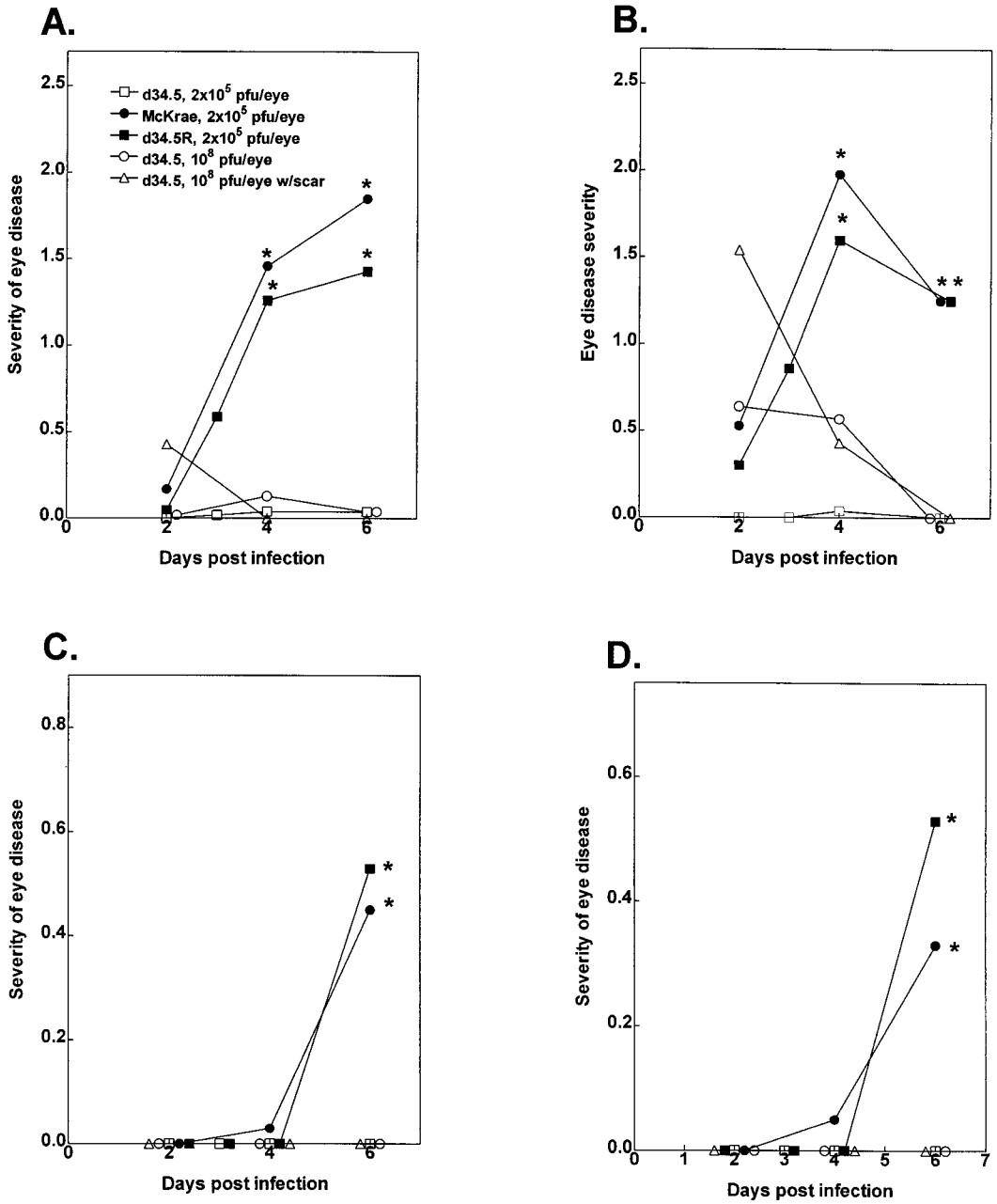

FIG. 3. Average severity of ocular disease induced by *d*34.5. Rabbits were infected with *d*34.5 with and without scarification and control rabbits were infected with *d*34.5R or the parental McKrae wild-type virus without scarification as indicated in panel A. All eyes were examined on days 2, 3, 4, and 6 postinfection and scored for eye disease as described in Materials and Methods. Epithelial disease (A), conjunctivitis (B), iritis (C), and clouding (stromal disease) (D) were each scored on a scale of 0 to 4. The mean severity of eye disease for each virus group at each time point was plotted as shown. The group infected with 2×10^5 PFU of *d*34.5 per eye consisted of 12 eyes. The 10^8-PFU groups consisted of 14 eyes each. The *d*34.5R group and the McKrae group each consisted of 20 eyes. The amounts of eye disease for both the *d*34.5R and the McKrae groups were significantly different (*) from those for all of the *d*34.5 groups at that time point ($P < 0.05$; Mann-Whitney rank serum test).

tion approached that of rabbits infected with marker-rescued virus. Finally, the cumulative spontaneous reactivation in rabbits infected with 10^8 PFU of *d*34.5 per eye following scarification (Fig. 5B) appeared to be the same as or even slightly higher than that for *d*34.5R. These results suggest that with *d*34.5 there is a dose-response relationship between the amount of input virus and spontaneous reactivation. Statistical analyses of these results are shown in Table 1.

