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Review

Varicella-zoster virus human ganglionic latency: a current summary

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Varicella-zoster virus (VZV) is a ubiquitous human herpes virus typically acquired in childhood when it causes varicella (chickenpox), following which the virus establishes a latent infection in trigeminal and dorsal root ganglia that lasts for the life of the individual. VZV subsequently reactivates, spontaneously or after specific triggering factors, to cause herpes zoster (shingles), which may be complicated by postherpetic neuralgia and several other neurological complications including vasculopathy. Our understanding of VZV latency lags behind our knowledge of herpes simplex virus type 1 (HSV-1) latency primarily due to the difficulty in propagating the virus to high titers in a cell-free state, and the lack of a suitable small-animal model for studying virus latency and reactivation. It is now established beyond doubt that latent VZV is predominantly located in human ganglionic neurons. Virus gene transcription during latency is epigenetically regulated, and appears to be restricted to expression of at least six genes, with expression of gene 63 being the hallmark of latency. However, viral gene transcription may be more extensive than previously thought. There is also evidence for several VZV genes being expressed at the protein level, including VZV gene 63–encoded protein, but recent evidence suggests that this may not be a common event. The nature and extent of the chronic inflammatory response in latently infected ganglia is also of current interest. There remain several questions concerning the VZV latency process that still need to be resolved unambiguously and it is likely that this will require the use of newly developed molecular technologies, such as GeXPS multiplex polymerase chain reaction (PCR) for virus transcriptional analysis and ChIP-seq to study the epigenetic of latent virus genome (Liu *et al,* 2010, *BMC Biol* 8: 56). *Journal of NeuroVirology* (2010) **16,** 411–418.

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Introduction

Varicella-zoster virus (VZV) is a pathogenic human alpha-herpesvirus that causes varicella (chicken pox) as a primary infection, following which the virus becomes latent in trigeminal, autonomic, and dorsal root ganglia (DRG) (Mitchell *et al*, 2003; Kennedy, 2002). After a variable period of time the virus can reactivate, either spontaneously or following a variety of triggering factors, especially in elderly and immunosuppressed individuals, to cause herpes zoster (shingles), an extremely painful vesicular skin eruption occurring along the distribution of one or more sensory dermatomes (Kennedy, 2002). The most important complication of herpes zoster is postherpetic neuralgia (PHN), a severe and persistent pain following zoster that is highly refractory to treatment. Risk factors for the development of PHN

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include age over 50 years, female gender, the presence of a prodrome, a severe disseminated rash, severe pain at presentation, and polymerase chain reaction (PCR)-detectable VZV viremia (Wareham and Breuer, 2007; Mueller *et al*, 2008). By contrast, herpes simplex virus type 1 (HSV-1), the prototype human alpha-herpesvirus, reactivates usually in younger individuals, tends to diminish with age, and generally multiple times, in contrast to zoster, which are seldom recurrent.

As well as causing PHN, VZV is associated with a wide variety of neurological complications, most important of which is VZV vasculopathy; viral infection of cerebral arteries, affecting small or large vessels or both (Steiner *et al*, 2007; Nagel *et al*, 2008; Gilden *et al*, 2009). VZV vasculopathy is more common in immunosuppressed individuals such as those with human immunodeficiency virus infection, and the linkage of VZV infection with stroke is being increasingly recognized. Other VZV complications include cranial nerve palsies, myelitis, segmental motor weakness, and zoster sine herpete where the characteristic dermatomal pain occurs in the absence of the zoster rash (Gilden *et al*, 1994). Since all these complications are associated with VZV reactivation, it is clearly of paramount importance to understand more fully the molecular basis of VZV ganglionic latency. Here we summarize key features of VZV latency, and also highlight some recent developments.

