Herpes Simplex Latent Infection: Quantitation of Latency-Associated Transcript-Positive Neurons and Reactivable Neurons

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Dorsal root ganglion neurons, which express herpes simplex virus (HSV) latency-associated transcript (LAT) during experimental latent infection, were investigated by in situ hybridization. The number of LAT-positive neurons was determined by examination of ganglion serial sections. In other latently infected mice, the number of ganglion neurons that reactivated HSV antigen after explant culture was determined in serial sections. LAT was detected in 100 percent of ganglia, with an average of 19.5 LAT-positive neurons per ganglion. After explant culture of latently infected ganglia (in the presence of colchicine to decrease spread of reactivated virus), HSV antigen was detected in 94 percent of ganglia, with an average of 13.1 positive neurons in the antigen-positive ganglia. The similar quantities of LAT- and antigen-positive neurons within ganglia support the hypothesis that LAT-positive neurons were the neurons from which HSV was reactivated.

Latent herpes simplex virus (HSV) infections occur frequently in neurons of peripheral nervous system sensory ganglia and are the substrate of recurrent HSV infections [1]. During latency, HSV deoxyribonucleic acid (DNA) is present in modified form [2,3], and, although viral proteins are probably not present, HSV-specified RNA has been detected by nucleic acid hybridization in latently infected human and experimental animal ganglia [4–10]. In early studies, purified radiolabeled HSV DNA was used to probe ganglion sections for ribonucleic acid (RNA) [4,5]. More recently, HSV viral RNA present in ganglion tissues has been investigated in detail by the use of cloned DNA restriction endonuclease fragments to probe ganglion tissues [6–11]. Viral RNA detected in ganglia has been termed HSV latency-associated transcript(s) (LAT) [9,10] or latency-related RNA [11]. LAT partially overlaps the viral ICP 0 gene but is transcribed from the HSV DNA strand which is complementary to the strand which encodes the ICP 0 gene [6–11].

Although most studies of HSV latency have investigated ganglia of the peripheral nervous system, HSV latency in central nervous system tissues may also occur. HSV has been isolated from latently infected tissues of the central nervous system, albeit at lower frequencies than from peripheral nervous system ganglia [12,13]. In some studies, it has not been possible to isolate HSV from central nervous system tissues [14,15], although the presence of LAT in these tissues was evident [14]. In addition, the detection of LAT but the relative lack of reactivable HSV has been noted after

Abbreviations: DNA: deoxyribonucleic acid  DRG: dorsal root ganglia  HSV: herpes simplex virus  LAT: latency-associated transcript  RNA: ribonucleic acid

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inoculation of mice with ICP 0 [16] or thymidine kinase [17] mutants of HSV. The presence of LAT but the relative inability to reactivate infectious HSV raises the question of whether LAT-positive ganglion neurons are the neurons from which HSV is reactivated.

In the present study, we quantitated the number of LAT-positive neurons by serially sectioning ganglia, followed by in situ hybridization of the sections. In other ganglia, the number of neurons that expressed HSV antigens after explant cultivation was determined and compared with the number of LAT-positive neurons. It was hypothesized that, if LAT-positive neurons were the cells which reactivated HSV, the number of LAT-positive neurons and antigen-positive neurons would be similar.

MATERIALS AND METHODS

Virus and Animal Inoculation

The KOS strain of HSV type 1, obtained from the American type culture collection and passed multiple times in cell culture [5], was grown and titered by standard means in primary rabbit kidney cells. Random-bred six- to eight-week-old male and female mice (Charles River Laboratories, Inc, Wilmington, MA) were anesthetized and one drop (approximately 50 μl) of virus suspension (10^7 plaque-forming units/ml) was dropped on to each rear footpad, which was then punctured multiple times with a needle through the virus drop. By this procedure, greater than 90 percent of mice were latently infected with the virus [18] without causing mortality [unpublished observation]. During the period of HSV latency (28–35 days post-footpad inoculation), mice were sacrificed, and dorsal root ganglia (DRG) were processed for in situ hybridization to detect LAT. Alternatively, DRG were explanted for four days in medium containing 5 percent heat-inactivated bovine serum, and subsequently processed for HSV antigen detection.

