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Virus-Induced Neuronal Apoptosis Blocked by the Herpes Simplex Virus Latency-Associated Transcript

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visualized with the FLAG-specific mAb M2 and TRITC-coupled anti-mouse IgG.
11. Plasmid pWS30 (*22*) was used for inactivation of *cag*A. For construction of a *cag*E$^-$ insertion mutant, the *Hp* 26695 chromosomal region (position 574639 to 579587) was amplified by polymerase chain reaction (PCR). The PCR fragment was cloned in pMin1, mutated by a Tn*Max* transposon derivative as described [A. F. Kahrs *et al.*, *Gene* **167**, 53 (1995)], and used for transformation of strain P12, resulting in the *cag*E$^-$ mutant. For construction of P12Δ*cag*A, region 579021 –to 579905 (*cag*A-upstream) and 583483 to 584934 (*cag*A-downstream) of the Hp 26695 genome were amplified by PCR with 18-nucleotide flanking sequences as primers. Fragments were cloned into pBluescript Xho I–Bam HI sites, separated by an *aph*A-3 gene cassette. For complementation, the *cag*A gene of 26695 was amplified by PCR and cloned into the pHel2 vector (Xho I–Bam HI) (*15*). The FLAG-tag (Sigma) sequence was fused to the *cag*A-downstream primer (5′-CGGGATCCTTACTTGTCATCGTCGTCCTTGTAGTCAGATTTTTGGAAACCACCTT-3). The 18-nucleotide *cag*A upstream primer starts at position 579701 (*19*). Transformation of *Hp* strains was performed as described [R. Haas *et al.*, *Mol. Microbiol.* **8**, 753 (1993)].
12. AGS cells (1×10^7) were infected with *Hp* P12, incubated for 4 hours (37°C, 5% CO_2), washed 5× with PBS*, and lysed in 500 μl of modified RIPA buffer [50 mM tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 1 mM PMSF, 1 mM orthovanadate, 1 μM leupeptin,1 μM pepstatin] (1 hour, 4°C, gentle shaking). The lysate was cleared (15,000 rpm, 4°C, 10 min) and the protein content adjusted to 3 mg/ml with PBS*. AK257 (1.7 μg/ml) was added to a total volume of 900 μl; after 1 hour at 4°C, 40 μl of Protein G–agarose (Roche) was added and the mixture incubated for a further 2 hours at 4°C. After a short centrifugation step (10 s, 6.000*g*) the supernatant was discarded, and beads were washed four times with 1 ml of PBS* and suspended in 40 μl of sample solution.
13. The COOH-terminal part of CagA was produced as a fusion protein with MS2-polymerase and an NH_2-terminal His_6-tag by using the *Escherichia coli* expression vector pEV40 [J. Pohlner *et al.*, *Gene* **130**, 121 (1993)]. The fusion protein was expressed and purified by Ni^{2+}–nitrilotriacetic acid affinity chromatography by standard procedures and used to immunize a rabbit to obtain antiserum AK257. AK175 was generated by immunization of a rabbit with whole heat-killed *Hp* bacteria. Immunoblotting was performed and alkaline phosphatase (AP)–coupled protein A was used to visualize the antibody bound by decomposition of nitro blue tetrazolium.
14. After standard infection of AGS cells with *Hp* strains (*10*), cells were washed three times with PBS$^+$, harvested with a cell scraper, and treated with PBS* containing 0.05% (w/v) saponin for 10 min at room temperature. Cellular debris and bacteria were separated from the soluble material by centrifugation (5 min, 6000*g*) and 0.2-μm pore diameter filtration of the supernatant. Pellets were resuspended in 30 μl of sample solution, and supernatants were precipitated with chloroform-methanol as described [D. Wessel and U. I. Flügge, *Anal. Biochem.* **138**, 141 (1984)] and resuspended in 30 μl of sample solution. Equal amounts (8 μl) of probes were loaded on 6 or 10% polyacrylamide gels.
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17. The sequence of the P12*cagA* gene was obtained by PCR amplification of the complete gene fragment from corresponding chromosomal DNA and sequencing of the cloned genes by primer walking. ATCC43526 CagA (GenBank accession number 2073135), CagA sequences of J99 (*18*) and 26695 (*19*).
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Virus-Induced Neuronal Apoptosis Blocked by the Herpes Simplex Virus Latency-Associated Transcript