Relevant VZV biology

The complete genomic DNA sequence from 24 VZV isolates have been submitted to the National Library of Medicine and are within the public domain of the National Institutes of Health (http://www.ncbi.nlm.nih.gov/pubmed/; accession numbers: AB097932; AB097933; AJ871403; AY548170; AY548171; DQ008354; DQ008355; DQ452050; DQ457052; DQ479953–DQ479963; DQ674250; EU154348; NC_001348; X04370). The ~125,000 (±0.5%) base pair VZV genome is 7-fold more stable than other members of the human alpha-herpesvirus subfamily (Muir *et al*, 2002). Although <600 single-nucleotide polymorphisms within the VZV genome exist and are used to identify five distinct currently circulating phylogenetic clades, the basic architecture of all VZV genomes remains the same (Tyler *et al*, 2007; Breuer *et al*, 2010). The VZV genome contains 68 unique open reading frames (ORFs); transcripts from each are detected in virus-infected cells in culture (Cohrs *et al*, 2003b; Kennedy *et al*, 2005). In addition, three novel VZV genes (ORFs 9A, 33.5, and S/L) have been identified (McMillan *et al*, 1997; Ross *et al*, 1997; Kemble *et al*, 2000); although evidence for multiple overlapping polyadenylated VZV transcripts exists, their biological significance has yet to be determined. Applying recombinant biological techniques to the VZV genome cloned into multiple cosmids (Cohen and Seidel, 1993; Mallory *et al*, 1997) or maintained as an bacterial artificial chromosome (Nagaike *et al*, 2004; Yoshii et al, 2007; Zhang *et al*, 2008; Wussow *et al*, 2009) has greatly advanced our understanding of individual VZV genes, but the study of VZV latency is largely in the descriptive phase due predominantly to the lack of a suitable small-animal model of virus latency and reactivation. In addition, most intracellular VZV assembled in tissue culture cells is targeted to late endosomes for degradation (Chen *et al*, 2004) resulting in particle:plaque-forming unit (PFU) ratios approaching 40,000:1 (Carpenter *et al*, 2009). As a result, high-titer cell-free VZV preparations are essentially impossible to obtain and much of our knowledge concerning the kinetics of VZV gene transcription is by analogy to HSV-1. Finally, even routine quantitation of VZV is problematic. Since VZV is highly cell-associated and essentially no infectious virus is released, plaque assay is typically performed by infecting monolayers of susceptible cells with dilutions of VZV-infected cells. This assay determines foci-forming units (plaques developing from foci of infected cells), which are substantially greater and inherently more variable than plaque-forming units (plaques developing from cell-free virus).

Localization of latent VZV in human ganglia

VZV is latent in at least 87% trigeminal ganglia (TG). The identity of the cell type harboring latent VZV was a controversial issue for many years, but it has now been established beyond reasonable doubt that latent VZV resides predominantly in neurons (Mitchell *et al*, 2003). The critical experiments that settled the dispute included (1) *in situ* PCR amplification followed by *in situ* hybridization demonstrating that VZV DNA was located predominantly in neuronal nuclei with only a few satellite cells infected (Kennedy *et al*, 1998); (2) enzymatic dissociation (LaGuardia *et al*, 1999); or (3) mechanical separation of the TG followed by PCR to detect VZV DNA exclusively in neuronal cells rather than satellite cells (Levin *et al*, 2003); and finally (4) laser-capture microdissection of human TG combined with PCR to demonstrated the presence of latent VZV almost exclusively in neurons rather than non-neuronal cells (Wang *et al*, 2005). Significantly, the authors of the original study (Croen *et al*, 1988) suggesting a non-neuronal site of latent VZV subsequently accepted that the overwhelming evidence indicates that latent VZV is localized in neurons as well as the rare satellite cell (Pevenstein *et al*, 1999). Estimates of the percentage of the total number of sensory neurons that are latently infected in

ganglia include figures of 1.5% (Levin *et al*, 2003) and 2–5% (Kennedy *et al*, 1998).

