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Original Article

Herpes Simplex Virus Type 1 Latency-Associated Transcript Reduces Human Neuroblastoma Cell Proliferation

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Abstract

Background and Aims: The latency-associated transcript (LAT) transcribed by latent Herpes Simplex Virus type-1 in neuron cells are able to influence their host cell pathways. While the most of previous studies were focused on anti-apoptotic effects of LAT, our investigation is making an effort to explore LAT potency on cell cycle pathway in neuroblastoma cell lines.

Methods: The evaluation of LAT expression was assayed by RT-PCR. Real-Time PCR of cell cycle critical gene controllers' transcripts expression like EP300, P15, RB, RBL1, RBL2, MAPK-1, cyclinA2 and SMAD2 along with other technical evaluation such as MTT and cell counting assay assessed the LAT effects on cell cycle.

Results: The LAT transfected cells gene expression showed the increase of EP300, P15, RBL1 and RBL2 along with decline in MAPK-1 and cyclinA2 in comparison to cells transfected by control vector. The cell counting and MTT assays determined that LAT brought cell cycle down rather than cells introduced by control plasmid.

Conclusion: our investigation revealed that LAT transcript is able to repress cell cycle in neuroblastoma cells.

Keywords: Latency associated transcript; Herpes simplex virus type 1 (HSV-1); Cell cycle

Introduction

Herpes simplex virus (HSV-1) infection makes latency in humans and experimental animal models. HSV-1 LAT is plentifully expressed throughout latency and plays a critical role in the virus latency and reactivation. In addition, previous investigations broadly revealed LAT effects on the cell fate pathways like apoptosis. LAT construct that is stably transduced in a neuronal cell line suppresses viral replication and reduces the immediate-early gene mRNA levels (1, 2). While the null LAT virus is able to make extensive apoptosis in rabbit trigeminal ganglia, the virus encoding wild type LAT blocks apoptosis in culture cells (3). Furthermore, the caspase activation in mouse neuroblastoma (Neuro-2A) cells infected by LAT-null mutant is increased versus wild-type HSV-1. In Neuro-2A, LAT accumulates Bcl-x(L) transcript, encoding an anti-apoptotic protein (4). LAT has also been shown to block apoptosis by inhibiting caspase-8 and caspase 3 activation pathways and DNA fragmentation (5, 6). caspase-8 is inactivated by inhibiting of cellular FLICE-inhibitory protein (c-FLIP), a potent inhibitor of caspase-8-mediated apoptosis (7).

The LAT functions in cells are due to its transcripts as regulatory RNAs that are origin

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of some microRNAs and small RNAs (sRNA). The mouse neuroblastoma cells are protected from apoptosis by sRNA1 and sRNA2. The expression of Icp0 protein, a viral immediate-early transcriptional activator, is reduced by miR-H2-3p which consequently promotes the latency process (8-10).

Naturally, neuron cell proliferation and cell cycle are arrested extremely during brain differentiation. Different studies showed that accurate regulation of neuron cell cycle is important in neurogenesis and maintaining of neuron cells phenotypes(11). The role of cyclin dependent kinase inhibitor proteins like P15 and p300 and other cell cycle regulator proteins such as, RBL1 (p107), RBL2 (p130) and RB are critical in G1/S phase regulation in neurons (12-15).

In spite of extensive studies about anti-apoptotic features of HSV-1 LAT, there is little information about the effect of this viral gene on cell cycle and cell proliferation. In this study the effect of LAT transcript in BE2(c) cell was inspected. BE2(c) cells are a selective colon of SK-N-BE2(c) cell line, continues cell culture originated from human neuroblastoma cells, a recurrent extracranial solid tumor in children, originated from the neural crest (16).

Methods

Cell and plasmids

BE-2(c) cells were cultured at 37°C and 5% CO2 under humidified atmosphere in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum, 100 unit/ml penicillin and 50 mg/ml streptomycin.

pcDNA3-LAT, a pcDNA3.1 containing EcoRV–BamHI digested fragment of HSV-1 genome, LAT, was a kindly gift from Dr. Bryan R. Cullen (Duke University Medical Center, Durham, North Caroline, USA). pcDNA3-LAT plasmid (LAT) and pcDNA3.1 plasmid (cont-vect), as control, were transformed in E.coli Dh5α strain and cultured in LB agar medium contained zeocin and ampicillin. The recombinant plasmid was extracted and confirmed by BamHI digestion. LAT transcript expression was evaluated by RT-PCR using exon junction primers (sense primer: GTT TCC AGG GCA CCG AC antisense primer: TCC TCC TCT GCC TCT TCC) that are able to identify spliced transcript rather than LAT gene.