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Latent infections with periodic reactivation are a common outcome after acute infection with many viruses. The latency-associated transcript (*LAT*) gene is required for wild-type reactivation of herpes simplex virus (HSV). However, the underlying mechanisms remain unclear. In rabbit trigeminal ganglia, extensive apoptosis occurred with *LAT*$^-$ virus but not with *LAT*$^+$ viruses. In addition, a plasmid expressing *LAT* blocked apoptosis in cultured cells. Thus, *LAT* promotes neuronal survival after HSV-1 infection by reducing apoptosis.

After primary infection of the eye, herpes simplex virus–type 1 (HSV-1) establishes a lifelong latent infection in neurons of the trigeminal ganglia (TGs), with sporadic periods of reactivation and recurrent disease. Recurrent ocular HSV (HSV-1 and HSV-2) is a leading cause of corneal blindness resulting from an infectious agent. Recurrent genital HSV is a serious sexually transmitted disease. Latent HSV infections affect 70 to 90% of adults.

During latency, a single viral gene—the *LAT* gene—is abundantly transcribed (*1, 2*). *LAT* is essential for the efficient reactivation of HSV from latency (*3*). The primary *LAT* is 8.3 kb and overlaps the important immediate early gene *ICP0* in an antisense direction. Thus, it was proposed that *LAT* may function through an antisense mechanism (*1, 2*). However, the first 1.5 kb of LAT alone is sufficient for wild-type levels of spontaneous reactivation (*4*), and this region does not overlap any known HSV-1 gene. *LAT* may enhance the establishment or maintenance of latency (*5–8*), thereby increasing the pool of latently infected neurons, which in turn results in increased levels of spontaneous and/or induced reactivation (*9*). Although studies with one *LAT*$^-$ mutant have suggested that a *LAT*-related function may suppress productive-cycle gene expression during acute and latent infection of mouse trigeminal ganglia (*10, 11*), no evidence was presented to show that *LAT* caused these effects directly, rather than through a pleiotropic effect. We recently reported on a mutant containing a partial deletion of *LAT* in which neurovirulence was increased (*12*). This finding suggested that *LAT* might protect neurons from being killed by HSV-1, thereby allowing HSV-1 to establish latency in more neurons.

To determine whether neurons were being protected by *LAT*, we used dLAT2903, a *LAT* null mutant derived from the McKrae strain of HSV-1 (*3*). This mutant contains a deletion that includes the *LAT* promoter and the 5′ half of the stable 2-kb *LAT*; this deletion (nucleotides −161 to +1667) results in the absence of *LAT* RNAs and does not overlap or interfere with the *ICP0* transcript. Like most *LAT* mutants, dLAT2903 has no known deficits other than being impaired for reactivation from latency. Thus, dLAT2903 is wild type for replication in mouse and rabbit eyes, HSV-1–induced eye disease, replication in TGs, and neurovirulence (*3*). However, with dLAT2903, large numbers of neurons positive for TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling) were seen in rabbit TGs on day 7 after infection (Table 1; 70% of sections, 100% of TGs), whereas in this experiment TUNEL-positive cells were not de-

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tected in TGs from rabbits infected with wild-type McKrae. Only small amounts of TUNEL-positive cells were detected in TGs from dLAT2903R, a rescued virus in which the deleted *LAT* region was restored. The results for dLAT2903-infected rabbits were significantly different from those for the wild type–infected and dLAT2903R-infected rabbits [$P < 0.001$ for sections, $P < 0.05$ for TGs; analysis of variance (ANOVA), Tukey comparison test for peak days of apoptosis]. Representative photomicrographs are shown in Fig. 1.

