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Failure of Thymidine Kinase-Negative Herpes Simplex Virus To Reactivate from Latency following Efficient Establishment

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Thymidine kinase-negative mutants of herpes simplex virus did not reactivate from latency in mouse trigeminal ganglia, even when their latent viral loads were comparable to those that permitted reactivation by wild-type virus. Thus, reduced establishment of latency does not suffice to account for the failure to reactivate.

Herpes simplex virus (HSV) has two distinct lifestyles, productive infection and latent infection. In animal models of HSV infection, the establishment of latency in sensory ganglia by wild-type (wt) virus is preceded by a period of viral replication at the site of inoculation and in ganglia. Nevertheless, viral mutants that are defective for viral replication can establish a latent infection, albeit with reduced efficiency (8, 9, 13, 15, 18, 23, 28, 34). For example, following corneal inoculation of mice, thymidine kinase-negative (TK^-) mutants are severely impaired for virus replication in trigeminal ganglia (4, 6, 10, 14, 16, 24, 29–33). However, these ganglia contain biologically active viral genomes that express latency-associated transcripts (LATs), indicating that TK^- mutants can establish latency (4, 6, 16).

Truly TK^- mutants derived from standard laboratory strains ordinarily do not reactivate from explanted murine ganglia (4, 30, 31) (although some derived from clinical isolates can reactivate inefficiently [7, 11]). This has generally been ascribed to a requirement for TK during reactivation (4, 6, 19, 24, 29–31). However, in the case of wt HSV and certain mutants, reactivation frequencies correlate with the numbers of viral genomes or latently infected neurons in ganglia or both (20, 21, 25, 26). Indeed, establishment of latency by TK^- mutants is less efficient than that of wt virus, as evidenced by a 6- to 50-fold reduction in total viral genomes, viral genome-positive neurons, total LATs, or LAT-positive neurons (1, 4, 14–16, 27, 33). Accordingly, the idea that TK is primarily required for reactivation rather than for establishment of latency has been called into question (33).

To address this issue, we first investigated whether the number of viral genomes per latent ganglion (latent viral load) established by a TK^- mutant is lower than the minimum latent viral load that permits wt virus to reactivate. Seven- to eight-week-old male CD-1 (Charles River Laboratories) or CD-1-derived Hsd:ICR (Harlan Sprague-Dawley) mice (similar results were obtained with mice from both suppliers) were anesthetized and inoculated on the eye via corneal scarification as previously described (18), with various doses of the wt strain KOS, ranging from 2×10^1 to 2×10^6 PFU, or with 2×10^6 PFU of *dl*sptk (4), a KOS-derived TK deletion mutant. Titers of virus at the site of inoculation and ganglia were assayed by swabbing eyes 1 day postinfection (p.i.) and by excising and homogenizing ganglia 3 days p.i. As shown in Fig. 1B, KOS did not detectably replicate on the eye when the inoculum was 2×10^1 or 2×10^2 PFU per eye. Viral replication was observed in 5 of 9 mouse eyes (55%) and 8 of 15 ganglia (53%) at the inoculum of 2×10^3 PFU per eye. At higher inoculating doses, KOS replicated in >90% of mouse eyes and ganglia. These data are similar to those obtained previously with KOS (9, 18). The TK^- mutant, *dl*sptk, replicated to wt titers at 1 day p.i. in the mouse eye but was severely impaired for replication in ganglia, as previously observed (1, 4, 11, 16, 31).

At 30 days p.i. (when latency was established), mice were sacrificed. One pool of ganglia was assayed for reactivation of virus as described previously, and a second pool was assayed for latent viral load using quantitative PCR, as previously described (1, 15–17). As shown in Fig. 1A and C, both the frequencies of reactivation from ganglia latently infected with KOS and the latent viral load increased with the dose of virus in the inoculum until the dose of 2×10^5 PFU per eye, at which dose a plateau was reached. The relationship between inoculum dose and reactivation is similar to that obtained in a previous study (18). (Although we did not observe reactivation from 12 ganglia following inoculation at 2×10^2 PFU per eye, Leib et al. [18] observed reactivation from 2 of 24 ganglia at this dose.) Notably, following inoculation with 2×10^3 PFU per eye, reactivation (6 of 12) and viral genomes (3 of 6) were observed in 50% of ganglia, similar to the percentage of ganglia in which replication was observed at that dose (Fig. 1A).