In Situ Hybridization

Lumbar 4 (L4) and L5 DRG were fixed in 3 percent paraformaldehyde and embedded in paraffin. Serial ten-micron sections were collected on to 3-aminopropyltriethoxysilane-treated slides. After deparaffinization in xylene and rehydration in graded ethanol, the slides were sequentially treated with 0.2 N HCl, proteinase K (5 μg/ml in 10 mM Tris, pH 7.4), 2 mM CaCl_2 and 0.25 percent acetic anhydride (in 0.1 M triethanolamine buffer, pH 8.0). Tissue sections were hybridized with 1–3 ng (approximately 10^5 cpm) of [35S]-labeled probe DNA. Prior to hybridization, DNA probes were denatured at 100°C for ten minutes, quenched on ice, and resuspended in sodium chloride-sodium citrate buffer (10 mM Tris, pH 7.4; 1 mM EDTA, Denhardt's solution, 10 percent dextran sulfate, 45 percent formamide), 100 μg/ml of mouse brain nucleic acids, and 100 μg of _M. luteus_ DNA. Hybridization was carried out at 46°C for 72 hours. After extensive washing, slides were coated with NTB-3 emulsion (Kodak, Rochester, NY), exposed for four days at 4°C, developed, and stained with hematoxylin.

Probes for in Situ Hybridization

The EC subfragment of the _Bam_ HI E fragment of HSV type 1 (Patton strain) and multiple other restriction endonuclease fragments were obtained from D.J. Spector and R.W. Hyman (The Pennsylvania State University College of Medicine). The restriction endonuclease fragments used are shown in Fig. 1. HSV DNA fragments
Map Units

A. HSV-1 genome in long segment inverted orientation

B. Subfragments of Bam HI-E fragment

C. Eco RI and Bam HI fragments

FIG. 1. Map of the HSV-1 genome and the EcoRI and BamHI restriction fragments used for in situ hybridization. A. The HSV-1 genome in the long segment inverted orientation [23]. The location of the Bam HI B fragment in this orientation and its size in kilobase pairs is indicated. The long unique (UL) and short unique (U,) regions are bounded by the long terminal repeat (TRL) and short terminal repeat (TR,) and by inverted repeats (IRL, IRB). B. Subfragments of the Bam HI-E fragment showing HpaI sites (H) and BamHI sites (B) with sizes in kilobase pairs. Diagram is derived from Spector et al. [24]. Positive hybridization was obtained only with the EC subfragment (solid bar). C. Other restriction fragments used in hybridization studies which gave negative results.

were isolated from plasmid vectors by electroelution, and 25- to 50-ng amounts were nick translated at 15°C with 60 μg of [35S]dCTP (1,000 Ci/mmol, Amersham). Unincorporated nucleotides were separated from nick-translated DNA by ethanol precipitation. Specific activities of [35S]-labeled probes were 1–2 × 10^6 cpm/μg.

Explant Culture and Antigen Detection

L4 and L5 DRG were removed and cultured in medium containing 5 percent heat-inactivated bovine serum for four days. To minimize axonal spread of reactived HSV, which would prevent identification of individual antigen-positive neurons, colchicine (50 μg/ml) was added to the explant culture medium. After incubation for four days, ganglia were washed in saline buffer and fixed in 3 percent paraformaldehyde. Following embedding, ten-micron serial paraffin sections were collected on gelatinized slides. Sections were deparaffinized and further treated with ice-cold methanol containing 0.5 percent H2O2. After hydration in phosphate-buffered saline containing 20 percent horse serum, sections were treated with polyclonal rabbit anti-HSV serum (1:100, Dako, Santa Barbara, CA). Biotinylated anti-rabbit IgG and avidin-peroxidase conjugate were added according to manufacturer's directions (Vector, Burlingame, CA), and sections were then treated with diaminobenzidine (0.25 mg/ml). After washing, sections were dehydrated and mounted.
**Counting Hybridization-Positive and Antigen-Positive Neurons**

Hybridization-positive neurons, indicating the presence of LAT, were identified by the presence of clusters of silver grains. In preliminary studies, individual hybridization-positive neurons were identified on either a single section or with lesser frequency on two adjacent sections. Therefore, to estimate the number of hybridization-positive neurons in DRG, the total number of hybridization-positive cells in sections of a ganglion was counted and this figure was divided by 1.25 to arrive at a corrected number of LAT-positive cells.