The total number of positive (spontaneously reactivated) cultures versus total cultures is shown in Table 1. In experiment A, the percentage of positive cultures increased with increasing amounts of input *d*34.5 virus from 1.4% (6 of 416) at 2×10^5 PFU per eye to 3.4% (16 of 468) at 2×10^6 PFU per eye ($P = 0.046$) to 11.1% (48 of 416) at 1×10^8 PFU per eye ($P < 0.0001$). In experiment B, only 0.3% (1 of 312) of the cultures of samples from eyes infected with 2×10^5 PFU of *d*34.5 per eye were positive. This was significantly less than the 6.3% (23 of 364) of cultures of specimens from eyes infected

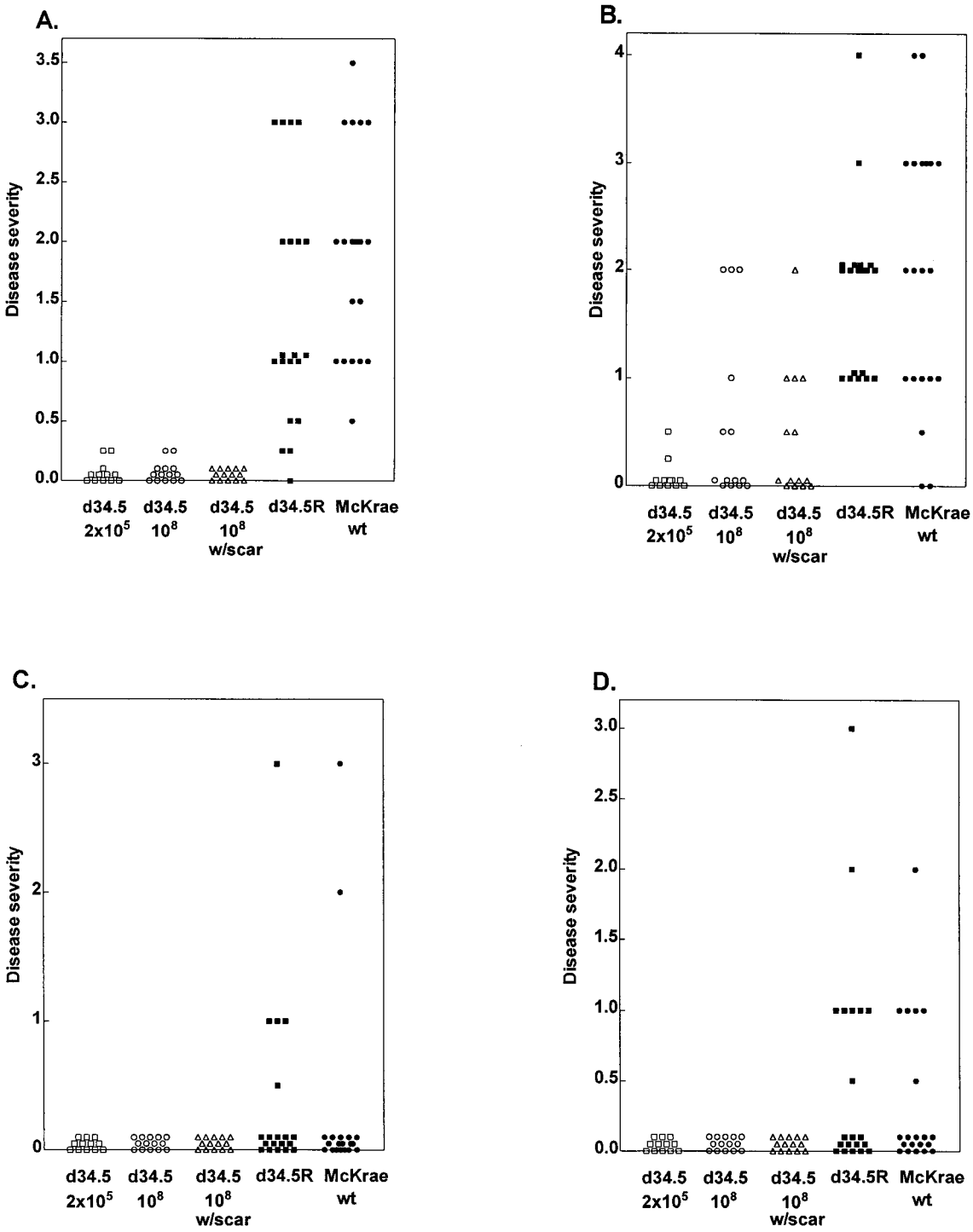

FIG. 4. Incidence and severity of ocular disease induced by *d*34.5. (A) Epithelial disease; (B) conjunctivitis; (C) iritis; (D) clouding. Each scattergram illustrates the incidence of disease severity for individual eyes. Each symbol represents a single eye, with the severity of disease for that eye shown on the *y* axis. All data are from the day of average peak severity of disease as determined from Fig. 3 (day 4 for conjunctivitis and day 6 for epithelial disease, iritis, and clouding). Within each disease parameter, results for *d*34.5R and McKrae were not significantly different from each other ($P > 0.3$; Mann-Whitney rank sum test), the results for infections with 10^8 PFU of *d*34.5 per eye (with or without scarification) were not significantly different from each other ($P > 0.6$), and results for all three *d*34.5 infections, regardless of dose or scarification, were less than those for *d*34.5R or McKrae ($P < 0.02$).

