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LATENCY OF VARICELLA ZOSTER VIRUS; A PERSISTENTLY PERPLEXING STATE

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1. ABSTRACT

Varicella zoster virus (VZV) is the herpesvirus which causes the childhood disease varicella, also known as chickenpox, and the adult disease herpes zoster, also known as shingles. These distinct diseases are separated by a lengthy period of latency, often lasting decades, in which the virus resides within the ganglia of the host. VZV latency and reactivation from it have, for the most part, been extraordinarily difficult to examine. This is due to the lack of a good animal model for the VZV latent state, the inability to experimentally reactivate VZV under any circumstances and the caveats and problems encountered in examining human ganglionic tissue. However, insights into features of the molecular events of VZV latency have been gleaned from its pathogenesis and from recent advances in molecular probing of human and animal ganglia. Evidence suggests that the latent VZV genome may express transcripts unlike those of closely related herpesviruses, and some evidence suggests an unusual site for the establishment of VZV latency. In this review, the current evidence for events occurring during the VZV latent state will be discussed, from a view of its pathogenesis as well as its molecular biology.

2. INTRODUCTION

VZV is a ubiquitous human herpesvirus which infects the majority of the worldwide population in epidemics, causing chickenpox. In the majority of healthy individuals, full recovery occurs with resulting lifelong protection against further varicella disease. However, VZV is never eradicated from the host but remains in the host ganglia in a dormant state that may last for decades. Re-emergence from latency occurs in approximately one fifth of the population, usually during their elderly years or when their immune status is compromised, resulting in the painful and debilitating disease known as herpes zoster, or shingles. Chickenpox can be contracted by the non-immune child from herpes zoster patients, and thus VZV is passed on over large generational gaps. The latent state is, therefore, key to the self-perpetuation of the virus in a strictly human host. It is also a major block to treatment and eradication of the virus; The latent state is currently completely refractile to antiviral therapy, and any eradication strategy needs to take into account the possible reactivation of VZV many decades after the initial infection.

This review summarizes our current understanding of events that may contribute to establishment and maintenance of VZV latency and reactivation from it. Much of the evidence is controversial, and it has become clear that this phase of pathogenesis is quite difficult to study. Two predominant factors that contribute to the difficulties in analyzing the VZV latent state are: 1) the poor growth of VZV in culture, which has made study of the virology and biology of the virus difficult; and 2) the lack of an animal model of VZV latency where reactivation can be achieved. However, advances in sensitive detection methods and reagent availability over the last few years have enabled some tempting evidence to be reported on the events that underlie the VZV latent state. This review will expand on previous reviews dealing with this controversial subject (1-3). For general aspects of VZV biology and disease, the reader is referred to several excellent and recent reviews (4-6).

3. VZV AS A HERPESVIRUS

VZV is a herpesvirus, classified in the suborder alphaherpesviridae. This suborder also contains the human herpes simplex virus types 1 and 2 (HSV-1, HSV-2), which are members of the genus simplexvirus, and the animal herpesviruses pseudorabiesvirus (PRV; also known as suid herpesvirus); equine herpesvirus types 1 and 4 (EHV-1, EHV-4) and simian varicella virus (SiVV; cercopithecine herpesvirus 9), which have been grouped with VZV into the genus varicellovirus (7). VZV is often compared to better studied and closely related herpesviruses for insights into its molecular biology, particularly HSV-1, but evolutionary analyses suggest a much more closer relationship of VZV with the three animal herpesviruses (8, 9). VZV contains one of the smallest genomes of the herpesvirus family, with only 125 kilobase pairs (Kbp) of potential coding sequence (10, 11). It contains at least 68 functional genes based upon the presence of open reading frames (ORFs), which are designated as ORF numbers starting with the most leftward end of the genome. Most VZV proteins show a degree of amino acid and/or functional conservation to the corresponding proteins of other alphaherpesviruses, indicating functional limitation on diversity as the alphaherpesviruses have evolved from a presumed common ancestor (9). The gene order of VZV is typical of the alphaherpeviruses (with some exceptions), being arranged in the same order, position and relative direction as the genes in PRV, HSV-1 and EHV-1 (9). Only four VZV genes have no counterpart in HSV-1, and VZV lacks several proteins found in HSV-1, particularly regarding the short regions of the respective genomes. One of the most intriguing is the lack of a homolog to glycoprotein gD, which is essential for HSV-1 infectivity and has homologs in PRV, EHV-1 and EHV-4.