More rabbits were infected (five rabbits per group) and killed on day 7 after infection to study additional aspects of enhanced apoptosis in rabbits infected with the LAT^- virus (Table 2). Fifty sections per group (five sections from each of 10 TGs) were examined. Extensive apoptosis (>25% TUNEL-positive neurons) was observed in 66% of the sections from dLAT2903 (LAT^-)–infected rabbits, versus only 4% of the sections from uninfected rabbits or rabbits infected with wild-type McKrae or dLAT2903R ($P < 0.001$; ANOVA, Tukey posttest). When all TGs with extensive apoptosis in at least one section were counted (Table 2), 90% of TGs from rabbits infected with dLAT2903 met this criterion, versus only 10 to 20% of TGs from uninfected rabbits or rabbits infected with wild-type McKrae or dLAT2903R ($P < 0.01$).

When we counted all TG sections with any amount of apoptosis in neurons, this threshold was met by 74% of the sections from rabbits infected with dLAT2903; this was significantly higher than for TGs from uninfected rabbits or rabbits infected with wild-type McKrae or dLAT2903R ($P < 0.001$) (Table 2). All 10 TGs from rabbits infected with dLAT2903 were positive according to this criterion, whereas only 5 of 10 TGs from rabbits infected with wild-type McKrae or dLAT2903R were positive ($P = 0.03$) (Table 2). The sporadic low levels of apoptosis detected in rabbit TGs infected with wild-type McKrae were expected because HSV-1 can induce apoptosis (*13–16*).

The results of these TUNEL assays suggest that apoptosis was more likely, by a factor of 2 to 16, in rabbit TGs infected with the LAT^- mutant. Because HSV-1 can induce apoptosis in infected tissue culture cells (*13–16*), we suspect that the apoptotic neurons detected in TGs by TUNEL were a direct result of the HSV-1 infection. However, because on day 7 (the time of peak apoptosis) viral antigens can no longer be detected in these TGs by immunofluorescent staining (*17*), it is possible, but unlikely, that some of the apoptosis may be due to a bystander effect.

A newly devised method for detection of apoptosis in tissue sections was used to confirm the TUNEL results. TG sections obtained 7 days after infection were stained with an antibody to poly(ADP-ribose) polymerase (PARP) (Anti-PARP p85 fragment pAb kit; Promega, Madison, WI), as described by the manufacturer. Anti-PARP p85 detects caspase-3 cleavage fragments of PARP, a hallmark of apoptosis (*18–20*). Representative photomicrographs are shown in Fig. 2. Extensive cleavage of PARP was detected in five of six TGs from LAT^--infected rabbits (>25% of neurons) but in none of the four TGs from wild-type–infected rabbits ($P = 0.048$, Fisher exact test). All six LAT^- TGs contained detectable levels of cleaved PARP staining, versus only one of four TGs from rabbits infected with wild-type virus ($P = 0.03$). To further confirm the similarity of the TUNEL and anti-PARP p85 results, we prepared seven pairs of sections, each pair from a different TG. One section of each pair was stained for TUNEL; the other section was stained for PARP p85. A correlation was seen between the percentage of positively stained neurons on the TUNEL and anti-PARP p85 sections ($R^2 = 0.62$, $P = 0.04$; linear regression; $P < 0.05$ indicates that the R^2 value was unlikely to have occurred by chance). Confirmation of the TUNEL results by anti-PARP p85 staining indicates that most of the TUNEL-positive cells were due to apoptosis rather than another mechanism of cell death or reaction with viral DNA ends. Thus, *LAT* appeared to decrease HSV-1–induced apoptosis in rabbit

Table 1. Apoptosis in rabbit TGs. Rabbits were infected with wild-type HSV-1 McKrae, dLAT2903 (LAT^-), or dLAT2903R (LAT^+) in both eyes (2×10^5 plaque-forming units per eye), as described (*3*). On the days indicated after infection, two rabbits were killed per group. TGs were harvested and sectioned, and five sections per TG (20 sections per group at each time point) were stained by the TUNEL assay that detects DNA ends produced during apoptosis (Klenow-FragEL DNA fragmentation detection kit; Oncogene, Cambridge, MA) as described by the manufacturer. Sections were read by light microscopy by two individuals who had no knowledge of the groups and were considered positive for apoptosis if one or more neurons were clearly positive. A TG was considered positive if one or more of its sections were positive. For each TG, the sections used were obtained from different areas spanning the TG.