with 1×10^8 PFU of *d*34.5 without scarification, 10% (37 of 364) of cultures for eyes infected with 1×10^8 PFU of *d*34.5 with scarification, or 7.7% (20 of 260) of cultures for eyes infected with 2×10^5 PFU of the marker-rescued virus *d*34.5R ($P < 0.0001$). There were no statistically significant differences among the two high doses of *d*34.5 and the marker-rescued *d*34.5R ($P > 0.05$). Although in experiment A the *d*34.5R-infected rabbits appeared to have a higher spontaneous reac-

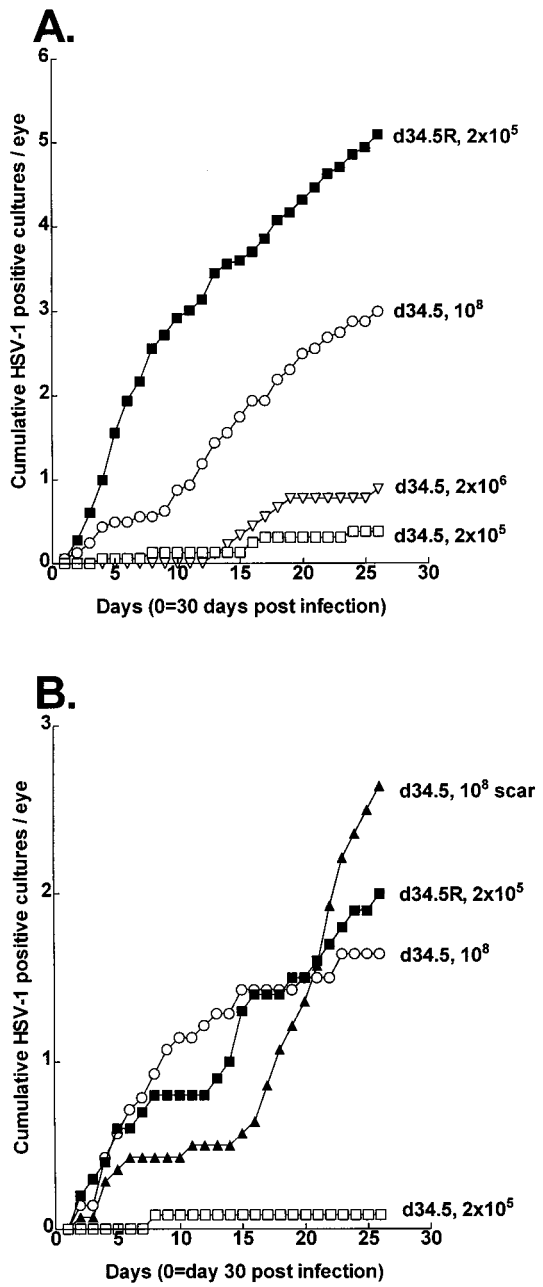

FIG. 5. In vivo spontaneous reactivation. Rabbits were infected with *d*34.5 or *d*34.5R. Beginning 30 days postinfection, tear film specimens were collected daily and the presence or absence of spontaneously reactivated virus was determined by plating on primary rabbit kidney cells as described in Materials and Methods. The average cumulative numbers of virus-positive tear film cultures per eye are plotted for each virus-infected group. Panels A and B show the results of two independent experiments. The numbers of eyes in each group were as follows: for panel A, *d*34.5 at 2×10^5 PFU, 16; *d*34.5 at 2×10^6 PFU, 16; *d*34.5 at 10^8 PFU, 18; and *d*34.5R, 18; panel B, *d*34.5 at 2×10^5 PFU, 12; *d*34.5 at 10^8 PFU, 14; *d*34.5 at 10^8 PFU, scarified, 14; and *d*34.5R, 10. The results of the low-dose *d*34.5 and *d*34.5R infections were previously published as part of a different report (12a). They were determined in parallel and at the same time as the other results shown here and are included for reference and as controls. Statistical analyses of the data are shown in Table 1. scar, scarification.

tivation rate than the unscarified rabbits infected with 10^8 PFU *d*34.5 per eye (19.6 versus 11.5%; $P < 0.05$; Fisher exact test), in experiment B there was no difference. There was also no difference in any of the additional statistical analyses reported below. Thus, the spontaneous reactivation rate in rabbits infected with the highest dose of *d*34.5 appeared comparable to that in rabbits infected with the wild-type marker-rescued virus.

Because the above analyses do not take into account the 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 the groups were compared by the Student *t* test (Table 1). The average incidence of positive cultures per eye is shown. In both experiments, the standard group infected with 2×10^5 PFU of *d*34.5 per eye had significantly less spontaneous reactivation than the group infected with 1×10^8 PFU of *d*34.5 per eye and the *d*34.5R (marker-rescued) group ($P < 0.05$). In addition, in each experiment, there were no significant differences between the *d*34.5R group and the groups infected with 10^8 PFU of *d*34.5 either with or without scarification ($P > 0.05$).

The number of eyes in each group that had at least one spontaneous reactivation was also determined (Table 1). In experiment A, only 25% (4 of 16) of the eyes infected with 2×10^5 PFU of *d*34.5 per eye and 17% (3 of 18) of the eyes infected with 2×10^6 PFU of *d*34.5 per eye reactivated at least once. These were each significantly less than for the *d*34.5R group (94% [17 of 18]; $P < 0.0001$) or the group infected with 10^8 PFU of *d*34.5 (88% [14 of 16]; $P < 0.0001$). In experiment B, the infection in only 1 of 12 eyes (9%) in the group infected with 2×10^5 PFU of *d*34.5 per eye reactivated. Again, this was significantly less than for either the high-dose *d*34.5 group or the *d*34.5R control group ($P < 0.05$, Fisher exact test, single sided). There were no differences among the high-dose *d*34.5 groups and the *d*34.5R control group in either experiment ($P > 0.25$).