Alphaherpesviruses as a group have a wide but variable host range with short reproductive cycles that cause rapid cytopathic effect and cell lysis (12). VZV growth in culture is restricted to a few human and primate cell lines and primary guinea pig embryo fibroblast cultures. Furthermore, VZV remains highly cell-associated in all tissue culture cells and is very difficult to obtain as stable cell-free virus stocks, for reasons that remain unclear. This has not allowed any experimentation involving single step high multiplicity infections, and has resulted in the biological exploration of VZV gene function lagging behind that of other human herpesviruses. Nevertheless, the realization that VZV genes are to some extent functionally conserved with other herpesviruses has provided a strong basis for the analysis of VZV gene function.

To set the stage for VZV, it is important to consider some of the features of latency in other alphaherpesviruses. Pathologically, alphaherpesviruses establish widespread infections of the skin and mucous membranes that may also extend to viscera and systemic organs (12). Some alphaherpesviruses have been shown to establish productive infections in cells of lymphocyte origin, including VZV (13, 14), enabling systemic transport to multiple peripheral sites. Alphaherpesviruses as a group have an affinity for the sensory ganglia of the host and all are able to establish latent infections within the ganglia, although some reports suggest that latency is established in additional non-neuronal sites for some alphaherpesviruses (15-17). Despite these pathological similarities and the consistent functional genomic organization across the alphaherpesviruses, the latent states of the members of the suborder show both common trends and surprisingly diverse features. While alphaherpesviruses establish latency in the ganglia, it appears that the triggers for reactivation are different; latent HSV-1, as well as PRV and EHV-1, can be readily reactivated from ganglia from the natural host and from animal models, whereas VZV has never been reactivated experimentally. Regarding transcription during latency, latent HSV-1 genomes express transcripts partly antisense to the regulatory protein ICP0, called latency associated transcripts (LATs; for a review on HSV-1 latency see references 18, 19). The most stable LATs are located in neuronal nuclei of latently infected ganglia and appear to be introns of a larger transcript. While it appears that LATs have some influence on reactivation efficiency, particularly in the rabbit model (20), they are not absolutely required for the establishment, maintenance and reactivation from latency in mouse or rabbit animal models. There is no encoded protein found to date associated with the HSV-1 LATs and the mechanisms by which they contribute to the HSV-1 latent state remains obscure. The animal herpesvirus PRV also expresses a latency transcript in neuronal nuclei (21-23) but this is larger, may be polyadenylated and overlaps the major immediate early protein. EHV-1 expresses latency associated transcripts in neuronal nuclei from a region of its genome corresponding to HSV-1 LAT (24) and also has been reported to express a latency associated transcript antisense to the major immediate early protein (15). In contrast to the lack of protein associated with HSV-1 LAT, bovine herpesvirus type 1 latency associated transcripts have been reported to encode a protein which inhibits cell cycle progression by interacting with cyclin A (25). From these examples, a common theme is that transcripts are found in neuronal nuclei of ganglia and are antisense to regulatory proteins. Differences include the size and genomic origins of the transcripts and the ability of such transcripts to encode proteins, suggesting that novel features governing latency have been acquired by each alphaherpesvirus as it has evolved.