BE-2(c) cells were seeded in a 6-well plate (1×10^6 cells per well) 24 hours before transfection of recombinant plasmids. The 80% confluence cells were subjected to transfection by lipofectamine 2000 according to manufacturer's protocol (invitrogene). The tests were performed in duplicate manner for each reaction.

Gene expression evaluation

BE-2(c) cells total RNA was extracted by QIAzol Lysis Reagent (QIAGEN) according to manufacturer's instructions 60 hours after transfection. Briefly, after cell lysis by QIAzol the aqueous phase containing total RNA was subjected to ethanol precipitation and the RNA pellet was washed by 70% ethanol and dissolved in 20 µl water. cDNA synthesis were performed using First strand cDNA synthesis kit (Fermentas) according to the manufacturer's protocol.

The assessment of cell cycle genes after transfection of LAT and cont-vect was carried out by Real-Time PCR. Based on the manufacture's protocol 6.25µl of master mix SYBR® *Premix Ex Taq*™ II (TAKARA), 0.8µM sense and antisense primers (Table 1), and 1 µl template cDNA were added in 12.5µl total volume of each reaction. The duplicated reactions were accomplished in a Corbett apparatus (Rotor-Gene 6000). Finally the Real Time relative quantification data, normalized with endogenous Hprt-1 mRNA, were analyzed by delta delta CT method. The Real Time PCR results were analyzed by REST 2009 (relative expression software tool V2.0.13, QIAGEN, GmbH). The assays were repeated for one more time.

Cell proliferation assay

To test the effect of LAT transcript on cell proliferation of cells were assayed. BE-2(c) cells were seeded in a 24-well plate at approximately 5×10^4 cells/well. After 24 hours cells were transfected by the recombinant vectors. The transfection media were exchanged by fresh DMEM after 6 hours. The

medium was removed from all wells and the cell monolayer was washed with phosphate buffered saline (PBS). The live trypsinized cells were counted using trypan blue vital dye as identifier of live cells. The assay was replicated in another time and the quantity of cell proliferation was calculated at 0, 12, 36 and 60 hours after transfection.

MTT assay

Proliferation and viability of BE-2(c) cells were examined by MTT method. BE-2(c) cells (5×10^4 cells/well) were seeded in a 24-well plate (duplicate for each sample) and incubated for 24 hours. Then the cells were subjected to transfection of LAT and cont-vector. The viability of cells was measured at 12, 36 and 60 hours after transfection. The cells culture media were discarded and 20 µL MTT solution containing 5mg/ml 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was added on cells and incubated for 3 hours at 37°C, then formazan crystals were diluted in 200 µL of pure dimethyl sulfoxide (DMSO) (Sigma-Aldrich), and the absorbance of each well was read at 570 nm (BCA mode in Eppendorf machines).

Statistical analysis

To evaluate cell proliferation and MTT assays results, the LAT group was compared with BE-2(c) cells and cont-vect groups by Mann-Whitney test. The results with $P<0.05$ was considered as significant. The results of gene expression by Real Time PCR were analyzed by Rest 2009 software in accordance with randomization techniques. According to Rest 2009 algorithm, statistical analysis of normalized results with $p=0.00$ is considered as significant.

Results

LAT transcript is expressed in cells

Total RNA of BE-2(c) cells transfected by LAT was extracted. The transcription of LAT construct produced a whole transcript that is spliced by omitting a 2kb intron. The expression of construct was confirmed by RT-PCR of LAT transcripts with exon junction primers (fig.1).

LAT transcript affected BE-2(c) cell cycle genes expression

Although LAT transcripts aren't able to code

Table 1. The human cell cycle and house-keeping primers were designed in exon junction manner to amplify just transcript. The annealing Tm of primers was adjusted at 58C.