Another method of analyzing possible differences in 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 each constitute one episode of spontaneous reactivation. The average numbers of spontaneous reactivations per eye in each group calculated in this manner are shown in Table 1. The numbers of episodes of spontaneous reactivation for the marker-rescued *d*34.5R virus were 2.1 and 1.3 for experiments A and B, respectively. Within each experiment, this was similar to the number of episodes for the high dose of *d*34.5 mutant virus (experiment A, 10^8 PFU of *d*34.5 without scarification, 2.0 episodes, $P = 0.15$; experiment B, 10^8 PFU of *d*34.5 with and without scarification, 1.1 episodes, $P = 0.67$ and 0.77). In contrast, the number of episodes in eyes infected with 2×10^5 or 2×10^6 PFU of *d*34.5 per eye was significantly less than that for *d*34.5R-infected eyes ($P < 0.0001$, experiment 1; $P = 0.02$, experiment 2). The combination of the above analyses indicates that spontaneous reactivation with the *d*34.5 mutant occurs in a dose-response manner. For low (standard)-dose infections, little spontaneous reactivation was seen, while with very high-dose infections (10^8 PFU per eye), spontaneous reactivation occurred at levels similar to that for the marker-rescued wild-type virus.

Estimate of establishment of latency by competitive PCR. Sixty days postinfection, total DNA was isolated from individual TG of rabbits latently infected with 2×10^5 PFU of *d*34.5, 1×10^8 PFU of *d*34.5, or 2×10^5 PFU of *d*34.5R per eye. The number of HSV-1 genomic copies per TG was then estimated

TABLE 1. Spontaneous reactivation in rabbits latently infected with different doses of *d*34.5[a]

Expt, virus, and dose (PFU/eye)	No. of positive cultures/total (%)	*P* vs *d*34.5 (2×10^5)[b]	Fraction of positive cultures/eye[c]	*P* vs *d*34.5 (2×10^5)[d]	No. of positive eyes/total (%)	*P* vs *d*34.5R[e]	No. of episodes of spont. react./eye[f] (*n*)	*P* vs *d*34.5R[e]
A								
*d*34.5								
2×10^5	6/416 (1.4)		0.01		4/16 (25)	<0.0001[g]	0.3 (16)	<0.0001[g]
2×10^6	16/468 (3.4)	0.046[g]	0.03	0.84	3/18 (17)	<0.0001[g]	0.2 (18)	<0.0001[g]
1×10^8	46/416 (11.1)	<0.0001[g]	0.12	0.0005[g]	14/16 (88)	0.59	2.0 (16)	0.15
*d*34.5R, 2×10^5	80/408 (19.6)	<0.0001[g]	0.19	<0.0001[g]	17/18 (94)		2.1 (18)	
B								
*d*34.5								
2×10^5	1/312 (0.3)		0.003		1/12 (8)	0.04[g]	0.1 (12)	0.02[g]
1×10^8	23/364 (6.3)	<0.0001[g]	0.06	0.004[g]	10/14 (71)	0.40	1.1 (14)	0.67
1×10^8, w/scar[h]	37/364 (10.2)	<0.0001[g]	0.1	0.01[g]	9/14 (64)	0.68	1.1 (14)	0.77
*d*34.5R, 2×10^5	20/260 (7.7)	<0.0001[g]	0.08	0.04[g]	5/10 (50)		1.3 (10)	

[a] Rabbits were bilaterally ocularly infected with the ICP34.5 deletion mutant *d*34.5 or the marker-rescued (wild-type) virus *d*34.5R as described in Materials and Methods. Experiments A and B were done independently at different times and with different groups of rabbits. Spontaneous reactivation was assessed beginning on day 30 postinfection by culturing tear films collected daily for 26 days as described in Materials and Methods.
[b] As determined by two-sided Fisher exact test, compared with the results for *d*34.5 at 2×10^5 PFU per eye.
[c] The fraction of time that each eye in each group contained reactivated HSV-1 (positive cultures for an eye/total cultures for that eye) was averaged for that group.
[d] As determined by two-sided Mann-Whitney rank sum test, using the fraction of time each eye was positive for reactivated virus.
[e] As determined by two-sided Fisher exact test, compared with the results for marker-rescued *d*34.5R.
[f] Total number of reactivations in each group, assuming that consecutive days of HSV-1-positive tear film cultures for a single eye are the result of a single reactivation event or episode. Spont. react., spontaneous reactivations.
[g] Significantly different from result for *d*34.5 at 2×10^5 PFU per eye ($P < 0.05$).
[h] w/scar, with scarification.

by competitive PCR assays as described in Materials and Methods. PCR assays were performed with 14 TG from rabbits latently infected with 2×10^5 PFU of *d*34.5 per eye, 15 TG from rabbits latently infected with 1×10^8 PFU of *d*34.5 per eye, and 11 TG from rabbits latently infected with *d*34.5R. The estimated number of HSV-1 DNA copies for each of the TG is shown in a scattergram (Fig. 6). Each symbol represents the estimated number of copies for one TG. The scattergram therefore shows the distribution and spread of the results. Visually, the range and distribution appear similar for all three viruses. The average numbers of genomic copies ($\pm$ standard deviations) were approximately $5.2 \times 10^5 \pm 2.8 \times 10^5$ per TG for *d*34.5R, $7.1 \times 10^5 \pm 3.8 \times 10^5$ per TG for *d*34.5 at 2×10^5 PFU per eye, and $5.8 \times 10^5 \pm 4.0 \times 10^5$ for *d*34.5 at 1×10^8 PFU per eye. The amounts of HSV-1 DNA were comparable for the three groups ($P = 0.93$, one-way analysis of variance).