Criterion	Day 3	Day 7	Day 10	Day 14
Sections with apoptotic neurons (%)				
dLAT2903	5	70	10	5
dLAT2903R	0	0	20	5
McKrae	0	0	0	0
TGs with apoptotic neurons (%)				
dLAT2903	0	100	25	25
dLAT2903R	0	25	25	25
McKrae	0	0	0	0

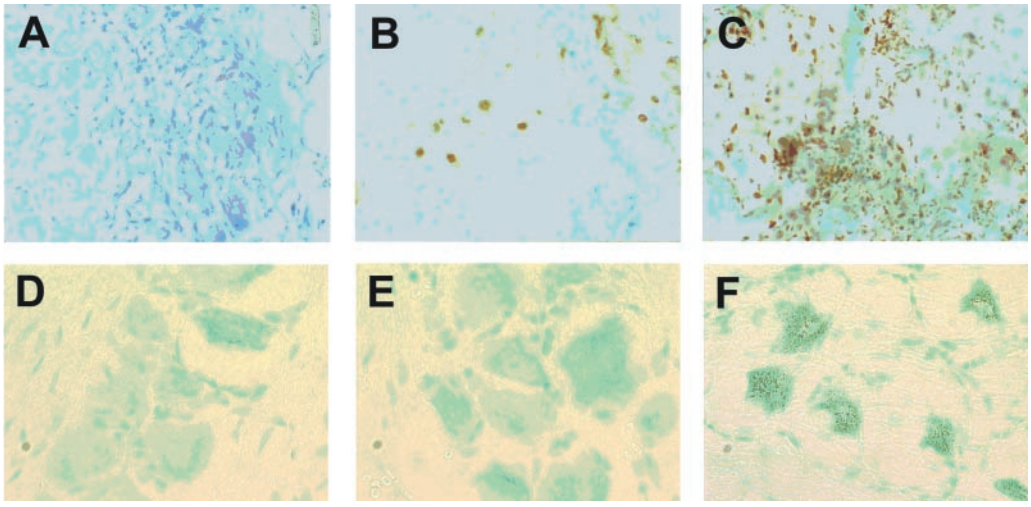

Fig. 1. Representative photomicrographs of TUNEL-stained TG sections (upper and lower rows represent two different experiments). Rabbits were infected, then killed 7 days after infection; TGs were harvested and sections were stained for apoptosis by TUNEL, as described in Table 1. (**A**) and (**D**), uninfected; (**B**) and (**E**), McKrae-infected; (**C**) and (**F**), dLAT2903 (LAT^-)–infected. In (A) to (C), positive staining is brown, counterstaining is blue-green; magnification, ~×150. In (D) to (F), positive staining is brown, counterstaining is green; magnification, ~×300. In (C), nonneuronal cells (apparently infiltrating immune cells), as well as neurons, show strong staining for apoptosis. In (F), brown dots in the nucleus of large neurons indicate a positive signal.