Name	Sequence
h-EP300-F	CCA CCC AAC CAG AGG AGA G
h-EP300-R	GGA TGG CAA TGG AAG ATA TAA TC
h-P15-F	CTG GTG GCT ACG AAT CTT C
h-P15-R	ATA ACT CCT CAG CAG ACA TTG
h-RB - F	AA TCA TTC GGG ACT TCT G
h-RB - R	ACT TCC ATC TGC TTC ATC
h-RBL1-F	AGC AGA GGA GGA TTC CTT G
h-RBL1-R	GGG CAC ATA ATC GCA TTG
h-RBL2-F	CGG AGC CAG GTG TAT AGA AG
h-RBL2-R	CGA GTA GGT GTG GGA GGA G
h-cyclin A2-F	TTG GTC CCT CTT GAT TAT CC
h-cyclin A2-R	TAC ATT TAA CCT CCA TTT CCC
h-MAPK1-F	CAT GGT GTG CTC TGC TTA TG
h-MAPK1-R	GTA GGT CTG GTG CTC AAA GG
h-SMAD2-F	AGC AGA ATA CCG AAG GCA GAC G
h-SMAD2-R	TTG AGC AAC GCA CTG AAG GG
h-Hprt1-F	CCT GGC GTC GTG ATT AGT G
h-Hprt1-R	TCA GTC CTG TCC ATA ATT AGT CC

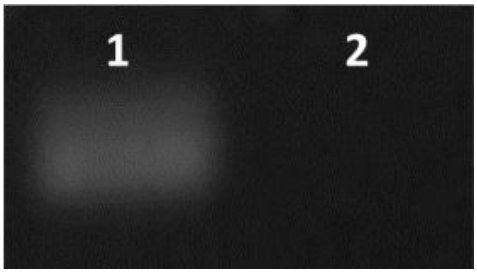

Fig. 1. LAT transcript expression was evaluated by RT-PCR of mature mRNAs (the spliced LAT mRNA without 2kb intron). To discriminate between probable DNA contamination of LAT plasmid gene and LAT transcript the RTPCR was carried out by exon-exon junction primers that are able to produce 119bp product (line-1), while these primers are not able to amplify the gene of LAT (line-2).

any protein, it is able to change its host fate. Many investigations have revealed that it could interfere in cellular pathways. To explore the effect of LAT on neuroblastoma cells, the BE-2(c) cells were subjected to transfection with plasmid containing 8.3 kb LAT gene and the pcDNA3 as a control then a set of genes involving in cell cycle were assayed. Sixty hours after transfection the cells were harvested to evaluate cell cycle genes expression by Real Time PCR. The analysis of relative quantification of cell cycle genes transcript levels by REST showed while the expression of cell cycle controller genes like EP300, P15, RBL1 and RBL2 were increased significantly, the cell cycle promoting genes like Mapk-1 indicated statistically significant decrease. The CyclinA2 expression was dropped off; however, the statistical analysis of results didn't demonstrate a significant difference. Other regulators of proliferation genes like RB and SMAD2 revealed a moderate rise, although it was not significant (fig. 2) Although LAT transcripts aren't able to code any protein, it is able to change its host fate. Many investigations have revealed that it could interfere in cellular pathways. To explore the effect of LAT on neuroblastoma cells, the BE-2(c) cells were subjected to transfection with plasmid containing 8.3 kb LAT gene and the pcDNA3 as a control then a set of genes involving in cell cycle were assayed. Sixty hours after transfection the cells were harvested to evaluate cell cycle genes expression by Real Time PCR. The analysis of relative quantification of cell cycle genes transcript levels by REST showed while the expression of cell cycle controller genes like EP300, P15, RBL1 and RBL2 were increased significantly, the cell cycle promoting genes like Mapk-1 indicated statistically significant decrease. The CyclinA2 expression was dropped off; however, the statistical analysis of

