

Quantitation of Herpes Simplex Virus Type 1 DNA and Latency-Associated Transcripts in Rabbit Trigeminal Ganglia Demonstrates a Stable Reservoir of Viral Nucleic Acids during Latency

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In this investigation we determined the dynamics of herpes simplex virus type 1 (HSV-1) DNA and latency-associated transcripts (LAT) in the latently infected rabbit trigeminal ganglion. Rabbit eyes were infected with either the McKrae strain or the 17Syn⁺ strain of HSV-1. Rabbits were sacrificed between 5 and 360 days after infection and their trigeminal ganglia were analyzed for the number of HSV DNA genomes and the number of neuronal cells expressing LAT. There was no statistically significant change in the number of HSV genomes or the number of neuronal cells expressing LAT in these ganglia between 20 and 360 days after infection. For both strains, the amount of HSV DNA averaged 16.8 genomes per 100 cells, and 9.2% of the neurons expressed LAT. There were 17 to 34 HSV genomes per LAT-expressing neuronal cell. The number of LAT-expressing neurons did not change over the 360 days. Spontaneous reactivation (HSV-1 recovery in tear film) and recurrence (HSV-1-specific epithelial lesions) occurred during the period of this study; however, these events did not alter the quantity of HSV-1 DNA or the number of LAT-expressing cells. These results suggest that after the latent infection is established, the viral DNA in the ganglia does not replicate to any measurable extent over long periods of latency, since no significant change in the number of HSV genomes occurs. The results also suggest that only a very small number of latently infected neuronal cells are needed to produce infectious HSV-1 during reactivation.

Herpes simplex virus type 1 (HSV-1), an alphaherpesvirus, is neurotropic and establishes lifelong latency in the nervous system. Primary HSV infection of the epithelium leads to entry of virus into axon terminals and viral transport to neuron cell bodies. Following ocular HSV infection, viral latency develops in the trigeminal, superior cervical, ciliary, and sphenopalatine ganglia. The viral genome probably persists in the nucleus as an episome in either a concatemeric or a circular form condensed into a chromatin-like structure (9). The HSV DNA can be quantitated by PCR, and the number of cells harboring the viral genome can be determined by in situ PCR hybridization (DNA-DNA) (15, 33, 34, 37). The number of cells in neural tissue that contain the latency-associated transcripts (LAT) can also be determined by in situ hybridization (DNA-RNA) (12, 13, 38, 42).

The HSV-1 McKrae and 17Syn⁺ strains were chosen for our study because they have been used in multiple related experiments with the rabbit eye model (25); the gene sequences, including those for LAT, are known; and HSV genetic constructs derived from the 17Syn⁺ and McKrae strains have been analyzed in the rabbit eye model (3, 14, 23, 35, 45). No data comparing and quantitating the HSV-1 DNA and LAT-expressing neurons in the rabbit ocular model during latency are available. Therefore, we quantitated by in situ hybridization the number of neuronal nuclei in the trigeminal ganglion (TG)

that contained LAT and quantitated HSV-1 DNA by PCR. The results for both strains revealed a stable reservoir of HSV DNA and LAT-containing neuronal cells resulting in a constant ratio of HSV genomes to LAT-expressing cells which was maintained during long periods of latency.

MATERIALS AND METHODS

Inoculation. The corneas of New Zealand White rabbits (2 to 2.5 kg) were inoculated either with 10⁶ PFU of HSV-1 strain McKrae following corneal scarification or with 0.5 × 10⁶ PFU of HSV-1 strain 17Syn⁺ without scarification. Slit lamp examination revealed dendrites at 3 to 7 days postinoculation (p.i.) in all rabbits. Rabbits were sacrificed between p.i. days 5 and 360, and the TG were excised and processed. One TG was used to quantitate HSV DNA, and the contralateral TG was used to quantitate LAT-expressing neurons.

Purification and standardization of viral DNA. Viral DNAs were purified from viral pellets by standard extraction procedures (39, 44). One genome equivalent of viral DNA was calculated to be 1.66 × 10⁻⁴ pg, assuming a mass of 10⁸ Da for HSV-1 (41). HSV-1 DNA was amplified with primers complementary to the HSV-1 gene for ribonucleotide reductase (RR), LAT, or thymidine kinase (see Table 1 for a list of primer pairs and probes). A 40-cycle PCR was performed on triplicate samples from a 10-fold dilution series of the viral DNA, and then the 40-cycle PCR was repeated with a 2-fold dilution series. The endpoint was defined as the last sample in the twofold dilution series that yielded a PCR product for the RR sequence in at least one of the triplicate. The data in Fig. 4 and 5 are derived from the RR primer pair results.