Detection of LAT RNA in TG of rabbits latently infected with *d*34.5. Rabbits were ocularly infected with 2×10^5 PFU of the marker-rescued virus *d*34.5R, 2×10^5 PFU of *d*34.5, or 1×10^8 PFU of *d*34.5 per eye following corneal scarification. Sixty days postinfection, the rabbits were euthanized and one TG was removed from each rabbit (Table 2). The TG were fixed, sectioned, and processed for in situ hybridization using a ^{32}P-labeled probe specific for the abundant 2-kb LAT RNA as described in Materials and Methods. A representative section from each group is shown in Fig. 7. Panels A and B show dark-field and bright-field images, respectively, of the same section from *d*34.5R latently infected TG. Similarly, panels C and D show *d*34.5 at 1×10^8 PFU per eye, and panels E and F show *d*34.5 at 2×10^5 PFU per eye. Dark-field microscopy allows increased efficiency in locating positive in situ hybridization results, which are then confirmed by bright-field microscopy. As previously reported, the black dots over the nuclei of neurons seen via bright-field microscopy indicate hybridization to the 2-kb LAT RNA (20). These images are representative of the overall trend. The *d*34.5R latently infected rabbit section (Fig. 7A and B) had more positive neurons than the section from the rabbit infected with 1×10^8 PFU of *d*34.5 per eye (Fig. 7C and D), which in turn had more positive neurons than the section from the rabbit infected with 2×10^5 PFU of *d*34.5 per eye (Fig. 7E and F). No LAT RNA was detected in any neurons from uninfected control rabbits (not shown).

Table 2 shows the number of sections examined from each

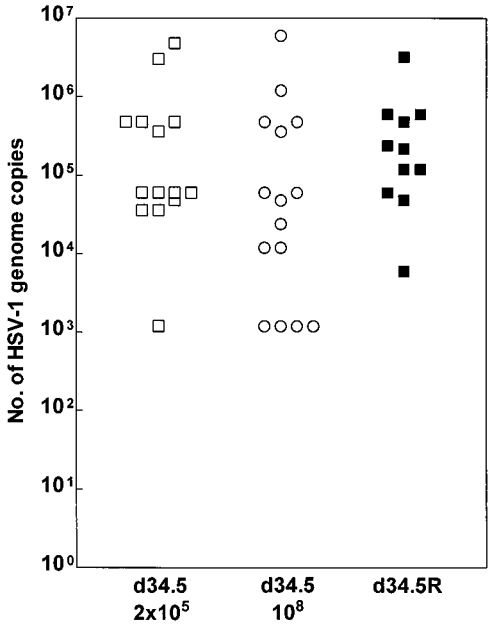

FIG. 6. Distribution of the number of copies of HSV-1 DNA in TG of latently infected rabbits as determined by competitive PCR. Competitive PCR assays were performed with 14 TG from rabbits latently infected with 2×10^5 PFU of *d*34.5 per eye, 15 TG from rabbits latently infected with 10^8 PFU of *d*34.5 per eye with scarification, and 11 TG from rabbits latently infected with *d*34.5R as described in Materials and Methods. The estimated HSV-1 DNA genome copy number for each TG is plotted on the scattergram. Each symbol represents the number of HSV-1 genome copies for one TG. The results of the low-dose *d*34.5 and *d*34.5R infections, although previously published (12a), were determined in parallel and at the same time as the high-dose results. There was no significant difference among the groups ($P = 0.93$; one-way analysis of variance).

TABLE 2. Establishment of latency based on the percentage of neurons positive for 2-kb LAT RNA as determined by in situ hybridization[a]

Latent virus	Results for individual rabbits[b]								Total results[c]				
	TG1		TG2		TG3		TG4						
	No. of sections	Positive/total neurons (%)	No. of sections	Positive/total neurons (%)	No. of sections	Positive/total neurons (%)	No. of sections	Positive/total neurons (%)	Positive/total sections[d] (%)	*P* vs *d*34.5R[e]	*P* vs high dose[e]	Positive/total neurons (%)	*P* vs *d*34.5R[f]	*P* vs high dose[f]
*d*34.5R	11	34/6,925 (1)	9	207/6,896 (3)	12	510/4,834 (11)			29/32 (91)		<0.0001	751/18,655 (4)		<0.0001
*d*34.5														
High dose	13	0/4,547 (0)	8	3/3,971 (0.1)	8	4/3,151 (0.1)	12	21/5,305 (0.4)	12/41 (29)	<0.0001		28/16,974 (0.2)	<0.0001	
Normal dose	12	0/6,061 (0)	11	0/3,851 (0)	14	8/6,667 (0.1)			2/37 (5)	<0.0001	0.007	8/16,580 (0.05)	<0.0001	0.002

[a] Rabbits were infected with 2×10^5 PFU of *d*34.5R (equivalent to wild-type McKrae) per eye or *d*34.5 (normal dose) or 1×10^8 PFU of *d*34.5 per eye (high dose). At 60 days postinfection, individual TGs were harvested, fixed, sectioned, and subjected to in situ hybridization with a ^{32}P-labeled probe specific for the 2-kb region of LAT RNA as described in Materials and Methods. Sections were quantitated for LAT RNA-positive and total neurons by light microscopy.