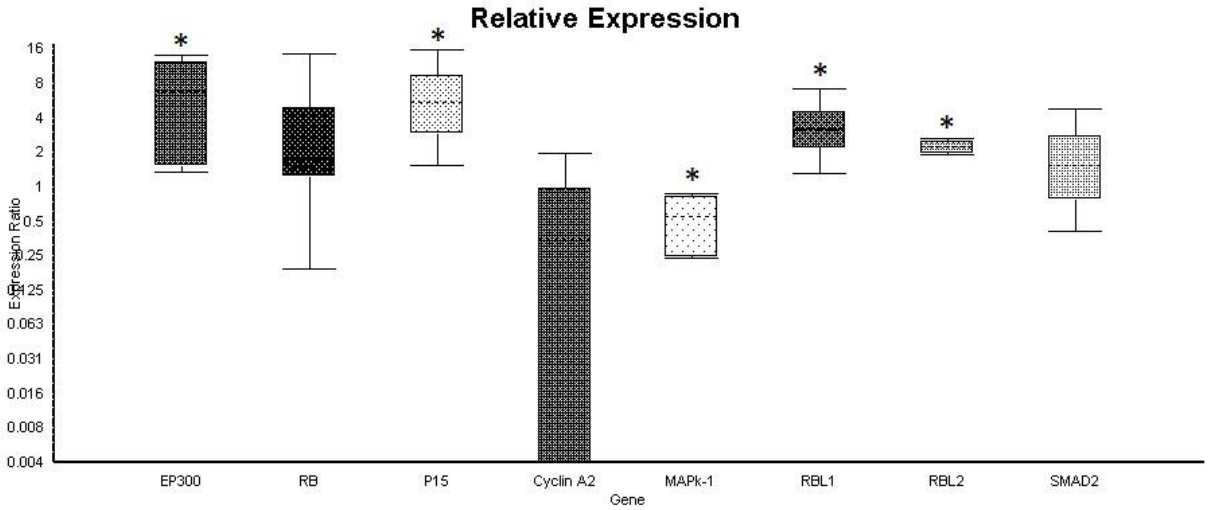

Fig. 2. The genes expression evaluation of LAT transfected BE-2(c) cells in comparison with cont-vect transfected cells by Real Time PCR was analyzed by REST software. EP300, P15, RBL1 and RBL2 transcripts increase and MAPk-1 gene expression drop were statistically significant (were shown by *) (P=0.00).