TG DNA extraction. Rabbits were sacrificed, and their TG were removed, frozen in liquid nitrogen, and ground with a pestle. DNA was phenol extracted, ethanol precipitated, and solubilized in TE buffer (44). The TG DNA was assayed in the PCR at concentrations between 1 and 25 ng per reaction mixture.

Amplification and analysis of HSV-1 and rabbit α -actin DNA. A 125-bp sequence of the rabbit α -actin gene and a 243-bp sequence of the HSV-1 RR gene were coamplified from the same DNA sample to determine the HSV DNA copy number per 100 neuronal cells (3, 19, 25). The procedures of Coen (5, 6) employing an internal standard were used for quantitation of HSV genomes. Coamplifications of the rabbit α -actin gene with either a 195-bp sequence of the

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TABLE 1. Characteristics of PCR components, genes, and products

Gene	Primer pair and sequence ^a	Sequence location (position) ^b	Size of PCR product (bp)	Reference
RR	RR-1 5'-ATGCCAGACCTGTTTTTCAA-3' RR-2 5'-GTCTTTGAACATGACGAAGG-3' Probe 5'-GGACACCAGCATGTCGCTCGCCGACTTTCA-3'	88517→ ←88759 88594→	243	7
TK ^c	TK-1 5'-CTGCAGATACCGCTCCGTATT-3' TK-2 5'-CATCTTCGACCGCCATCCCAT-3' Probe 5'-GTCAAGCTGCCATAAAGGTAT-3'	47053→ ←47326 ←47255	273	32
LAT	SK-1 5'-GACAGCAAAAATCCCTGAG-3' SK-2 5'-ACGAGGGAAAACAATAAGGG-3' Probe 5'-CACACCAGCGGGTCTTTTGTGTTGG-3'	120702→ ←120896 ←120762	195	32
LAT	SKI-1 5'-GACAGCAAAAATCCCTGAG-3' SK-1.5 5'-CACACCAGCGGGTCTTTTGTGTTGG-3' α Actin-1 5'-AAGATCTGGCACCACACCTT-3' α Actin-2 5'-CGAACATGATCTGGGTCATC-3' Probe 5'-CTCGGTCAGCAGAGTGGGGTG-3'	120702→ ←120762 ←120762	61	46
			125	3

^a The topmost primer in each pair is the upstream (mRNA sense) primer, and the bottom primer is the downstream (mRNA antisense) primer.

^b Location is based on the complete sequence of 17Syn⁺. Only the location in the long internal repeat is indicated for the LAT gene. Arrows designate the orientation of the oligonucleotide's 3' end.

^c TK, thymidine kinase.

HSV-1 LAT gene or a 273-bp sequence of the HSV-1 thymidine kinase gene were also performed for selected samples.

The PCR mixture contained the following: 50 μ M deoxynucleoside triphosphates (dNTPs), 200 ng of each HSV-1 primer, 40 ng of each actin primer, *Taq* buffer (Promega Corp., Madison, Wis.), 1.5 mM MgCl₂, 1% glycerol, 1.5 mM MgCl₂, and 1 to 25 ng of template DNA. This mixture was heated for 5 min at 97°C, and then *Taq* polymerase (2.50 U; Promega Corp.) was added. The final volume was 100 μ l in the 30-cycle PCR (5 cycles of 2 min at 94°C, 2 min at 56°C, and 30 s at 72°C, followed by 25 cycles of 1.25 min at 94°C, 1.5 min at 56°C, and 10 s at 72°C). Optimal conditions were determined as described by Hill et al. (19, 25).

Reaction mixtures containing DNA standards corresponding to 10⁻² to 320 copy numbers of HSV-1 DNA cells were amplified. Two negative controls included a reaction mixture minus template DNA and a reaction mixture with TG DNA from an uninfected rabbit. Quantitation of HSV-1 and actin DNA in rabbit TG was done with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Details of the dot blotting, hybridization, and calculation have been reported previously (19, 25, 26). The calculation of copy numbers was performed according to the procedures described by Coen (5, 6) and Lubin et al. (31), and a standard curve is shown in Fig. 3.

In situ hybridization. Excised TG taken on selected days after infection were fixed in 4% paraformaldehyde for 18 to 24 h at 4°C and processed by procedures described by Stroop et al. (43). Tissues were paraffin embedded, and 7- μ m-thick sections were cut. PCR was used to synthesize a ³H-DNA probe for use in the in situ hybridization by incorporating tritiated dNTPs into the reaction mixtures. The probe solution (35 μ l per section) was applied, and slides were heated to 94°C for 5 min and then incubated overnight at 44°C. Excess probe was removed by washing, and autoradiography was performed with Kodak NTB-2 nuclear emulsion (Biotech International, Inc., New Haven, Conn.). After 10 days of

incubation at 4°C, sections were stained with Harris hematoxylin and eosin (Harleco Eosin Y, 1% Alcoholic Solution; EM Corp., Chestnut Hill, Mass.).