Antigen-positive cells were seen on one, two, or three adjacent sections, with most antigen-positive cells being seen on two sections. Approximately twice as many were seen on three sections as on one section. Therefore, to estimate the number of antigen-positive cells, the absolute count was corrected by dividing by 2.5.

To determine proportions of LAT- and antigen-positive neurons within ganglia, we used 3,500 as an estimate of the number of neurons present in mouse L4 and L5 DRG. Investigators have recorded varying numbers of neurons in DRG [19], and 3,500 is an estimate from our counts in which nucleoli were counted in serial eight-micron sections [unpublished results].

**RESULTS**

LAT was detected in all DRG (13/13) from mice sacrificed 28–35 days after footpad inoculation of HSV. The number of LAT-positive neurons ranged from 4–57 per DRG (mean, 19.5; refer to Table 1). Grains were localized over the nuclei of neurons (Fig. 2), as noted initially with purified HSV DNA probes [5] and more recently with cloned restriction endonuclease fragment probes [6–9]. Hybridization was detected only with the BamHI EC subfragment of the E fragment, similar to results obtained by others using the BamHI B fragment [6–9]. Positive results with the E fragment would be predicted since repeat sequences are the same in both the BamHI B and E fragments, and hybridization has been reported with BamHI subfragments limited to repeat sequences [9,14]. In this report we considered LAT to be present if hybridization was detected with the EC subfragment of the BamHI E fragment. The proportion of LAT-positive neurons in our serial section study with the EC subfragment of the E fragment supported and extended the results obtained by others with the BamHI B fragment in which ganglion sections were sampled [6–9].

After explant culture for four days in the presence of colchicine, discrete antigen-positive neurons were easily recognized (Fig. 3A–E), whereas in cultures without colchicine the spread of antigen within ganglia was apparent (Fig. 3F). Apparent axonal spread of HSV and spread to Schwann cells (Fig. 3G) precluded accurate

| TABLE 1 | LAT-Positive and HSV Antigen-Positive Neurons in Mouse Dorsal Root Ganglia |
|-----------------|-----------------------------|-----------------|-----------------|
| **Number of Ganglia Positive/Total (%)** | **Number of Positive Neurons in Positive Ganglia, Mean ± SD** | **Proportion of Positive Neurons in Positive Ganglia, Mean (range)** |
| LAT-positive | 13/13 (100) | 19.5 ± 16.8 | 0.006 (.001–.02) |
| HSV antigen-positive | 31/33 (94) | 13.1 ± 18.5 | 0.004 (.001–.02) |

*Proportions were calculated based on an estimate of 3,500 neurons per ganglion (see text).
*After explant for four days in the presence of colchicine, 50 μg/ml
FIG. 2. LAT expression as detected by positive hybridization with the BamHI EC fragment. A. Low-power photomicrograph showing three LAT-positive neurons. B-D. Higher-power photomicrographs showing a single LAT-positive neuron in only one of three serial ten-micron sections.

counting of cells in which reactivation had occurred. Following colchicine treatment of explant cultures for four days, HSV antigen was detected in 31/33 (94 percent) of DRG, and the number of antigen-positive neurons ranged from 0–76 (mean, 12.4; refer to Table 1). In HSV antigen-positive ganglia, the range of positive neurons was 2–76 (mean, 13.1). The only cells clearly seen to be antigen-positive were large and small neurons. Estimates of neuron size were arrived at by determination of the number of ten-micron sections in which peroxidase reaction product was present in individual neurons; most positive neurons were observed in two adjacent sections.

A comparison of the number of LAT-positive neurons and HSV antigen-positive neurons is shown in Table 1. Although some differences in the average number of antigen- and LAT-positive neurons are apparent, there was no significant difference between the two when neurons from positive ganglia were compared ($p > .05$).

DISCUSSION

It was hypothesized that if LAT-positive neurons in latently infected ganglia were the cells from which HSV was reactivated, the number of LAT-positive and antigen-positive neurons would be similar. Only slight differences between the number of LAT-positive and antigen-positive neurons were observed, and these are probably explained by biological variability and by differences in the experimental methodologies used. Specifically, the number of HSV antigen-positive cells is probably a minimum estimate of the neurons which reactivated virus.