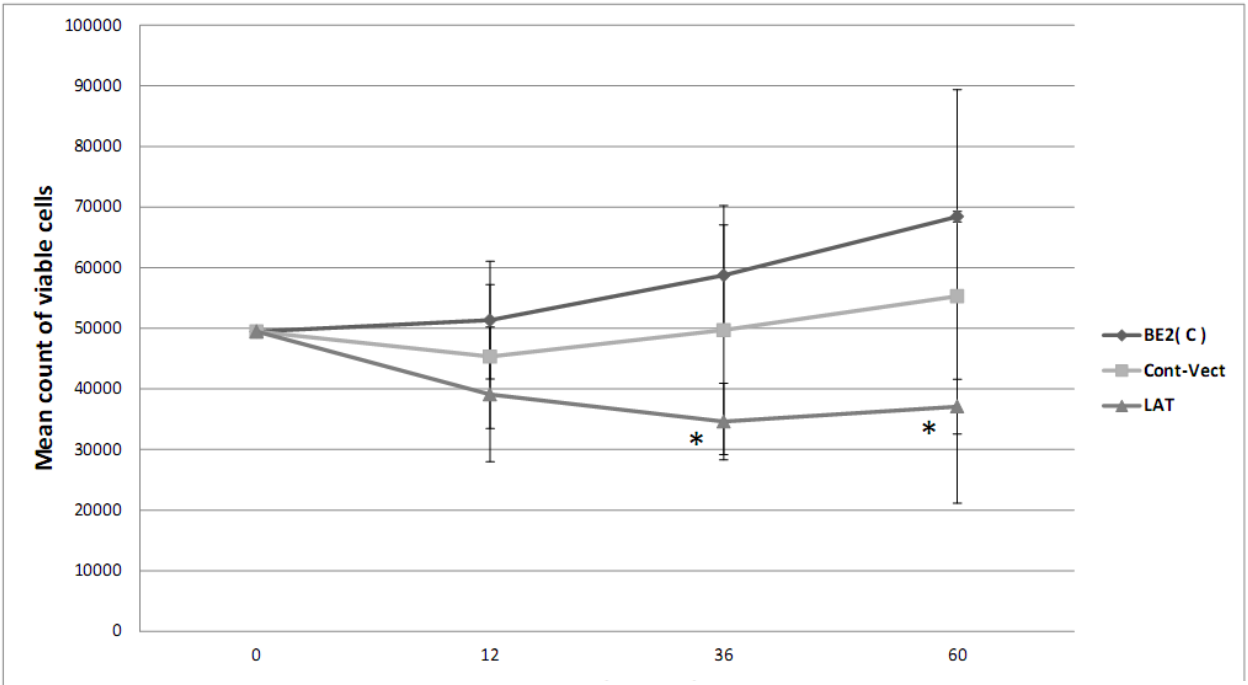

Fig. 3. The proliferation of LAT transfected cells in comparison with control cells were analyzed by cell counting at 0, 12, 36 and 60 hours after transfection of recombinant plasmids. The LAT induced cells population was decreased significantly 36 and 60 hours from transfection in comparison to BE-2(c) cells.

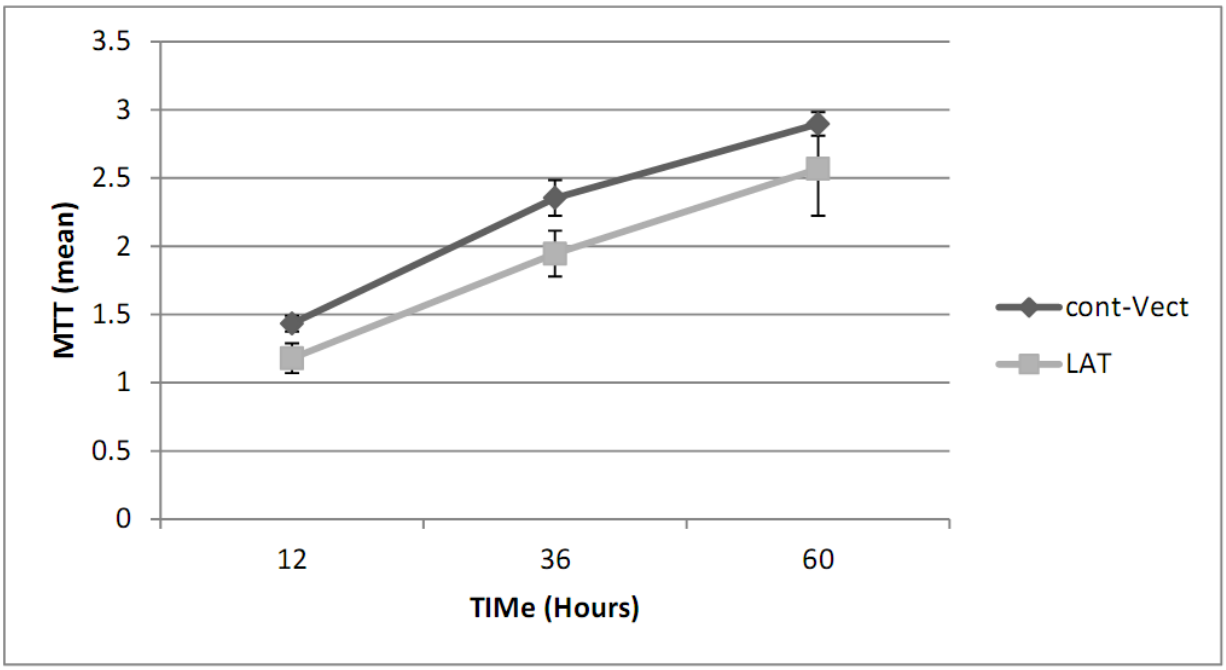

Fig. 4. The neuroblastoma cell lines, BE-2(c) cells that were subjected to LAT and cont-Vect transfection; 12, 36 and 60 hours after transfection were lysed. The duplicated test results showed that although the viable cells population in LAT induced cells declined, the fall in vital cell count in comparison to cont-vect induced cells wasn't statistically significant ($p<0.05$).

results didn't demonstrate a significant difference. Other regulators of proliferation genes like RB and SMAD2 revealed a moderate rise, although it was not significant (fig. 2).