4. PATHOGENESIS OF VZV LATENCY

4.1. Primary disease - varicella

A primary infection with VZV causes varicella, or chickenpox, a disease largely acquired during childhood in westernized societies (for a extensive review of clinical disease, see references 4, 26, 27). Acquisition occurs largely through the respiratory route via droplet spread and occurs in epidemics, particularly in schools. Grose (28) has proposed that VZV follows a model of infection which is shared for most exanthemous viruses. Specifically, an initial infection results in virus growth in the regional lymph nodes which then feeds a primary viremia within 24 hours of contact. Subsequent factory sites of viral replication are established in organs such as the spleen and

the liver, and a secondary lymphocyte mediated viremia delivers virus systemically to the cutaneous epithelial cells, where replication causes deep necrotic lesions of the epidermis and dermis. During varicella, a strong humoral and cellular immunity develops which clears all actively replicating and antigen-presenting VZV infected cells from the bloodstream, skin and ganglia. Latently infected cells are not recognized by the immune system either because of the absence of cellular antigens required for immune recognition, or alternatively because of the total absence of viral antigen. Immunity is protective against subsequent disease following further household contact with VZV and second bouts of varicella are rare. However, evidence suggests that immunity may not necessarily prevent reinfection (29, 30)

There seems little doubt that VZV latency is established in the sensory nerve ganglia of the host, as VZV DNA can readily be detected in human autopsy ganglionic tissue long after primary infection (31-37). Virus likely reaches the ganglia through the peripheral nerve and through hematogenous routes. Evidence suggesting entry at the peripheral route stems for the fact that reactivation of the live attenuated varicella vaccine occurs at the site of inoculation (38) and that most zoster cases are located largely at the predominant sites of varicella lesions (39). However, extensive infection of ganglia in fatal varicella cases suggests that infection of ganglia from peripheral blood mononuclear cells (PBMC) carrying VZV probably occurs (40, 41). It is not known how viral transport of VZV to the ganglionic nucleus occurs, but it may be similar to HSV-1 where transport is achieved through axonal flow within the neuron (42, 43).

4.2. Reactivated disease - Zoster

Reactivation of latent VZV leads to zoster, also known as shingles, and occurs in approximately 20% of VZV seropositive subjects (for review of clinical zoster and its complications, see references 3, 44-46). Zoster presents as a deep vascularized skin rash restricted to a regional area (dermatome) which is infiltrated by a single nerve group usually located on the trunk or the head. On occasion, particularly in circumstances of immune suppression, zoster can become systemic. Zoster incidence rises with age and has become more important in our society as the retired population and life expectancy has increased. An immune compromise status is also a major contributor to increased zoster incidence, whether it be due to cancer, aggressive cancer treatment, infectious disease or more extensive use of transplantation. For example, zoster is a leading presenting sign of the declining immunity associated with human immune deficiency virus (HIV) infection.

Three features of zoster hint at unusual mechanisms of VZV latency and reactivation. First, the restricted geography of zoster, which is scattered in grape - like clusters but limited to a single dermatome, suggests that the peripheral lesions originate from multiple nerve endings rather than from one or a few focal sites of peripheral infection. This implicates an extensive neuron to neuron spread of VZV at the site of latency upon reactivation, most likely due to active replication in the ganglia. Subjects examined who have had active zoster upon death contain dorsal root ganglia with extensive signs of VZV infection in both neuronal and non-neuronal cells (33, 41, 47). For comparison, reactivated HSV-1 lesions tend to be small and focal, suggesting that few neurons become involved. This difference was originally proposed by Croen *et al* and Meier *et al* (2, 41, 48) to substantiate a model in which reactivation from VZV latency requires considerable ganglionic replication and spread from satellite cells to gain access to multiple neurons (see below). In contrast, reactivation of latent HSV-1 in the latent neuron is all that is required for peripheral access.

A second unusual feature is the chronic pain that often complicates clinical zoster. Post herpetic neuralgia (PHN) is the most common complication of zoster and may last for weeks, months or even years following resolution of clinical disease (for review of PHN see references 3, 49). There are also reports of pre-herpetic neuralgia prior to vesicular eruption, which may reflect inflammation and ganglionic replication prior to peripheral disease (50). PHN is likely a result of necrosis and inflammatory responses to demyelination and cell damage during ganglionic replication. For comparison, such long term pain is not usually experienced with reactivated HSV-1 disease.