Calculation of the percentage of LAT⁺ neurons. Masked observers counted the number of LAT⁺ neurons and the total number of neurons. Neuronal cells were distinguished by the size of their cell body (28). Only LAT⁺ neurons with a visible nucleus were counted. For each of the 12 to 16 sections from each TG, at least three independent masked observations were made. The number of neurons per section ranged from 400 to 1,200. The percentage (frequency) of LAT⁺ neurons per TG was calculated for each section, and the averages for all sections yielded the frequencies of LAT⁺ neurons per TG.

RESULTS

The data in Fig. 1 and Table 2 are the percentages of LAT-expressing neurons in the TG taken on p.i. days 10 through 360 from rabbits latently infected with the McKrae strain. A total of 49 ganglia from 49 rabbits were analyzed. The mean \pm standard error of the mean for all time points was 9.02% \pm 0.77% LAT-expressing neurons. The means ranged from a high of 14.0% at 100 days p.i. to a low of 5.7% at 151 days p.i. The level of LAT-expressing neurons from rabbits infected with the 17Syn⁺ strain, based on frequencies for 42 trigeminal ganglia from 42 rabbits, was 9.41% (Fig. 2 and Table 2). The means in Fig. 2 ranged from a high of 16.2% at p.i. day 45 to a low of 5.0% at p.i. day 250. There was no statistically significant difference between any days ($P > 0.2$) or between the two strains ($P > 0.4$).

Quantitative analysis revealed that one TG cell contained 8 pg of DNA (data not shown). This assay determined that the PCR amplification of rabbit TG DNA for the α -actin sequences was positive for samples containing 4 pg of TG DNA

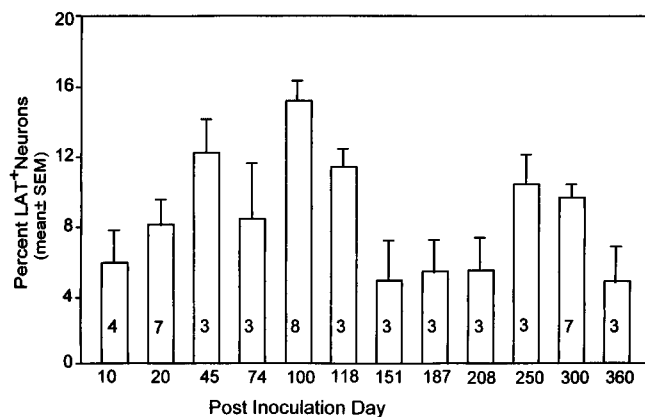


FIG. 1. Percent LAT-expressing neurons versus p.i. day for strain McKrae. The numbers in the columns are sample sizes. SEM, standard error of the mean.

TABLE 2. Summary of results for quantitation of HSV genomes and LAT⁺ cells in rabbit TG

HSV-1 strain	% LAT ⁺ neuronal cells (no. of ganglia analyzed) ^{a,b}	No. of HSV genomes per 100 cells (no. of ganglia analyzed) ^{a,c}
McKrae	9.02 \pm 0.77 (49)	15.85 \pm 1.23 (46)
17Syn ⁺	9.41 \pm 0.80 (42)	17.92 \pm 1.27 (38)
Cumulative result	9.20 \pm 0.54 (91)	16.80 \pm 0.93 (84)

^a Data are means \pm standard errors of the means.

^b LAT data are from p.i. days 10 to 360.

^c HSV DNA data are from p.i. days 20 to 360.

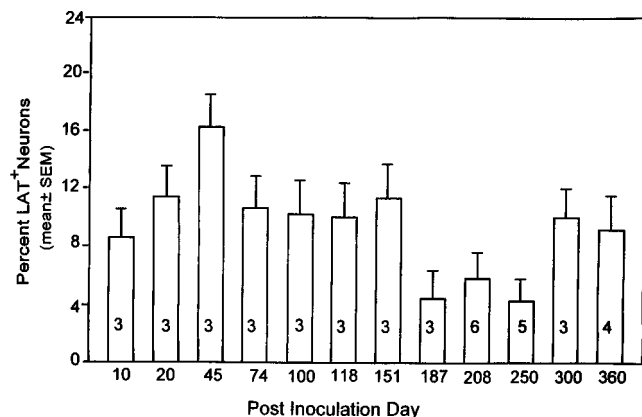


FIG. 2. Percent LAT-expressing neurons versus p.i. day for strain 17Syn⁺. The numbers in the columns are sample sizes. SEM, standard error of the mean.

