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Commentary

Latent HSV 1 virus in trigeminal ganglia: the optimal site for linking prevention of Alzheimer's disease to vaccination

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If the pathogenetic cascade occurring in the human brain eventuating in dementia of the Alzheimer type is proven to be triggered by latent Herpes simplex virus Type 1 in the human central nervous system, the suggestion by Itzhaki et al. [1] that mice vaccinated with mixed HSV-1 glycoproteins incorporated into immunostimulating complexes (ISCOM) carrier adjuvant can be protected against latent HSV-1 might have enormous therapeutic implications for cognitive decline amongst the elderly. Itzhaki's opinion would be strengthened if a polymerase chain reaction (PCR) assay carried out on the murine Trigeminal Ganglia (TG) had verified the presence of herpes infection/latency in the animals. How do we know that all the animals were infected? The discussion of ear erythema and its apparent association with latency in the TG while not being associated with infection in the mouse brain does not seem to correlate with Table 1, in which only 12 of the 80 mice are described. Is erythema that common an event? PCR of the TG (particularly for Latency Associated Transcripts, LAT) would clarify that there was latency without erythema. Additionally, a reactivation assay by dexamethazone or other means might have been performed. If reactivated virus travels from the TG to other regions of the brain, the unvaccinated (control) group may have shown detectable HSV-1 in greater than the 41% observed, and the ability of the ISCOM to prevent reactivation would be more rigorously proven.

Recent data from our laboratory, however, would argue that causal affiliation between the pathology of Alzheimer's disease (AD) and reactivation of latent HSV-1 is more robustly tested by investigations of the human trigeminal (Gasserian) ganglia. Neuropathologists have for many decades been impressed with the predilection of Alzheimer neuronal lesions for the mesial temporal lobe [2]. Such very remarkable, earliest and eventually most severe involvement of nerve cells in the medial portion of the human temporal cortex prompted our prediction, "Whatever the eventual explanation for the focality of such lesions in the mesial temporal lobe, those researchers who discover the biological basis for this predilection and focal selectivity will then be within grasp of comprehending the essential etiology for this devastating neurodegenerative disorder" [3].

In 1982, reflecting upon how strikingly the quantitatively pathognomonic neuronal lesions of AD (neurofibrillary tangles, senile plaques, granulovacuolar degeneration, Hirano bodies and neuronal loss) favor the limbic system, one of us speculated that reactivation of latent Herpes simplex virus in human trigeminal ganglia might be involved in Alzheimer's disease [4]. Early anatomical and physiological studies had indicated that fibers from the trigeminal ganglia innervate meninges and vessels within the middle and anterior cranial fossae, especially in the same subfrontal and mesial temporal regions preferentially afflicted in acute Herpes encephalitis. Those limbic regions are critical for memory processing and recall. Explantation and co-cultivation techniques had demonstrated Herpes simplex virus in many human trigeminal ganglia, which we found also re-

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veal a life-long lymphocytic infiltration in the absence of any pathological changes in those sensory nerve cells [5]. Such lymphocytes could represent a histological marker of latent Herpes virus, which when reactivating has already been well established as the ganglionic source of recurrent Herpes labialis. Ball suggested that reactivation of the same dormant viral material travelling centripetally instead might be the cause of the "degenerative" lesions typical both for Alzheimer's disease and of the normal aged human brain.

Our laboratory harvested trigeminal ganglia, hippocampal, and brainstem samples from 40 subjects (21 demented patients confirmed by neuropathological examination as AD; 15 aged-matched negative controls; and 4 demented individuals with causes other than AD), from which Deatly et al. utilized *in situ* hybridization to detect Latency-Associated Transcripts (LAT). We discovered that the abundant latency-associated transcripts (LAT) of HSV-1 were detectable at a significantly higher incidence in the trigeminal ganglia of Alzheimer victims than in controls, employing as probes the purified EcoR1 EK fragment from a Herpes simplex virus Type-1 clone and a mixture of fragments from the rest of the genome [6]. However, the few tissue sections available from the severely afflicted hippocampal cortex of the demented subjects failed to show any evidence for viral RNA. Nevertheless, latent HSV-1 RNA was detectable in 81% of the trigeminal ganglia from the cases of Alzheimer's disease, but in only 47% of those from control patients ($P < 0.05$). Latency transcripts were not found in tissue sections from pons or medulla of Alzheimer demented individuals.