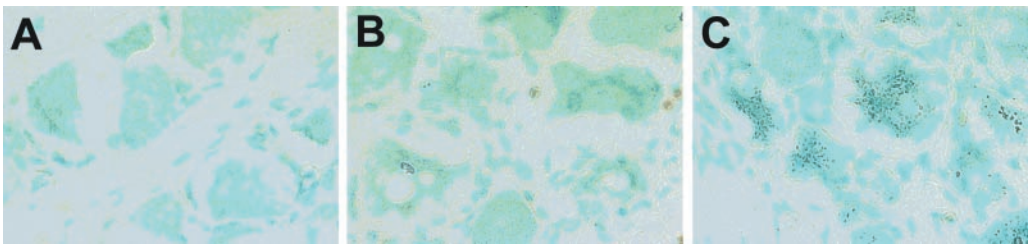

Fig. 2. Photomicrographs of TG sections stained with anti-PARP p85 (magnification, ~×300). Rabbits were infected, then killed 7 days after infection; TGs were harvested and sections were stained for apoptosis using the Anti-PARP p85 fragment pAb kit (Promega). Brown dots in the nucleus and cytoplasm of large neurons indicate positive staining. Counterstaining is blue-green. Representative photomicrographs are shown. (**A**) Uninfected cells; (**B**) wild-type McKrae–infected cells; (**C**) dLAT2903 (*LAT*$^-$)–infected cells.

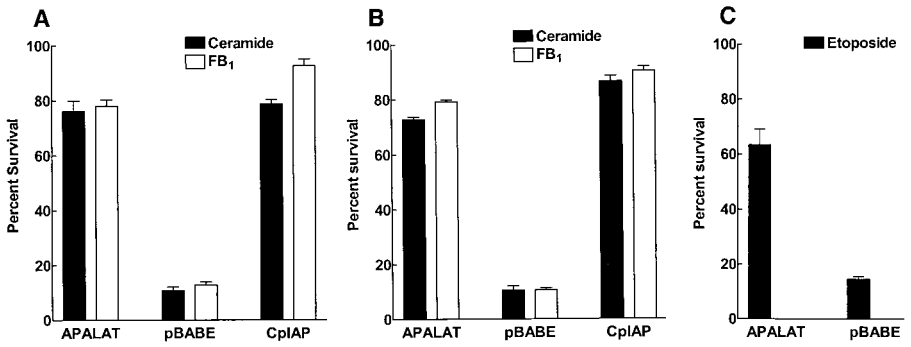

Fig. 3. In vitro inhibition of apoptosis by a *LAT* plasmid. IMR-90 cells (**A**), CV-1 cells (**B**), or neuro-2A cells (**C**) were cotransfected with 1 μg of pCMV-β-gal (a β-galactosidase expression plasmid) and 5 μg of APALAT (*5*). APALAT contains an Apa I–Apa I *LAT* restriction fragment (*LAT* nucleotides 301 to 2659) inserted into pBABE Puro, a mammalian expression vector containing a retrovirus LTR, Poly(A) addition signals, and the puromycin resistance gene regulated by the SV40 early promoter. Procedures for calcium phosphate transfection and maintenance of cultures were as described (*26*). Twenty-four hours after transfection, 10 μM C_6-ceramide (Calbiochem, San Diego, CA) (*23–25*) or 25 μM fumonisin B_1 (FB_1) (*26, 27*) were added to IMR-90 and CV-1 cultures, and 15 μM etoposide (Sigma) was added to neuro-2A cultures. After 48 hours, β-gal$^+$ cells were identified by staining the fixed cells with Bluo-gal (Gibco-BRL) for 24 hours; β-gal$^+$ cells were counted in five fields. The number of β-gal$^+$ cells in control cultures treated with phosphate-buffered saline represents 100% survival. Data are averages from four independent experiments. Cells were cotransfected with pCMV-β-gal and APALAT (APALAT), pCMV-β-gal and the empty expression plasmid instead of APALAT (pBABE Puro), or pCMV-β-gal and a plasmid expressing the baculovirus *CpIAP* (inhibitor of apoptosis) gene instead of APALAT (CpIAP). Similar results were obtained with a *LAT* restriction fragment not containing a Poly(A) signal.

Table 2. Rabbits were infected and TGs harvested 7 days after infection. Five well-spaced sections were stained for apoptosis and examined from each of 10 TGs per group (50 sections per group), except for the uninfected control group, in which only 47 sections were examined.