Abundance of latent VZV DNA in ganglia

The extent of the latent VZV burden in human ganglia is an important question, since a link has been demonstrated between HSV burden during latency and virus reactivation (Sawtell, 1998; Sawtell *et al*, 1998; Hoshino *et al*, 2007, 2008). The VZV burden during latency has been addressed in several studies using different techniques, but there is, to date, no clear consensus on this issue, with widely varying reports of VZV copy numbers (Kennedy, 2002; Azarkh *et al*, 2010). For example, an early report using quantitative PCR in 1993 found 6 to 31 copies of the VZV genome per 10^5 cells (Mahalingam *et al*, 1993), but a subsequent study using real-time quantitative PCR reported a mean copy number 9046 (range 577–55,543) per 10^5 TG cells (Cohrs *et al*, 2000). The study by Pevenstein *et al* (1999) used real-time quantitative fluorescent PCR to show that there were 7.7×10^4 VZV DNA copies per TG, and that there are 8.1×10^4 neurons and 8×10^6 satellite cells per TG, with an overall estimate of 8 VZV copies per cell. The reasons for such wide discrepancies are likely to be related to the heterogeneity of the individual from whom the ganglia have been taken (Azarkh *et al*, 2010). In terms of the physical configuration of the VZV genome during latency, it was shown by Clarke *et al* (1995) that the genome termini are adjacent and that the physical state of latent VZV is extrachromosomal and circular, as has also been reported in HSV latency.

Both VZV and HSV can be latent in the same ganglion and indeed the same cell. The study mentioned above by Pevenstein *et al* (1999) detected not only VZV genomes in TG but also latent HSV-1 and HSV-2 in the same ganglia, with TG neurons containing an average of 28 or more HSV-1 genomes. This ability to detect both VZV and HSV in the same TG was also reported by Cohrs *et al* (2000) who found that of a total of 17 subjects, VZV DNA was detected in all of them and HSV-1 DNA was detected in 12 of these. Interestingly, where the same subject's ganglia contained both VZV and HSV-1, there was no significant correlation between the copy numbers of the two viruses. Further, a recent study showed that in a study of TG from 207 cadavers, all of the latter that were positive for HSV-1 DNA were also positive for VZV DNA, again demonstrating the very high frequency of colocalization of these two herpesviruses in human ganglia (Inoue *et al*, 2010). In this study, VZV and HSV-1 became latent in bilateral TG and were not affected by gender. Relevant to these findings, it has been shown using double fluorescence and *in situ* hybridization (ISH) that a small number of neurons in human TG contained both HSV-1 and VZV, and latency-associated transcripts for both viruses were detected in common areas within the ganglia (Theil *et al*, 2003a).

VZV gene expression during human ganglionic latency

Studies of viral gene expression are important both because the identity of latency-associated genes may indicate the molecular mechanisms involved in maintaining latency, and also because of the potential to identify viral targets for potential antiviral therapy (Kennedy, 2002). All studies to date have clearly indicated that viral gene expression during latency is highly restricted with the detection of just a small number of open reading frames (ORFs). The techniques used to identify VZV transcripts in latently infected TG and DRG include Northern blot hybridization, cDNA libraries, PCR, ISH, and, more recently, quantitative PCR (Mitchell *et al*, 2003; Azarkh *et al*, 2010). The overall consensus at present is that transcripts mapping to ORFs 21, 29, 62, 63, and 66 are detectable in latently infected ganglia (Figure 1) (Cohrs *et al*, 1994, 1995, 1996, 2003a; Kennedy *et al*, 1999, 2000; Azarkh *et al*, 2010). There is also some less consistent evidence for ORF 4 expression during latency (Croen *et al*, 1988; Kennedy *et al*, 2000). In the large study using ISH by Kennedy *et al* (2000), VZV ORF 63 was the most frequently detected transcript in latently infected TG, and the restricted pattern of gene expression in fixed ganglia was markedly different from the more extensive pattern detected in explanted TG grown *in vitro*. Although it seems likely that during latency there is selective expression of multiple genes, it is not known at present how extensive this viral gene expression actually is, since currently only about 11 ORFs out of a potential 71 have actually been studied in detail. Recent work has, however, given us some understanding of the relative abundance of expressed viral genes. Thus, quantitative analysis using real-time PCR of individual TG showed that VZV ORF 63 was consistently the most abundant and frequently expressed transcript in individual ganglia, with a relative >2000-fold expression (Cohrs *et al*, 2000). In the same study, HSV-1 latency-associated transcripts (LATs) were consistently detected in ganglia containing latent HSV-1. The conclusion that VZV gene 63 expression is the hallmark of VZV latency is confirmed by a subsequent study from the same laboratory that reported a very high abundance of ORF 63 transcripts compared to other detected transcripts in VZV latency (Cohrs and Gilden, 2007). The finding that multiple VZV genes are transcribed in latently infected human ganglia is paralleled in the simian model of varicella virus latency. Transcripts mapping to simian varicella virus