In repeated, intriguing investigations by polymerase chain reaction studies, Itzhaki and colleagues have published data reporting that HSV-1 in latent form is present in a high proportion of both elderly normal and AD patients' neocortex, with virus positivity as high as 74% of AD cases and 63% of age-matched elderly normal controls (mean age 80 years; range 58–95) [7]. Although Itzhaki and co-workers found a significant association between the Apolipoprotein E–E4 allele and the presence of HSV-1 in brains of Alzheimer patients but not age-matched normals [8], the viral DNA was said to be usually present in temporal or frontal cortex, or in a few hippocampal samples, but never in occipital cortex; yet to our knowledge Itzhaki's laboratory has never examined tissue from human trigeminal ganglia.

More recently, Pevenstein et al. examined trigeminal ganglia from 15 normal individuals of younger age (ranging from 18 to 73 years; mean = 38.6 years; no subjects in their 50's, only 2 in their 60's and only 1 in his 70's), by real-time quantitative fluorescence reverse-transcriptase polymerase chain reaction, to quantify the numbers of copies of latent Herpes simplex virus Type 1 and Type 2 genomes, and of latent varicella-zoster virus [9]. Using primers and probes from the HSV-1 glycoprotein G (gG1) region, these investigators found that eight of the 15 ganglia (53%) were positive for the HSV-1 gG1 gene. Age correlation was not reported. Straus and colleagues could extrapolate from their data to estimate the total copy numbers and distributions of those 3 viruses in human trigeminal ganglia. As they recovered a mean of 478 micrograms DNA per ganglion, based on their estimate of 15.6 picograms of DNA per cell, the average ganglion contained upwards of 3×10^7 cells. They therefore estimated that on average, each latently infected ganglion contained 8.7×10^5 HSV-1 genomes. Since our own laboratory had reported human trigeminal ganglia contain an average of 8.1×10^4 nerve cells [5], and since HSV-1 persists exclusively in neurons, Straus's group projected that each latently infected neuron contains at least 11 copies of HSV-1 DNA, assuming all nerve cells are infected. While *in situ* hybridization studies for HSV-1 latency-associated transcripts had suggested that only 1 to 4% of neurons are positive, more recent PCR and *in situ*-PCR analyses had demonstrated that 3 to 10 times that percentage are positive (e.g., reference 10). Such newer data therefore indicated to Straus and colleagues that latently infected trigeminal nerve cells contain an average of 28 or more HSV-1 genomes, a copy number similar to those of Epstein-Barr virus DNA copies (episomes) carried in B lymphocyte cell lines. Pevenstein et al. therefore estimated that each human ganglion contains an average of 2,902 HSV-1 G genomes per 10^5 cells ($\pm$1,082 S.E.M.).

One of the major discoveries in the studies of Herpes simplex virus latency was the latently associated transcripts (LAT). This unique transcript is made antisense to all of the other genes in the herpes genome [11]. Although the exact mechanism for LAT is not known, most investigators believe that it plays a significant role in viral reactivation since most LAT null mutants in animal models have significantly reduced reactivation [12]. Furthermore, numerous studies have shown that a large number of viral genomes can establish neuronal latency in the absence of significant viral replication within the neurons. This suggests that nerve cells can be infected by many HSV virions and yet still enter the latent state. Yet another tempting affiliation with AD is the striking homology of a Herpes simplex virus glycoprotein B fragment to the Alzheimer's A-beta amyloid peptide, recently reported by LaFerla and co-workers [13]. Such potential interaction between HSV-1 and a human host carrying the ApoE4 allele might suggest that the ability to disrupt a direct interaction between viral glycoproteins and apolipoproteins would offer a novel therapeutic approach [14].