[b] Each TG is from a different rabbit. Approximately 15 sections were mounted on slides from one TG of each of four rabbits latently infected with *d*34.5R, four rabbits latently infected with the normal dose of *d*34.5, and five rabbits latently infected with the high dose of *d*34.5. The sections were spaced throughout each TG to give a representative sampling of the entire TG and to ensure that portions of the same neuron were not contained on multiple slides. Since the in situ hybridization procedure often results in tissue sections and parts of tissue sections falling off the slides, the number of sections and the number of neurons on each section varied. Only TG in which at least 2,000 neurons could be counted were included.

[c] Totals of the individual TG results for each group.

[d] Sections were considered positive if at least one unequivocal LAT RNA-positive neuron was detected.

[e] Two-sided chi-square test. A *P* of <0.05 is considered statistically significant.

[f] Two-sided Fisher exact test. A *P* of <0.05 is considered statistically significant.

TG and the number of LAT RNA-positive neurons detected in each TG compared with the total neurons in that TG. As summarized in Table 2, 751 of 18,655 neurons (4%) examined in sections from TG latently infected with *d*34.5R contained detectable amounts of LAT RNA. This was approximately 80 times higher than the percentage of positive neurons in TG from rabbits latently infected with 2×10^5 PFU of *d*34.5 per eye (8 of 16,580 [0.05%]; $P < 0.0001$) and 20 times higher than the percentage of neurons in TG from rabbits latently infected with 1×10^8 PFU of *d*34.5 per eye (28 of 16,974 [0.2%]; $P < 0.0001$). TG from rabbits infected with the high dose of *d*34.5 had approximately four times more LAT RNA-positive neurons than did TG from rabbits infected at the normal dose of *d*34.5 ($P = 0.002$).

The in situ hybridization results were also analyzed using the number of positive sections versus total sections rather than neurons. Sections were considered positive if at least one unequivocally positive neuron was detected. Consistent with the analysis of total neurons, the *d*34.5R latently infected group had more LAT RNA-positive sections than either of the *d*34.5 groups. In the *d*34.5R-infected group, 91% (29 of 32) of the sections were positive for LAT RNA. This was approximately 18 times higher than the result for the standard-dose *d*34.5 group (2 of 37 [5%]; $P < 0.0001$) and approximately three times higher than the result for the high-dose *d*34.5 group (12 of 41 [29%]; $P < 0.0001$). The approximately sixfold difference between the high- and standard-dose *d*34.5 groups was also significant ($P = 0.007$). The results of these analyses suggest that *d*34.5 established latency at a rate directly related to the initial dose of virus used for infection.

DISCUSSION

High-dose infection with *d*34.5 separates corneal disease and virulence from spontaneous reactivation. We previously constructed the ICP34.5 deletion mutant, *d*34.5, and showed that infection of rabbit eyes with a standard amount of *d*34.5 (2×10^5 PFU per eye) resulted in no corneal disease, a decrease in death from 48 to 0%, and a >10-fold decrease in spontaneous reactivation (14). In the present study, we found that high-dose ocular infection with *d*34.5 (10^8 PFU per eye, with or without scarification) still produced no corneal disease and no deaths but resulted in wild-type levels of spontaneous reactivation. This finding separates (at least partially) the phenotypes of corneal disease and virulence from that of spontaneous reactivation.

ORF P. While this work was in progress, a potential open reading frame (ORF P) on the opposite strand of ICP34.5 with a presumptive mRNA transcribed independently of LAT RNA was reported (6, 22). Since ORF P is completely deleted in *d*34.5, one or more of the effects attributed to deletion of ICP34.5 in this report could formally be due to deletion of ORF P.

Quantitative and competitive PCR analysis versus in situ hybridization. We previously used quantitative PCR assays to estimate the number of latent HSV-1 genomic copies per TG in rabbits ocularly infected with the standard dose of *d*34.5 (14). Although it appeared that there were approximately three- to fivefold fewer genomic copies in the TG of *d*34.5-infected rabbits than in wild-type-virus-infected rabbits, the differences were not statistically significant. This was likely due to the large apparent variation in HSV-1 DNA copy number seen from TG to TG, even within the same group of rabbits, in this type of analysis (5, 13, 14).

In the current study, we showed that although spontaneous reactivation is greatly reduced following standard-dose infec-