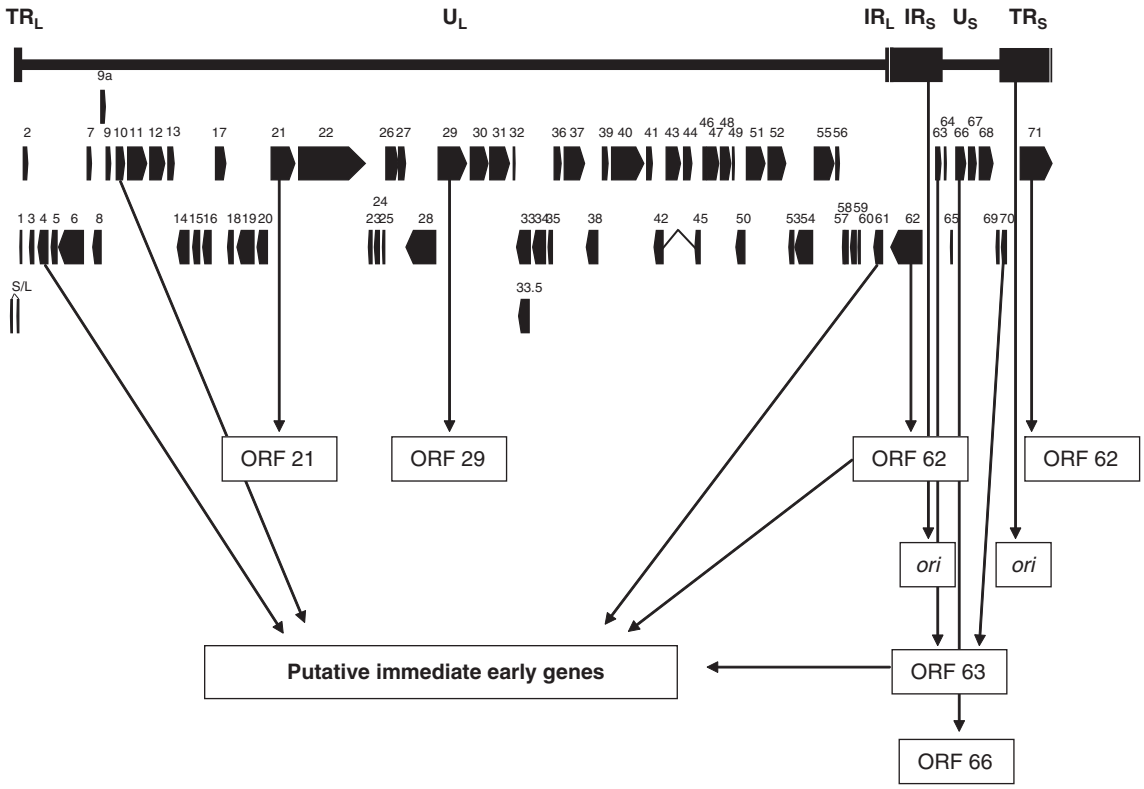

Figure 1 The double-stranded VZV genome is composed of unique long (U_L) and unique short (U_S) DNA segments covalently joined through internal repeats located at the ends of the long (IR_L) and short (IR_S) unique segments. The repeats are present in an inverted form at the virus genome termini (TR_L, TR_S). The 71 predicted and three experimentally discovered open reading frames (ORFs) are depicted in their approximate location and their direction of transcription. The VZV ORFs detected in RNA extracted from latently infected human ganglia by DNA sequence analysis are highlighted with respect to the location of the two origins of virus DNA replication (*ori*) and putative immediate-early VZV genes.

(SVV) ORFs 21, 62, 63, and 66 are present in latently infected trigeminal and thoracic ganglia (Messaoudi *et al*, 2009). However, unlike VZV where no transcripts have been detected mapping to ORF 61 (sense or antisense), SVV ORF 61 antisense transcripts are abundantly detected during latency (Ou *et al*, 2007), indicating subtle differences between these highly related neurotropic alpha-herpesviruses.

An obvious question is which function of VZV ORF 63 is important in the latency process. A clue to this was recently provided by a study that reported that ORF 63 inhibits apoptosis of primary human neurons, with the implication that this gene may promote neuron survival during primary and reactivated infections, thereby driving the virus towards a latency process (Hood *et al*, 2006). Inhibition of virus-induced apoptosis in neurons is a theme seen in other alpha-herpesviruses and may be a key element in the maintenance of virus latency. In the case of herpes simplex virus type 1, apoptosis is blocked by the function of the latency-associated transcript (Perng *et al*, 2000). In the case of bovine herpesvirus type 1, a virus protein expressed during latency provides the antiapoptotic function (Shen and Jones, 2008).