With the auspices of the Oregon Brain Bank founded in 1990 in affiliation with the NIA Alzheimer Center at Oregon Health Sciences University, Portland, our laboratory has successfully accessioned pairs of trigeminal ganglia and accompanying CNS brain samples from over 220 autopsies, most retained at −80°C for molecular investigations.

1. Preliminary data

Our laboratory has just completed a highly preliminary pilot investigation on 56 trigeminal ganglia, from 18 *control*

subjects, ages stillborn to 104 years, whose neuropathology showed no neurodegenerative or other significant disease; 24 *Alzheimer's disease* patients dying at ages 48 to 79 years with dementia Alzheimer type confirmed pathologically by published criteria; and 14 *non-AD* but demented patients ages 47 to 79 years, whose neuropathology diagnoses were other than Alzheimer's disease. From animal models, it has been shown that the HSV-1 latency associated transcript (LAT) is the only locus within the Herpes genome to be abundantly transcribed during latency. The exact mechanism by which LAT affects establishment, maintenance, and reactivation is under intensive investigation [15]. Hill et al. have shown, in the rabbit eye model, that the genome copy number and the number of neuronal cells containing LAT are very stable over a longer period of time [16]. We have therefore employed fluorescent real-time, polymerase chain reaction (PCR) of the HSV-1 gG region to quantify the HSV-1 genomes per cell. To quantify the latency-associated transcripts (LAT), we used real-time reverse transcriptase polymerase chain reaction (RT-PCR) to measure the quantity of LAT from the RNA recovered post-mortem in these human brains. Using these probes in this very small preliminary series, we observed that while nearly all the subjects' ganglia tested thus far do contain (dormant) Herpes virus DNA, only a portion of those individuals are also expressing the latency associated transcripts' RNA.

2. Laboratory methods

Primers used are based upon sequence data from Professor I. Steiner. The forward primer is 21 nucleotides in length, corresponding to nucleotides 208–228 in the LAT/ORF2 region: CCAACAGACAGCAAAAATCCC. The reverse primer is also 21 nucleotides long, corresponding to 278–298 in LAT/ORF2: GAAAAGTGAAAGACACGG GCA. The Taqman probe, internal to the primers, is a 28-mer and corresponds to nucleotides 240–267 in the LAT/ORF2 region.

The HSV-1 gG (glycoprotein G) DNA is quantitatively determined using (a) oligo probe: 5′ FAM–CCC CGC CCA GCG TCT TGT CAT T–3′ TAMRA (b) a forward primer, 23 nucleotides long: GGT GTC CCC GGA AAG AAA TAT AT and (c) a reverse primer, 20 nucleotides long: CTG CAT CTG CGT GTT CGA AT (IDT Incorporated, Coralville, IA). Advice in these latter items is provided by Todd Wisner, courtesy Professor David C. Johnson's laboratory at OHSU.

2.1. Synopsis of RNA/DNA extraction from trigeminal ganglia

Samples are disrupted in buffer containing GITC and homogenized. The majority of proteins are removed by salt precipitation.

The QIAGEN RNA/DNA Kit procedure is based on selective purification of RNA and genomic DNA by anion exchange on QIAGEN resin in a QIAGEN-tip. The cellular extract is prepared, the conditions adjusted to allow separation of RNA and DNA, and the extract loaded onto the QIAGEN-tip. Under the conditions used, total RNA and a portion of the genomic DNA present in the sample bind to QIAGEN resin while the remaining DNA passes through in the first flow-through fraction. Residual proteins, metabolites, and low-molecular-weight impurities are removed by washing the QIAGEN-tip with a medium-salt buffer. Pure RNA is eluted in a high-salt buffer while DNA remains bound to the resin. The RNA is then concentrated and desalted by isopropanol precipitation.

For simultaneous isolation of genomic DNA and RNA from the same sample, the first flow-through is reapplied to the QIAGEN-tip after elution of the RNA in order to bind the rest of the genomic DNA. The column is washed again, and the genomic DNA is eluted. Concentration and desalting of the RNA and DNA by isopropanol precipitation can then be performed in parallel. Quantitative recovery of DNA and RNA was confirmed in our pilot study with UV-spectrophotometry.