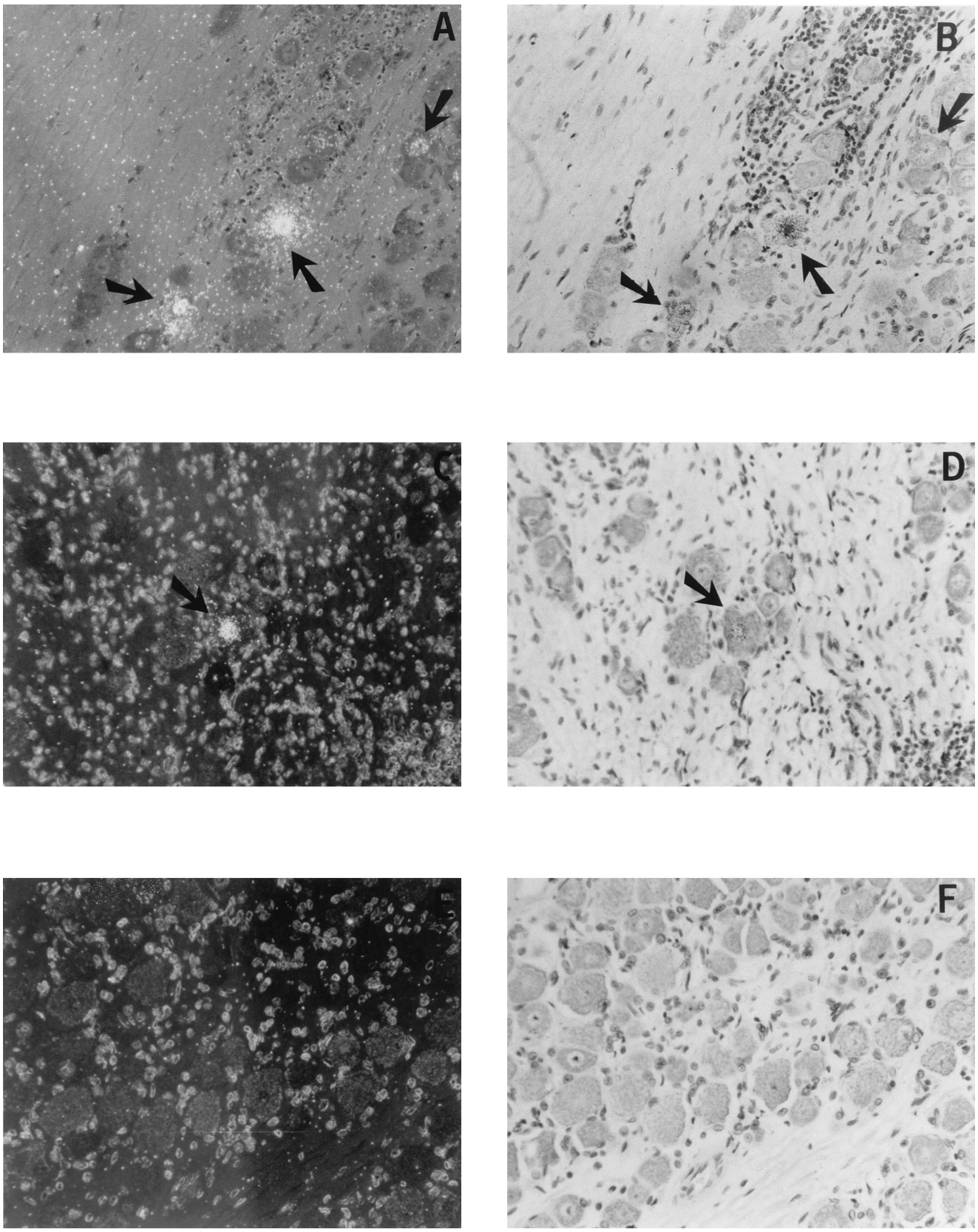

FIG. 7. Visualization of the 2-kb LAT RNA transcript by in situ hybridization. Rabbits were infected with 2×10^5 PFU of *d*34.5R per eye (A and B), 1×10^8 PFU of *d*34.5 per eye with scarification (C and D), or 2×10^5 PFU of *d*34.5 per eye (E and F). Sixty days postinfection, TG were removed, fixed, and sectioned. Sections were hybridized to a ^{32}P-labeled oligonucleotide probe specific for the 2-kb LAT RNA and exposed for autoradiography, and the numbers of LAT RNA-positive and -negative neurons on each slide were determined as described in Materials and Methods. Hybridization to the 2-kb LAT RNA (arrows) is indicated. Representative sections are shown. Dark-field (A, C, and E) and bright-field (B, D, and F) images of the same sections are shown. The numbers of positive neurons and sections are given and analyzed in Table 2.

tion with *d*34.5, at high infectious doses *d*34.5 reactivated at wild-type levels. Since *d*34.5 replicated poorly in eyes and TG (14), the simplest hypothesis would be that standard-dose infection with *d*34.5 results in decreased establishment of latency and therefore in reduced spontaneous reactivation, while high-dose infection results in a higher rate of latency and therefore a more normal rate of spontaneous reactivation. We attempted to observe these predicted results by means of competitive PCRs. Theoretically, competitive PCRs should be more accurate and therefore more sensitive than the quantitative PCRs which previously were unsuccessful at demonstrating a significant difference between levels of wild-type and *d*34.5 DNA. However, the results of our competitive PCR assays agreed with our previous findings (i.e., we were unable to show any statistically significant differences). Thus, using two different PCR methods to estimate the number of latent HSV-1 genomic copies per TG, we could not definitively demonstrate that *d*34.5 established latency less efficiently than wild-type virus or that high-dose *d*34.5 infection resulted in a higher rate of establishment of latency than standard-dose *d*34.5 infection. Since we were surprised by these results, we decided to see if an alternative method of estimating the relative amount of HSV-1 latency (namely, in situ hybridization for LAT RNA) would produce similar results.