Is it possible that latent VZV gene expression is more widespread that currently appreciated? To answer this question it will be necessary either to use novel technology to facilitate the search or else a very painstaking and lengthy analysis of TG for all 71 ORFs using conventional methodology. Both of these approaches are currently being used in our laboratories. It is our common experience that VZV gene microarray analysis, though excellent for studying viral gene expression during acute lytic infection of susceptible cells (Cohrs *et al*, 2003b; Kennedy *et al*, 2005), does not possess the sensitivity necessary to detect very low abundant VZV transcripts expected in human ganglia. A promising new assay for detecting gene expression is multiplex PCR (GeXPS; Beckman Coulter), which uses chimeric PCR primers containing both gene-specific and universal DNA sequences (Nagel *et al*, 2009). This technique is proving to be very promising, as it possesses the required sensitivity to detect latent viral transcripts. The parallel approach that one of us is currently using is to analyze gene expression of all 71 ORFs in latently infected ganglia by multiple individual reverse transcriptase (RT)-PCRs in a small group of human ganglia. Although the latter

approach is time-consuming and tedious, it is to be hoped that results obtained from these two approaches will be complementary and greatly extend our understanding of VZV gene expression during latency. These ongoing studies in both the authors' laboratories strongly suggest that during VZV ganglionic latency the number of expressed transcripts is not limited to the classical five latency-associated ones.