As in these first 56 cases, on all of the additional cases already extant in the Brain Bank, we have full demographic information, including age, gender, number of hours' post-mortem interval, brain weight, neuropathological diagnosis, and quantitative histopathological grading of severity of nerve cell abnormalities in the Alzheimer victims. We also have on hand a small 3rd cohort of brain autopsies of demented individuals dying from causes other than Alzheimer's disease, including Parkinson's disease, Huntington's disease, and frontotemporal dementia of the non-Alzheimer type. Analysis of this latter cohort ensures that significant differences discovered in the Alzheimer victims do not represent merely some non-specific effect of brain shrinkage, prolonged nursing home infirmity, etc.

3. Preliminary observations

Four of the 56 cases proved not useful, as there was insufficient tissue sample to generate 500 ng of genomic DNA.

Whether using the gG or the LAT DNA primer pairs, analyses of the informative 52 cases studied thus far show that HSV-1 would be potentially detectable in 43 of these 52 cases. The other 9 cases did not yield sufficient quality DNA or RNA for comparable analysis, based upon amplification of other internal housekeeping standards (e.g., 18-S ribosomal RNA). Given post-mortem intervals for these brain autopsies ranging between 2 and 144 hours (mean, 16 hrs.), it is not unexpected that 17% of such a series might reflect significant tissue decay.

We used the 43 cases with proven quality of genomic DNA and cDNA prepared from RNA samples to study the expression of gG and LAT genes at the genomic and RNA

level. Thirty of the 43 intact cases are positive for HSV-1 genomic DNA (by both gG and LAT genes), indicating that a surprisingly high (nearly 70%) proportion of the cases do contain virus. The other 13 of the 43 tested under similar conditions are negative for any evidence of HSV-1 infection.

While 6 of the 30 HSV-1 positive cases are negative for any LAT mRNA, 24 of the 30 HSV positive cases do show variable concentrations of LAT mRNA expression. Employing an HSV-1 standard curve generously provided by Dr. MeeiLi Huang, corrected copy numbers demonstrate an approximately 200-fold variation in relative Latency-associated transcript mRNA signal, with a span not unlike that recently published in 17 normal subjects' ganglia [17]. (No gG mRNA was detected in any ganglia, suggesting there is no evidence for reactivation in these samples.) Thus, in our initial series, fully 80% of the infected ganglia are transcribing latent HSV-1 mRNA.

If (.8 × .7 or) 56% of all adults in a larger series were to show HSV-1 latency expression, then a population study [18] finding 47% of all East Bostonians past age 85 years develop Alzheimer's disease would not be surprising, if HSV-1 plays a significant role in its pathogenesis.

4. Possible significance

Thus far, we have already observed that the strength of the latency potential for reactivation of herpes virus within the LAT-positive cases varies considerably, by several orders of magnitude. This means that statistically, we need to analyze a considerably larger number of such cases, before we can determine the precise significance of these highly preliminary observations, specifically to determine whether age, gender, ApoE genotype, or diagnosis of Alzheimer dementia correlates either with absence/presence of LAT-positivity or with strength of such positivity. No such correlations are as yet apparent. Statistical evaluations of all data, including logarithmic transformation, are being analyzed using both parametric and non-parametric methods.

If it should transpire that those individuals also expressing the LAT RNA in their ganglia are significantly more elderly than, more often E4 genotypes, or significantly more frequently demented from Alzheimer's disease than, those subjects whose ganglia contain only HSV DNA, further studies of latent HSV-1 signals from hippocampal and other neocortical samples of the same subjects may then also be compared with their age, gender, ApoE genotype and diagnoses. At that point, the hint arising from the Itzhaki murine model of potential efficacy for a vaccine preventing Herpes latency in human brains might generate enormous interest, not only for prevention of the relatively rare acute encephalitis of HSV-1 origin, but more importantly for that expanding cohort particularly of very elderly North Americans destined to develop the lesions of Alzheimer's dementia. If by contrast the expression of LAT RNA in trigeminal ganglia shows no correlation with clinical or neuropathological category, it will be considerably more difficult to support the Itzhaki claim.

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