Criterion	dLAT2903	dLAT2903R	McKrae	Control
Sections with extensive apoptosis	33/50 (66%)	2/50 (4%)	2/50 (4%)	2/47 (4%)
TGs with extensive apoptosis	9/10 (90%)	1/10 (10%)	2/10 (20%)	1/10 (10%)
Sections with any apoptotic neurons	37/50 (74%)	11/50 (22%)	8/50 (16%)	17/47 (36%)
TGs with any apoptotic neurons	10/10 (100%)	5/10 (50%)	5/10 (50%)	10/10 (100%)

TGs on day 7 after infection.

To more directly test the hypothesis that *LAT* has an antiapoptotic activity, we used a quantitative assay (*21, 22*) in which cells are cotransfected with a β-galactosidase expression plasmid (pCMV-β-gal) and an expression plasmid containing the gene of interest, in the presence of an inducer of apoptosis. If the gene of interest (in this case, *LAT*) reduces apoptosis, the number of β-gal$^+$ cells will be higher. Apoptosis was induced by the sphingoid base C_6-ceramide (*23–25*), the mycotoxin fumonisin B_1 (FB_1) (*26, 27*), or the anticancer drug etoposide. Primary human lung cells (IMR-90), monkey kidney cells (CV-1), and murine neuroblastoma cells (neuro-2A cells) were used for these studies.

An Apa I–Apa I *LAT* restriction fragment (nucleotides 301 to 2659 of the 8.3-kb primary *LAT*) containing the entire stable 2-kb *LAT* region from HSV-1 strain KOS (*5*) was inserted into pBABE Puro, a mammalian expression vector containing a retrovirus long terminal repeat (LTR), polyadenylate [Poly(A)] addition signals, and the puromycin resistance gene regulated by the SV40 early promoter. The resulting plasmid (APALAT) was cotransfected into the respective cells along with pCMV-β-gal. IMR-90 and CV-1 cells were subsequently treated with C_6-ceramide or FB_1 and neuro-2A cells with etoposide. Relative to cultures cotransfected with the empty pBABE Puro plasmid, the *LAT*-containing plasmid increased cell survival in all cultures (as judged by the number of β-gal$^+$ cells) regardless of the agent used to induce apoptosis (Fig. 3) ($P < 0.0001$; ANOVA, Tukey posttest). Although the baculovirus antiapoptotic gene *CpIAP* (*28*) appeared to inhibit apoptosis slightly more efficiently than did *LAT*, the differences were not significant ($P > 0.05$).

Ceramide-induced apoptosis is blocked by activating protein kinase C (*23, 25*), whereas blocking the tumor necrosis factor pathway (TNF/FAS) inhibits FB_1-induced apoptosis (*26, 29*). Etoposide inhibits topoisomerase II, thus leading to higher levels of DNA damage and p53-dependent apoptosis (*13, 14, 30*). The ability of *LAT* to block apoptosis induced by each of these agents therefore suggests that *LAT* interferes with a downstream effector of apoptosis that is common to many apoptotic pathways. In addition, the ability of the APALAT fragment to block apoptosis mapped this function to within a region comprising only 28% of the primary *LAT*. The ability of this region to promote efficient establishment and subsequent reactivation from latency (*5*) strengthens the likelihood that *LAT*'s antiapoptosis function plays an important role in the latency reactivation cycle. The antiapoptotic activity of *LAT* did not appear to be HSV-1 strain specific, because we saw this activity with strain McKrae–derived viruses and strain KOS *LAT*.

Previous studies have reported that under different conditions, herpes simplex virus can induce or inhibit apoptosis (*15, 31, 32*). At least two other viral genes, *ICP27* and *US3*, can protect certain cells against virus-induced apoptosis in tissue culture (*31, 32*). Our results show that *LAT* can suppress apoptosis of neurons (either in vivo or in vitro) and that this function may explain the importance of *LAT* in herpes simplex virus latency and reactivation. Interestingly, we recently found that the latency-related (*LR*) gene of bovine herpes virus (BHV-1) also inhibits apoptosis in transient transfection assays (*33*). BHV-1 and HSV-1 are only distantly related. The

BHV-1 *LR* and the HSV-1 *LAT* have greatly different sizes and little or no sequence similarity. Although the BHV-1 *LR* antiapoptosis function correlates with expression of an LR protein (*33*), the *LAT* antiapoptosis function may be mediated by the *LAT* RNA, because *LAT* does not appear to encode a protein (*34*).