Because our assay for HSV DNA had a limit of detection of 10 viral genomes per ganglion (due to our sampling only 10% of the ganglion), we sought to increase sensitivity of detection of latent HSV by measuring LATs, since there are $\sim 10^5$ LATs per viral genome in latently infected ganglia (2, 3, 16, 17). Using a quantitative reverse transcription-PCR assay for LATs as described previously (2, 3, 16, 17), we detected LATs only in ganglia in which we also detected viral DNA, even when six more cycles of PCR amplification were applied to the LAT assay (data not shown). Thus, we detected latent DNA and

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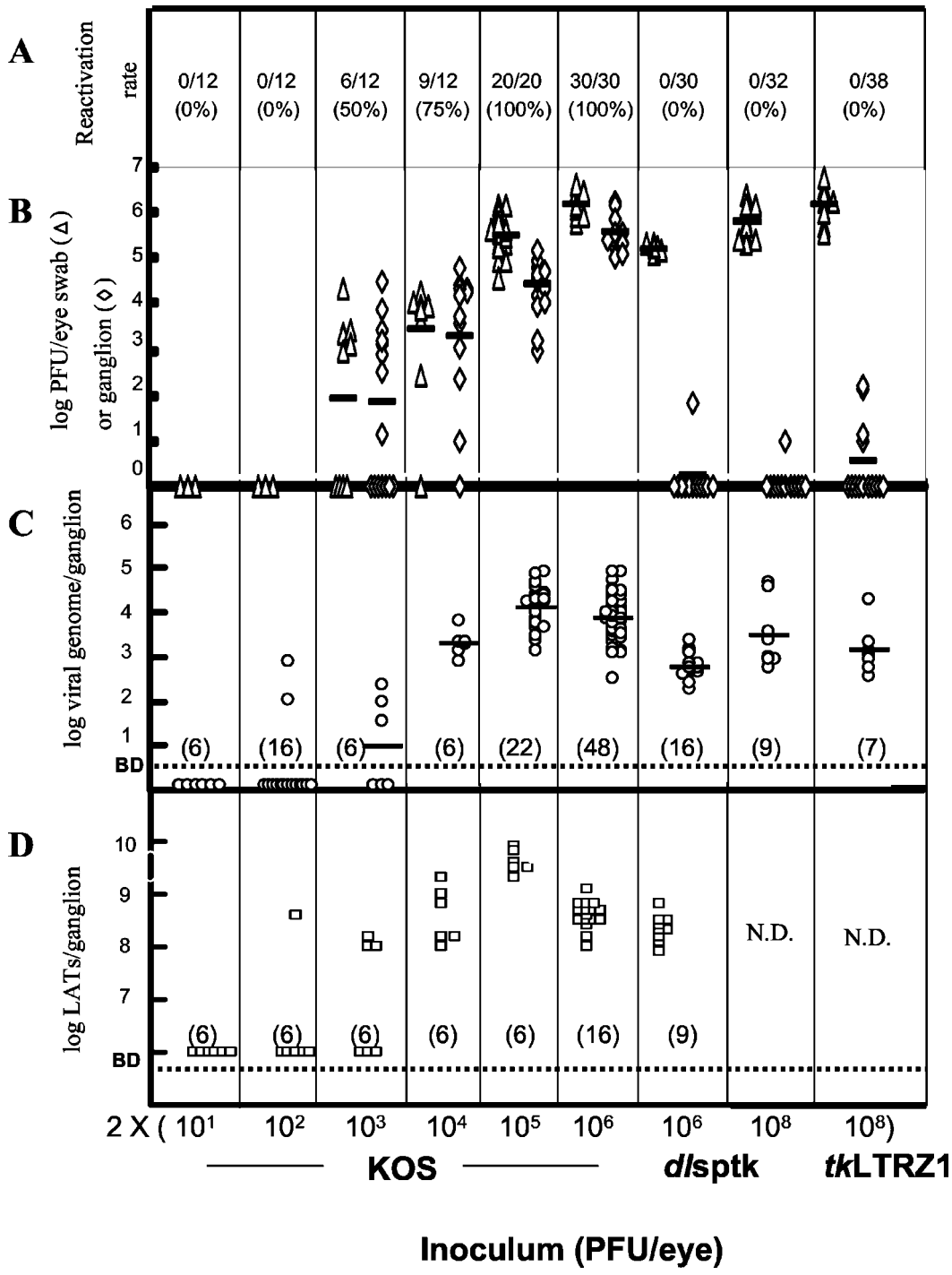