During latency in rabbits, mice, and humans, the 2-kb LAT RNA can be detected in abundance by in situ hybridization in approximately 1 to 10% of neurons in the TG (19, 20). Although not all latently infected neurons contain this LAT RNA, the amount of LAT RNA has long been used as a rough indicator of the amount of HSV-1 latency. Specifically, it is presumed that the amount of latency is directly related to the percentage of LAT RNA-positive neurons. We used this notion to estimate the comparative amount of latency following infection with different doses of *d*34.5 and with *d*34.5R. In contrast to the PCR studies which did not detect any differences in the estimated levels of establishment of latency, the in situ hybridization results clearly indicated that, as judged by positive neurons and positive TG sections, (i) wild-type *d*34.5R established latency at a rate 18 to 80 times higher than the standard dose of *d*34.5; (ii) *d*34.5R established latency at a rate 3 to 20 times higher than the high dose of *d*34.5; and (iii) the high dose of *d*34.5 established latency at a rate 4 to 6 times higher than the standard dose of *d*34.5.

There are several possible explanations for the apparent discrepancy between the DNA PCR results for total ganglionic DNA and the in situ hybridization results for LAT RNA. (i) Many groups that have used DNA PCR analysis to estimate the number of latent HSV-1 genomes in large numbers of TG have found a wide variation even among TG from the same experimental group. Variations of 1,000- to 100,000-fold within groups have been reported (5, 13, 14). Regardless of whether these findings are artifactual or represent authentic variation, the resulting high standard deviations make it extremely difficult to achieve statistical significance between different experimental groups. (ii) Ocular infection with standard and high doses of *d*34.5 and the standard dose of wild-type virus may generate similar numbers of total latent HSV-1 genomes per TG, but the neuronal distribution may differ. To be consistent with both the PCR and the in situ analyses, wild-type virus would have to produce latency in a large number of neurons, with each neuron containing only a few viral genomes. In addition, low-dose *d*34.5 infection would have to produce latent infection in only a small number of neurons, each with a large number of viral genomes. The high-dose *d*34.5 infection would then have to fall between these extremes. Such a scenario would suggest that ICP34.5 affects the neuronal distribution of latency. (iii) In the *d*34.5 mutant, the rate of synthesis of 2-kb LAT RNA or its stability may be reduced. In a low-dose infection, this effect would have to be more apparent than in a high-dose infection. This would not be unprecedented, since, for example, ICP0 null mutants grow very poorly at low multiplicities of infection but replicate reasonably well at high multiplicities (17). In this situation, the total amount of latent genomic DNA and its neuronal distribution would be similar, but with low-dose *d*34.5 infection there would be a large reduction of the 2-kb LAT RNA, so that we detected it in only 0.05% of the neurons. With the high-dose *d*34.5 infection, LAT RNA would also be reduced, but less so than with the low-dose infection, such that we detected LAT in four times more neurons (0.2%). With wild-type infection, LAT RNA would not be impacted, and we therefore detected the 2-kb LAT RNA in 4% of neurons. This scenario would mean that ICP34.5 affects the amount of 2-kb LAT RNA during latency. (iv) Finally, as alluded to above, these types of PCR analyses may just not have the sensitivity or reproducibility, when total DNA in extracts of TG is used, to detect the types of differences encountered in this system. This may be a result of the low copy number (<1) of HSV-1 genomes per cell genome in the TG.

Correlation between establishment of latency and spontaneous reactivation. If we hypothesize that the in situ results are a better reflection of the relative rates of establishment (and maintenance) of latency than the PCR results, then the following arguments and conclusions can be drawn.

(i) ICP34.5 was involved in establishment of, but not reactivation from, latency. Both standard- and high-dose infections of *d*34.5 established latency in fewer neurons than did wild-type-virus infection. Nonetheless, high-dose *d*34.5 infection reactivated at a wild-type rate. Since a reduced amount of latent virus lacking ICP34.5 and ORF P was able to reactivate at wild-type levels, neither ICP34.5 nor ORF P could have been important for spontaneous reactivation.

(ii) Establishment of latency and therefore the ultimate spontaneous reactivation of *d*34.5 was directly related to the amount of virus detected in tear films. Between days 3 and 10 postinfection, the amount of *d*34.5 detected in eyes was between 10- and 100-fold greater in eyes infected with the high dose than in those infected with the standard dose of *d*34.5. Furthermore, the average amount of wild-type virus (*d*34.5R) was approximately 100- to 1,000-fold greater than the high dose of *d*34.5. Since *d*34.5 replicates very poorly in rabbit eyes, the amount of virus detected in the *d*34.5-infected eyes may reflect viral replication or residual input virus or a combination of the two.

(iii) Once a threshold level of latency was reached, additional latency did not produce an increase in spontaneous reactivation. The high-dose *d*34.5 infection resulted in expression of 2-kb LAT RNA in only 0.2% of neurons and 29% of sections (levels approximately 3- to 20-fold lower than with wild-type infection). Nonetheless, spontaneous reactivation was at wild-type levels. In contrast, the standard-dose *d*34.5 infection resulted in only 0.05% LAT RNA-positive neurons and 5% LAT RNA-positive sections (four- to sixfold less than with the high dose) and a decrease in spontaneous reactivation of 8- to 20-fold compared with that of the high-dose *d*34.5 infection (1.4 versus 11.5% in experiment A and 0.3 versus 6% in experiment B; Fig. 5). Thus, an increase in LAT RNA expression from 0.05 to 0.2% of the neurons (5 to 29% of sections) corresponded to a large increase in spontaneous reactivation, while an increase in LAT RNA from 0.2 to 4% of the neurons (29 to 91% of sections) did not correspond to any change in the observed spontaneous reactivation. This supports the hypothesis first proposed by Gordon et al. (3, 4) that

there is a threshold level of latency required for efficient reactivation and that past this threshold, additional latency has little impact on the rate of spontaneous reactivation.

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