While the quantities of LAT-positive neurons in ganglia can be considered to be
FIG. 3. HSV antigen detected after explant culture of latently infected DRG.  A–E. DRG explanted for four days in medium containing colchicine (50 μg/ml), showing neurons expressing antigen; spread of antigen within the ganglion is limited. Serial ten-micron sections show two antigen-positive neurons in each of three sections. F. DRG explanted for four days in medium without colchicine, showing extensive spread of antigen in a ganglion. G. Higher magnification, showing antigen spread in axons and Schwann cells.
accurate estimates, those of HSV antigen-positive neurons after ganglion explant culture are probably underestimates. To count antigen-positive cells accurately after explant culture, it was necessary to minimize the effect of HSV spread within explants following HSV reactivation in latently infected neurons. Therefore, we added colchicine to the reactivation medium, to decrease axonal spread of HSV and to permit maintenance of explant cultures for four days. Explant cultures without colchicine permitted the detection of individual antigen-positive cells only in cultures of up to two days. Beyond two days in culture, cell-to-cell spread of HSV was evident, and quantitation of the number of individual antigen-positive cells was precluded.

Although the use of colchicine permitted the evaluation of four-day explant cultures, it is probable that occasional neurons that could have reactivated virus later were missed. Therefore, it is likely that the number of antigen-positive neurons in ganglia is slightly higher than the estimate in the present study. This underestimation would decrease the difference between LAT- and antigen-positive neurons. It is also probable that colchicine inhibited HSV reactivation or subsequent HSV replication within neurons, to explain partially the relatively low number of antigen-positive cells. In cell culture studies, colchicine was found to decrease the yield of HSV slightly when low multiplicities of infection were used, and amounts of HSV in reactivating explant cultures were also decreased [unpublished observations]. It also remains possible that some LAT-positive cells were destroyed after explantation, or that for other reasons not all LAT-positive cells reactivated HSV.

Estimates of the number of neurons reactivating HSV antigen have not been previously published, although an estimate of the number of HSV-positive neurons was determined in an infectious center study [20]. The results obtained in the present study indicated a lesser number of antigen-positive neurons, possibly related to methods of detection but also probably related to the strain of virus and/or mouse tested. In addition, we noted large differences in antigen distribution in ganglia, related to the titer of virus inoculated and whether trigeminal ganglia (after corneal inoculation of virus) or dorsal root ganglia (after footpad inoculation of virus) were studied [unpublished observation].

The results of this study support the conclusion that LAT-positive neurons are probably the cells from which HSV reactivates, although the role of LAT during latency and its possible relationship to HSV ICP 0 expression remain unclear. Speculation of an interaction with HSV ICP 0 has centered on possible antisense inhibition [10,11]. While such a mechanism is possible, the demonstrations that HSV mutants defective for ICP 0 [16] and for LAT expression [21,22] can establish latent infections need to be taken into account. In addition, the detection of LAT but the relative lack of reactivable HSV in central nervous system tissues [14] and after inoculation with thymidine kinase mutants of HSV [17] suggest that, although LAT-positive neurons probably reactivate HSV, LAT expression alone is not synonymous with HSV latency. LAT expression with limited reactivation of HSV might indicate abortive or incomplete latency.