LAT transcripts were able to impede BE-2(c) cells proliferation

The proliferation and cell cycle growth rate of LAT expressing cells were assayed by MTT assay and cell viable counting. The neuroblastoma cell line, BE-2(c) cells, were cultured and harvested at 0, 12, 36 and 60 hours after transfection of either LAT or control vector. The measurement of viable cells and comparison of LAT group with cont-vect transfected group and BE-2(c) cells without any treatment showed that 36 and 60 hours after transfection the cell rise rate in LAT group dropped dramatically (Fig. 3).

The MTT assay of the two group's cells showed that while the proliferation and viability of BE-2(c) cells transfected by LAT is depleted at 12, 36 and 60 hours after transfection in comparison to control group, during the time both of them revealed a heightening trend (Fig. 4) The proliferation and cell cycle growth rate of LAT expressing cells were assayed by MTT assay and cell viable counting. The neuroblastoma cell line, BE-2(c) cells, were cultured and harvested at 0, 12, 36 and 60 hours after transfection of either LAT or control vector. The measurement of viable cells and comparison of LAT group with cont-vect transfected group and BE-2(c) cells without any treatment showed that 36 and 60 hours after transfection the cell rise rate in LAT group dropped dramatically (Fig. 3).

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Discussion

In this study we have evaluated the effects of LAT transcript on neuroblastoma BE-2(c) cell cycle. The experiment has provided an opportunity to study the mitosis controller points and interfering genes in cell proliferation pathways after transfection of LAT construct in BE-2(c) cells.

MAPK-1 is a critical member of ERK family that has a capacity to involve in G0-G1 transition and promoting the cell proliferation (17). Other studies showed that MAPK-1 enhances neurogenesis and controls timing of differentiation in neuron cells (18, 19). Our results revealed that in response to LAT transcript the MAPK-1 transcript was significantly reduced in BE-2(c) cells. Other studies showed that the inhibition of MAPK causes to increase P15 transcript and protein, the heightening of P15 expression in our experiment was also able to verify MAPK-1 repression(20, 21).,

The assessment of other cell cycle controller genes transcripts including EP300 and P15 demonstrated the elevation of these genes expression in response to LAT transcript transfection in neuroblastoma cells. Ep300 (in P53 dependent manner) and P15 directly induce Cyclin/CDK inhibition in cell cycle (22, 23). In neuron cells Ep300 in cooperation of Sp1 induces CDK inhibitor during cell differentiation and declines neurogenesis rate (24). P15 as a member of CDK inhibitors by controlling the R point induces G1 arrest in neuron cell cycle (25).

The gene expression results in our experiment demonstrated while heightening of RB wasn't significant, The RBL1 and RBL2 expression was increased significantly. The RB family including RB, RBL1 and RBL2 is introduced as tumor suppressor genes that control cell cycle in S phase of DNA replication (26, 27). In neural cells maturation the RB family plays a critical role specially in promoting cell differentiation (15).

The Real Time PCR results of gene expression showed that the transcription levels of cyclinA2 declined when the SMAD2 expression grew but both of these variations weren't statistically significant. CyclinA2 as a controller point in cell cycle promotes the mitosis by triggering DNA synthesis and binding to cdk2 and cdc2 (28). Smad2 in cooperation with other members of Smad family is able to activate p15, p21, and p57and repressing some proto-oncogenes such as c-myc (29-31).

Consequently evaluation of cell cycle interfering genes expression in different points of proliferation pathway revealed significant increase in p15, Ep300, RbL1 and RBL2 expressions along with dramatic drop of MAPK-1 and cyclinA2. These genes remarkably are changed by the LAT transcript of HSV-1 directing to repress of cell cycle and neurogenesis.

Herpes simplex virus type 1 latency associated transcript is plentifully expressed through virus latency. Extensive investigations have explored the LAT features while most of their reports revolved around anti-apoptotic attributes of LAT. Our experiments based on different assessments comprising MTT, cell proliferation and gene expression analysis showed that LAT transcript is able to reduce BE-2(c) cells proliferation. Using BE-2(c) cells as a neuronal derived cells revealed that LAT by arresting the cell and inhibition of apoptosis is able to manipulate cell fate in a moderate manner and tries to maintain the latency state of the virus.

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