Our results suggest that *LAT* promotes neuronal survival after HSV-1 infection by reducing apoptosis. This hypothesis is supported by studies indicating that LAT^- mutants establish latency less efficiently than does the wild type (*5, 6, 35, 36*) and that a mutant expressing an altered *LAT* has increased neurovirulence (*12*). In general, stress is associated with reactivation and increased corticosteroid levels. Because corticosteroids induce apoptosis (*37, 38*), stress and viral gene expression during reactivation may induce apoptosis. Because *LAT* facilitates reactivation (*3, 39, 40*), inhibition of apoptosis by *LAT* would increase the probability that productive infection (i.e., productive reactivation from latency) succeeds. Whether reactivation was successful or not, LAT^+ neurons may have a better chance to survive and resume latency. Thus, a *LAT* antiapoptosis function could allow *LAT* to enhance reactivation by (i) enhancing the establishment and maintenance of latency, thereby providing more latently infected neurons in which future reactivations could occur; (ii) facilitating productive reactivation by protecting against apoptosis in neurons in which reactivation occurs; and (iii) facilitating the resumption of latency after a reactivation insult by protecting neurons against apoptosis.

It is unlikely that *LAT* is the only factor that promotes neuronal survival, because terminally differentiated neurons must have a well-devised mechanism to prevent programmed cell death. Furthermore, *LAT* may have additional mechanisms by which it enhances reactivation. Nonetheless, our results strongly suggest that suppressing apoptosis is an important mechanism by which *LAT* enhances HSV-1 reactivation. In addition, the ability of *LAT* to prevent HSV-1–induced apoptosis may be important in preventing the virus from causing extensive neuronal damage and subsequent neuronal disorders.

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Porphyrin and Phthalocyanine Antiscrapie Compounds

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The transmissible spongiform encephalopathies (TSEs) are fatal, neurodegenerative diseases for which no effective treatments are available. The likelihood that a bovine form of TSE has crossed species barriers and infected humans underscores the urgent need to identify anti-TSE drugs. Certain cyclic tetrapyrroles (porphyrins and phthalocyanines) have recently been shown to inhibit the in vitro formation of PrP-res, a protease-resistant protein critical for TSE pathogenesis. We now report that treatment of TSE-infected animals with three such compounds increased survival time from 50 to 300%. The significant inhibition of TSE disease by structurally dissimilar tetrapyrroles identifies these compounds as anti-TSE drugs.

The TSEs are a group of rare, fatal neurodegenerative diseases that include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in deer and elk, and Creutzfeldt-Jakob disease (CJD) in humans. The onset of the BSE epidemic in cattle in Great Britain raised concerns that humans could be at risk through exposure to contaminated cattle by-products. In 1996, a previously unknown form of CJD (variant CJD or vCJD) was identified in young people in Great Britain (*1*). The hypothesis that the most likely cause of vCJD was exposure to BSE-contaminated materials has since been supported by several different studies (*2*). At present, over 40 cases of vCJD have been confirmed, and there is some concern that the number of cases could be on the rise (*3*). Thus, with the potential exposure of millions of people to BSE and the onset of vCJD, the need for effective anti-TSE drugs has become acute.

A critical event in TSE pathogenesis is the conversion of the normal protease-sensitive host prion protein, PrP-sen, to a partially protease-resistant form (PrP-res) that is closely associated with disease pathogenesis. Studies have shown that there is a close correlation between compounds that inhibit PrP-res formation in vitro and compounds that inhibit TSE disease (*4*). Therefore, PrP-res is an obvious target for therapeutic intervention. The conversion of PrP-sen to PrP-res involves changes in

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