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REFERENCES

1. Tenser R: Herpes simplex and herpes zoster. Neurol Clin 2:215–239, 1984
2. Rock DL, Fraser NW: Detection of HSV-1 genome in central nervous system of latently infected mice. Nature 302:523–525, 1983
3. Efstatiiou S, Minson AC, Field HJ, Anderson JR, Wildy P: Detection of herpes simplex virus-specific DNA sequences in latently infected mice and in humans. J Virol 57:446–455, 1986
4. Galloway DA, Fenoglio CM, McDougall JK: Limited transcription of the herpes simplex virus genome when latent in human sensory ganglia. J Virol 41:689–691, 1982
5. Tenser RB, Dawison M, Ressell SJ, Dunstan ME: Detection of herpes simplex virus mRNA in latently infected trigeminal ganglion neurons by in situ hybridization. Ann Neurol 11:285–291, 1982
6. Stevens JG, WagnerEK, Devi-Rao GB, Cook ML, Feldman LT: RNA complementary to herpes virus gene mRNA is prominent in latently infected neurons. Science 235:1056–1059, 1987
7. Rock DL, Nesburn AB, Ghiasi H, Org J, Lewis TL, Lokensgard JR, Wechsler SL: Detection of latency-related viral RNA’s in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. J Virol 61:3820–3826, 1987
8. Gordon YJ, Johnson B, Romanowski E, Araullo-Cruz T: RNA complementary to herpes simplex virus type 1 ICP 0 gene demonstrated in neurons of human trigeminal ganglia. J Virol 62:1832–1835, 1988
9. Spivack JG, Fraser NW: Expression of herpes simplex virus type 1 latency associated transcripts in the trigeminal ganglia of mice during acute infection and reactivation of latent infection. J Virol 62:1479–1485, 1988
10. Wagner EK, Devi-Rao G, Feldman LT, Dobson AT, Zhang Y-F, Flanagan WM, Stevens JG: Physical characterization of the herpes simplex virus latency-associated transcript in neurons. J. Virol 62:1194–1202, 1988
11. Wechsler SL, Nesburn AB, Watson R, Slanina SM, Ghiasi H: Fine mapping of the latency-related gene of herpes simplex virus type 1: Alternative splicing produces distinct latency-related RNAs containing open reading frames. J Virol 62:4051–4058, 1988
12. Cook ML, Stevens JG: Latent infections following experimental viraemia. J Gen Virol 31:75–80, 1976
13. Tenser RB, Hsiung GD: Pathogenesis of latent herpes simplex virus infection of the trigeminal ganglion in guinea pigs: Effects of age, passive immunization and hydrocortisone. Infect Immun 16:69–74, 1977
14. Deatly AM, Spivack JG, Lavi E, O’Boyle DR III, Fraser NW: Latent herpes simplex virus type I transcripts in peripheral and central nervous system tissues of mice map to similar regions of the viral genome. J Virol 62:749–756, 1988
15. Tullo AB, Shimeld C, Blyth WA, Hill TJ, Easy DL: Spread of virus and distribution of latent infection following ocular herpes simplex in the non-immune and immune mouse. J Gen Virol 63:95–101, 1982
16. Leib DA, Coen DM, Bogard CM, Hicks KA, Yager DR, Knipe DM, Tyler KL, Schaffer PA: Immediate early regulatory gene mutants define stages in the establishment and reactivation of herpes simplex virus latency. J Virol 63:759–768, 1989
17. Tenser RB, Hay KA, Edris WA: Latency associated transcript but not reactivatable virus is present in sensory ganglion neurons after inoculation of thymidine kinase negative mutants of herpes simplex virus type 1. J Virol, in press
18. Tenser RB, Edris WA, Hay KA: Herpes simplex virus latent infection: Reactivation and elimination of latency after neurectomy. Virology 167:302–305, 1988
19. Schmalbruch H: The number of neurons in dorsal root ganglia L4-L6 of the rat. Anat Rec 219:315–322, 1987
20. Price RW, Walz MA, Wohlenberg C, Notkins AL: Latent infection of sensory ganglia with herpes simplex virus: Efficiency of immunization. Science 188:938–940, 1975
21. Javier RT, Stevens JG, Dissette VB, Wagner EK: A herpes simplex virus transcript abundant in latently infected neurons is dispensable for establishment of the latent state. Virology 166:254–257, 1988
22. Steiner I, Spivack JG, Lirette RP, Brown SM, MacLean AR, Subak-Sharpe JH, Fraser NW: Herpes simplex virus type I latency associated transcripts are evidently not essential for latent infection. EMBO J 8:505–511, 1989
23. Roizman B: The cellular organization of the herpes simplex virus genomes. Annu Rev Genet 13:25–57, 1979
24. Spector DJ, Jones TR, Parks CL, Deckhut AM, Hyman RW: Hybridization between a repeated region of herpes simplex virus type I DNA containing the sequence (GGC)n and heterodisperse cellular DNA and RNA. Virus Res 7:69–72, 1987