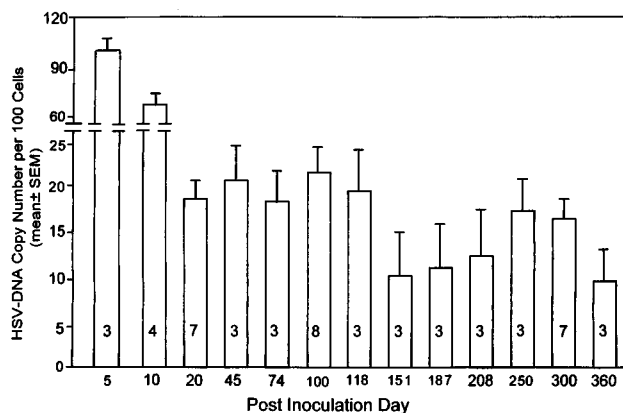


FIG. 4. HSV DNA copy number versus p.i. day for strain McKrae. The numbers in the columns are sample sizes. SEM, standard error of the mean.

but not for those containing 2 pg of DNA. Thus, by definition, 4 pg of TG DNA represents one copy of the rabbit genome. Since one genome copy represents one-half of a cell's chromosomes, 8 pg of TG DNA must represent a whole set of chromosomes; this value was used to calculate the number of HSV genomes per 100 cells. Figure 3 is the result of a typical standard curve derived from analysis of PCRs containing RR and α -actin primer pairs. The plotted results of PhosphorImager quantitative analysis demonstrated that the assay was linear on the log-log scale. The data in Fig. 3 were obtained by using variable amounts of HSV DNA and 25 ng of TG DNA. Virtually identical results were obtained with 1 or 10 ng of TG DNA. Standard curves were employed in all assays for the data presented in Fig. 4 and 5.

The data in Fig. 4 give the HSV DNA copy numbers per 100 TG cells from rabbits latently infected with HSV-1 strain McKrae. The data were obtained by analyzing the contralateral TG from the rabbits for which data are shown in Fig. 1. The mean standard error of the mean of values for p.i. days 20 to

360 was 15.85 ± 1.23 genome equivalents per 100 cells (Table 2). Since approximately 5 to 10% of the cells in the rabbit TG are neurons (28), the copy number of HSV genomes per neuronal cell would be 1.6 to 3.2. The mean number of HSV genomes per 100 cells in the TG from rabbits latently infected with 17Syn⁺ for p.i. days 20 to 360 was 17.92 ± 1.27 , and so the copy number per neuronal cell would be 1.8 to 3.6 (Fig. 5). There was no statistically significant difference between either p.i. days or strains ($P > 0.3$), excluding p.i. days 5 and 10. The average HSV DNA copy number for both strains on all days between p.i. days 20 and 360 was 16.8 per 100 cells.

In Fig. 4 and 5, the HSV DNA copy numbers per 100 cells for p.i. days 5 and 10 are expressed on the histograms, but they were not used in calculating the average copy number for the latency period between p.i. days 20 and 360 (Table 2). The very high DNA copy numbers obtained at p.i. days 5 and 10 represent the acute phase, during which infectious HSV-1 is present. At p.i. day 20 and subsequently, all the TG were latent and did not contain infectious virus. For the two HSV-1 strains studied, 9.2% of the neuronal cells contained LAT (Fig. 1 and 2). The results shown in Fig. 4 and 5 indicate that there were 17 to 34 copies of HSV DNA per LAT-expressing neuronal cell.

DISCUSSION

This is the first report to quantitate the percentage of LAT-expressing neuronal cells in latently infected rabbit TG following ocular HSV-1 inoculation. We found that 9.2% of the

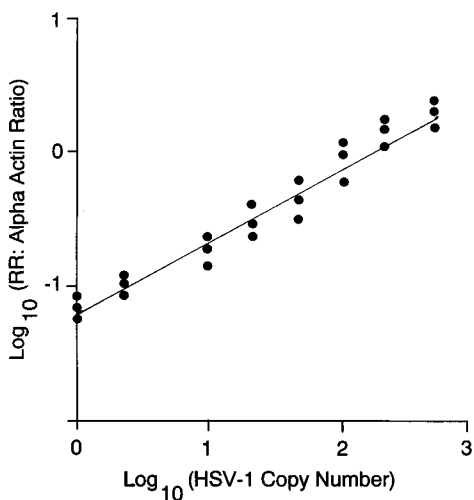


FIG. 3. RR standard curve. The radiolabeled blots from a typical standard curve for a PCR mixture containing RR and α -actin primer pairs were quantified by using storage phosphor technology. Dilutions (10- and 2-fold) of purified and quantified HSV-1 strain 17Syn⁺ DNA were mixed with 25 ng of rabbit TG DNA. Similar results were obtained with 1 and 10 ng of rabbit TG DNA. The log₁₀ ratios of RR to α -actin were plotted against the log₁₀ number of HSV genomes. The best-fit line was generated by linear regression analysis.