VZV proteins in latently infected human ganglia

There have been several reports of VZV protein expression in latently infected ganglia. Initially, *in situ* immunocytochemistry using monospecific, polyclonal antibody raised in rabbits against VZV gene 63 protein was used to detect the protein in the cytoplasm of neurons within latently infected human TG (Mahalingam *et al*, 1996). This finding has been confirmed in several subsequent studies; however, there remains some discussion pertaining to the frequency at which the virus protein is present during latency. Kennedy *et al* (2000) found gene 63 protein in neurons in a minority of ganglia tested, although a subsequent study, also using immunocytochemistry (Grinfeld and Kennedy, 2004), detected gene 63 protein in a higher percentage of neurons as well as evidence for proteins encoded by VZV genes 21, 29, and 62; however, the patchy staining pattern prevented accurate quantitation. These studies contrast with the much higher percentages of immunostaining for VZV proteins 4, 21, 29, 62, and 63 in the cytoplasm of neuronal and non-neuronal cells reported in TG by Lungu *et al* (1998). Some clarification of the gene 63 protein issue has recently been provided in a rigorous analysis by Zerboni *et al* (2010). In their study, VZV gene 63 protein was detected in <2.8% of TG neurons, a frequency much lower than previously reported. Additionally, the protein was detected in only one ganglion. The authors suggest that the presence of VZV gene 63 protein during latency is a rare event not necessarily associated with latency but may indicate early stages of VZV reactivation. Although all studies thus agree that VZV gene 63–encoded protein is detectable in latently infected TG, further studies will be required to define this issue in more detail, and it is likely that discrepancies in the percentages of cells staining positive reflect in large part methodological differences. Further, a problem with all such studies is that the ganglia are usually not collected until days after the individual has died. Therefore it is possible that the conclusions of such studies are clouded by the fact that in certain circumstances VZV protein expression may be the result, at least in part, of viral reactivation associated with death.

Recent novel observations during VZV ganglionic latency

Three other aspects of VZV latency have been addressed recently. First, there is now evidence for epigenetic regulation during latency. Gary *et al* (2006) showed that the promoters of VZV ORFs 62 and 63 are associated with the histone protein H3K9 (Ac) and thus maintained in an euchromatic state during ganglionic latency. Overall, their data indicated that the expression of the two latency-associated VZV genes, ORFs 62 and 63, is regulated epigenetically through chromatin structure. Second, the question of microRNA (miRNA) expression during herpes virus latency has now been addressed. When human TG latently infected with VZV and HSV-1 were studied by Umbach *et al* (2009), it was found that five, mostly LAT-derived, HSV-1 miRNAs were detected, including two novel miRNAs, miR-H7 and miR-H8, in the ganglia. By contrast, no VZV miRNAs were detected in the ganglia despite the presence of VZV genomic DNA. These findings presumably reflect underlying differences between the two types of viral latency. Third, it is now recognized that VZV latency may be influenced by the host immune response. Thus, Theil *et al* (2003b) reported the presence of a chronic inflammatory response in TG latently infected with HSV-1 and VZV. The immune cell infiltration in ganglia correlated with the presence of both the LAT and sometimes with VZV protein, and was thought likely to maintain viral latency and influence viral reactivation. Subsequently, Hufner *et al* (2006) reported that such an immune response occurs in latently infected human TG but not DRG, despite the fact that the VZV protein 62 was present in both types of ganglia. The authors suggested that T-cell infiltration is associated with antigen recognition during viral latency in the TG but not the DRG.

Conclusions

Despite increasing understanding of several issues in VZV ganglionic latency over the last decade, there still remain many aspects that require clarification and questions that need to be answered. One of the key questions is the precise role VZV gene 63–encoded protein plays in the latency process. Although it is established that expression of this gene is a hallmark of VZV latency, we still do not know which of its proposed gene functions are specifically latency related. Another issue is whether VZV gene expression during latency is truly restricted to just five virus genes. If it proves not to be the case, as we suspect, this would indicate a potential scenario of simultaneous latency and low-level viral reactivation. The extent and significance

of epigenetic regulation of latency-associated VZV gene transcription also remains to be determined. We also don't know whether VZV latency is restricted to particular neuronal subpopulations in TG and DRG, a possibility that seems quite plausible in view of the anatomical and pharmacological heterogeneity of ganglionic neurons. Further studies of the immune response associated with VZV latency are also important for our understanding of primary neuronal infection, the establishment of latency, and the steps resulting in virus reactivation. It is to be hoped that answers to these questions no doubt associated with technical advances will yield significant therapeutic targets to prevent and/or mitigate disease associated with VZV reactivation.

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