FIG. 1. Relationship between inoculum dose, reactivation, acute HSV replication, latent viral load, and LAT expression. Mice were infected with the doses of KOS, *dl*sptk, or *tk*LTRZ1 as indicated on the *x* axis. (A) Virus reactivation was assayed 30 days p.i., and the number of reactivations/number of ganglia assayed are shown. The reactivation percentages are shown in parentheses. (B) Titers of virus in tear film (open triangle) and ganglia (open diamond) were assayed in samples obtained by swabbing eyes 1 day p.i. and by excising and homogenizing ganglia 3 days p.i. Each point on each scattergram represents an individual sample. (C) Thirty days p.i., ganglia were harvested and subjected to quantitative PCR assays to determine the numbers of viral genomes per ganglion. Each circle on the scattergram represents $\log_{10}$ viral DNA per ganglion of an individual sample. (D) Selected ganglia samples were assayed for LATs using quantitative reverse transcription-PCR. Each square on the scattergram represents $\log_{10}$ LATs per ganglion value of an individual sample. In panels B and C, bars represent mean values for each group. In panels C and D, the number of samples assayed for each group is shown in parentheses and the dashed line delineates the detection limit for each assay. N.D., not done.

LATs in the same proportion of ganglia at each inoculating dose (Fig. 1C and D). At doses of 2×10^3 and above, the proportion of ganglia from which virus reactivated was similar to that in which latent HSV was detected molecularly. At a dose of 2×10^2, although there were a few ganglia in which we could detect HSV molecularly, no reactivation was observed. This difference is most likely due to chance ($P = 0.162$, Fisher's exact test). Similar to the viral load, the amount of LATs in latently infected ganglia increased with the dose of virus in inocula until the dose of 2×10^5 PFU, at which dose a plateau was reached. (The surprisingly high level of LATs in the ganglia latently infected with 2×10^5 PFU was unexpected, and the reason is unknown).

In contrast, following inoculation with *dl*sptk at 2×10^6 PFU per eye, although no reactivation was observed in 30 ganglia, 16 of 16 (100%) ganglia contained viral DNA and LATs (Fig. 1C and D). Moreover, the average latent viral load was 12-fold higher in these ganglia than in ganglia latently infected with KOS at 2×10^3 PFU per eye, in which reactivation was detected at a frequency of 50%. Therefore, the failure of *dl*sptk to reactivate is not due to its failure to establish latency as measured by the latent viral load.

We also investigated whether increasing the latent viral load of a TK^- mutant to levels similar to that where wt virus reactivates efficiently would overcome the block to reactivation. In an initial experiment, we used the immunosuppression protocol of Halford and Schaffer (8, 9), which enabled HSV *ICP0* mutants both to attain wt latent viral loads and to increase their reactivation frequency in mouse ganglia. However, this method increased neither the acute replication of *dl*sptk in six ganglia we titrated, the reactivation in 12 ganglia we assayed (similar to the results of a previous study) (24), nor the latent viral load in 14 ganglia we analyzed (data not shown). We therefore turned to inoculation with high doses of virus, which has previously resulted in wt levels of spontaneous reactivation of an *ICP34.5* null mutant in rabbits (23) and low levels of reactivation from explanted mouse ganglia by a TK deletion mutant derived from a clinical isolate (11). We inoculated mice with 2×10^8 PFU of *dl*sptk or *tk*LTRZ1, a KOS-derived mutant with the *Escherichia coli lacZ* gene inserted into the *tk* coding region (5), per eye. We used this mutant, in part, because *dl*sptk has recently been shown to express lower levels of the UL24 protein, which is encoded mainly by long transcripts whose start sites lie within the deletion of this virus (22). Although these lower levels of UL24 protein evidently do not affect viral replication in cell culture or in the mouse eye, they could conceivably affect replication or reactivation in mouse ganglia. The insertion in *tk*LTRZ1 is well upstream of the start site for this transcript. We tested the expression of the UL24 protein by *tk*LTRZ1 in Vero cells using Western blot analysis with a rat polyclonal antiserum as previously described (22), and it was very similar to that of the wt at both 5 and 10 h p.i. (Fig. 2, top panel). As a control the blot was probed with an anti-glycoprotein D (gD) monoclonal antibody (Fitzgerald), which verified that similar levels of infection were attained and similar amounts of protein were loaded and transferred (bottom panel).