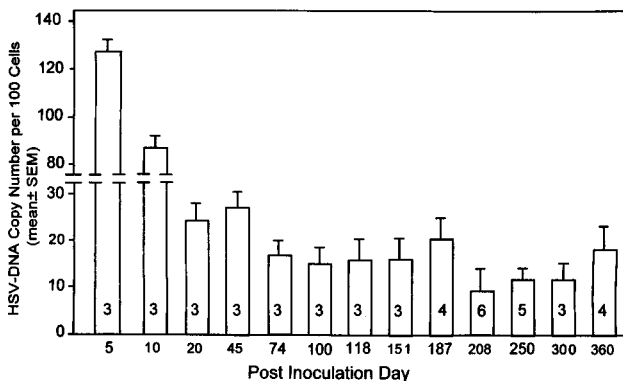


FIG. 5. HSV DNA copy number versus p.i. day for strain 17Syn⁺. The numbers in the columns are sample sizes. SEM, standard error of the mean.

neuronal cells make LAT and that the LAT⁺ cells contain 17 to 34 copies of HSV DNA. Our LAT results with rabbits are similar to those of Ecob-Prince et al. (11–13), who determined that 9% of the neuronal cells in the mouse dorsal root ganglia expressed LAT. Their data also suggested no association between an alteration in the percentage of LAT⁺ cells and viral reactivation induced by neurectomy. This finding is supported by the results of Bourne et al. (4), who reported that the amount of LAT did not affect viral reactivation in the guinea pig vaginal model. No correlation between the percentage of LAT⁺ neurons and *in vivo* reactivations has been reported.

Numerous studies (3, 10, 27, 29, 35, 37, 40, 41) have reported the quantitation of HSV-1 DNA in TG. Even though the procedures varied, values between 10 and 1,000 HSV genome equivalents per 100 cells were estimated in most studies. No one has reported any *in vivo* changes in HSV DNA in sensory ganglia relative to time (duration of latency) and/or reactivation (spontaneous or induced). Recently, Kramer and Coen (29) have estimated a value of 100 genome equivalents per latently infected neuronal cell from mouse TG with no difference at 30 or 60 days p.i. Rock and Fraser (37) showed that levels of HSV DNA in mouse TG were similar between 2 and 5 months.

Almost 100% of all rabbits latently infected with HSV-1 strain McKrae or 17Syn⁺ and at least 80% of the eyes of these rabbits undergo ocular reactivation from 20 to 360 days following inoculation. This conclusion is based on the recovery of virus from eye swabs and/or recurrent corneal epithelial lesions (1–3, 8, 16–25, 30, 35, 45). The average duration of a reactivation episode is 2 days. Over half of all rabbits undergo two or more episodes of spontaneous reactivation. We postulate that the actual spontaneous ocular reactivation frequency is higher but that many episodes are missed because of the limitations of the viral assay. Regardless of the frequency or duration of HSV reactivation, no changes in HSV DNA or in LAT-expressing neurons in the TG occurred during the long period of latency.

The values for both the percentage of LAT⁺ cells and the HSV DNA per cell could be underestimates. Inherent in the determination of numbers of LAT⁺ cells was a system of counting those cells whose nuclei were visible in the section. Some cells whose nuclei could be positive but were not visible by autoradiography in this section could be included in the count of neuronal cells but not in the count of LAT⁺ cells. Thus, the number of LAT⁺ cells could be an underestimate. The system used calculated only the number of HSV DNA-containing cells that also contained LAT. Cells with HSV DNA that lack LAT have been found to compose a significant portion of the neuronal population during latency (15, 17, 33, 34, 36, 37). While our values could be underestimates for both LAT and HSV DNA, the results are consistent throughout the period of latency and provide a precise, if not accurate, determination of events occurring during latency and reactivation.

Experiments are in progress to determine if other HSV-1 strains phenotypically different from McKrae and 17Syn⁺ will yield similar results. Other planned experiments will assess the effects of repeated induced ocular reactivation in the presence and absence of high doses of an antiviral agent.

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