Following inoculation of 10^8 PFU, the level of virus replication in the eye was comparable between mice infected with *dl*sptk and *tk*LTRZ1 mutant viruses or wt virus at 2×10^6 PFU per eye (Fig. 1B). Both mutant viruses were severely impaired for replication in ganglia during acute infection (Fig. 1B). At 30 days p.i., ganglia were removed and subjected to quantitative PCR and reactivation assays. Interestingly, the latent viral loads of the two mutants were increased by high-dose inoculation to levels comparable to that with KOS inoculated at 2×10^4 PFU per eye (Fig. 1C), a dose which permits 75% (9 of 12) of KOS-infected ganglia to reactivate (Fig. 1A). Indeed, the mean viral loads achieved by the mutant viruses were not significantly different from those in ganglia from mice infected with 2×10^6 PFU of wt virus, from which virus reactivated at a frequency of 100% ($P > 0.1$ for *dl*sptk versus KOS; $P = 0.07$ for *tk*LTRZ1 versus KOS [Student's t test]). Additionally, some mutant-infected ganglia had loads close to the highest attained by wt virus. Yet no reactivation was observed in a total of 70 ganglia latently infected with the TK^- mutants following inoculation with 2×10^8 PFU per eye (Fig. 1A).

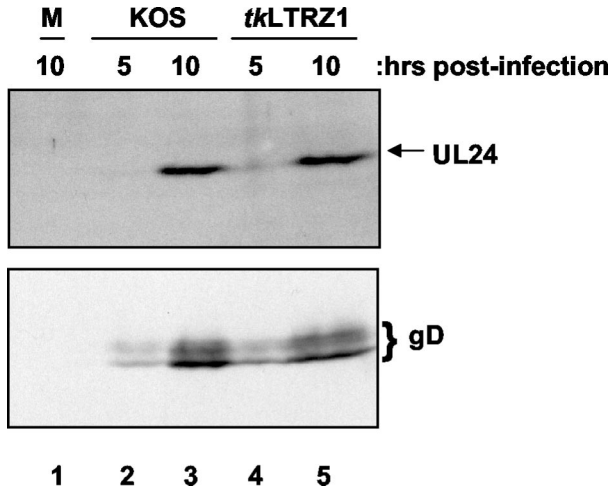

FIG. 2. Expression of UL24 protein from *tk*LTRZ1 virus. Vero cells were either mock infected (M) (lane 1) or infected at a MOI of 10 with KOS (lanes 2 and 3) or *tk*LTRZ1 (lanes 4 and 5). Total cell lysates were harvested at the indicated hours postinfection, resolved on a sodium dodecyl sulfate-polyacrylamide gel, and analyzed by Western blotting with anti-UL24 serum (top panel). As a loading control the membrane was subsequently stripped and incubated with a monoclonal antibody directed against gD (bottom panel). The positions of UL24 and gD are indicated to the right of the panels.

Our having achieved relatively high latent viral loads with TK^- mutants is akin to results in a previous report (33) that showed that one such mutant could achieve a wt profile of latent genome copy numbers per neuron. There were, however, several differences between the two studies: (i) the earlier study did not examine reactivation; (ii) we used high-dose inoculation on the cornea rather than combined snout and corneal inoculation; and (iii) we used a TK^- mutant that does not exhibit reduced *UL24* expression.

In this study, we found that TK^- mutants did not reactivate from latency even when their latent viral loads were comparable to those that permit relatively efficient reactivation of wt virus. This contrasts with, for example, *ICP0* mutants that can reactivate from latency, even when latent viral loads are severalfold lower than those of the wt (8). For *tk*LTRZ1, the inability to reactivate is clearly due to the *tk* mutation, because

rescue of the mutation restores reactivation (12) and there are no mutations other than the engineered *lac*Z insertion in the *Bam*HI fragment corresponding to the fragment used in marker rescue (A. Griffiths and D. M. Coen, unpublished results; GenBank accession no. AY326452 and AY326453). Moreover, the mutation does not affect expression of UL24 protein (Fig. 2). Thus, we infer that the inability of TK^- mutants to reactivate is largely due to the requirement for TK in reactivation rather than in boosting the efficiency of establishment of latent infection.

Acyclovir-resistant mutants, mostly *tk* mutants, can arise during drug therapy and can pose a significant problem in immunocompromised patients. In at least one instance, such mutants have been shown to establish and reactivate from latency in a patient (11). How these mutants cause human disease, given their substantial attenuation in animal models as illustrated in this report, is an interesting question that requires further understanding of how TK is required for reactivation and how certain clinical isolates (7, 11) can circumvent this requirement.

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