Finally, the frequency of zoster is unusual in that some 80% of VZV seropositive individuals never show clinical evidence of reactivation. This suggests that either entry into latency or reactivation from it are highly inefficient processes or, alternatively, that reactivations of VZV are more frequent but are well controlled by the immune surveillance mechanisms prior to the development of peripheral disease. With HSV-1 infection, reactivation can occur multiple times in the presence of an active and fully functional immunity, and asymptomatic reactivation often occurs. Furthermore, while HSV-1 incidence decreases with age, zoster incidence increases dramatically, suggesting triggers for reactivation are quite different for these two viruses.

4.3. Reactivation from latency-the role of the host immune response

Studies have clearly indicated a critical role for the cellular arm of the immune response in the control of VZV primary and reactivated disease. The generation of active immunity to VZV correlates to recovery from it, and the increased severity of varicella in the immune compromised clearly indicates an essential role in limiting primary disease (reviewed in reference 51). The increased incidence following induced or acquired immune compromise also suggests that there may be a considerably higher incidence of VZV reactivation in the immune healthy individual which is effectively controlled by immune surveillance mechanisms but is not checked in the immune compromised patient. Experimental data supporting frequent reactivation of VZV is scant, largely due to technical aspects of study design and the means to document silent reactivations. However, evidence of subclinical VZV viremia has been demonstrated by PCR in

peripheral blood lymphocytes of a high fraction of bone marrow transplant recipients (52), and some evidence of transient asymptomatic viremia in elderly patients has been demonstrated (33, 52-55). It may, therefore, be possible that VZV is like other herpesviruses in that frequent and periodic asymptomatic reactivation occurs.

An attractive model, proposed by Hope Simpson (56) suggested that a continual boosting of the immune response occurs on a frequent basis. He concluded that boosting of immunity stemmed either from external exposure to wild-type VZV and/or from well-contained and frequent subclinical reactivations within the host. Clinical zoster was proposed to occur when protective immunity falls below the critical level needed to contain disease. Evidence has suggested that exogenous subclinical infections occur following wild-type virus exposure post varicella, as T cell responses in VZV-immune adults are boosted upon exposure to children with chickenpox (30), and nasal washings of immune patients can be shown to contain VZV by PCR following contact with wild-type varicella (29). While the Hope Simpson model has not been experimentally proven, it does fit some of the current epidemiological patterns of zoster and its relationship to the level of VZV specific cellular immunity. This model also has implications for prevention strategies; for example, it would suggest that the extended use of the live attenuated varicella vaccine for the elderly to increase immunity to a protective level against zoster. It would also suggest that a live VZV vaccine unable to establish and reactivate from latency, or a subunit VZV vaccine, may not generate long term protection because the immune response would not receive internal boosting from subclinical reactivations.

4.4. Latency of the live attenuated varicella vaccine

A live attenuated varicella vaccine (LAVV) is now licensed in the United States and many parts of Asia and Europe (for a recent consideration of the VZV vaccine, see references 57, 58). LAVV was originally developed in Japan by M. Takahashi and co-workers, using semipermissive tissue culture passage to attenuate the virus. Healthy recipients of the vaccine show only mild and occasional side effects and most show no adverse clinical reactions. Recipients of the vaccine worldwide now number in the hundreds of thousands, and it is currently recommended by most pediatricians. LAVV has proven quite effective, protecting 85-95% of childhood vaccinees from acute clinical varicella. However, it is not a vaccine without problems and concerns. Evidence has shown that LAVV can enter latency and reactivate to cause clinical zoster (38, 59). In addition, there is evidence of superinfection by and reactivation of wild type VZV strains following vaccination (60). LAVV has been suggested to stimulate a much weaker immune response than a wild type infection, and there is a concern of immunity waning in vaccinees as they age. Despite these concerns, long-term follow up studies to date have suggested a considerably lower rate of reactivated disease in LAVV recipients. Future improved vaccines that are unable to reactivate from latency might be argued to be a desirable direction of future VZV vaccine research. However, as argued above, the Hope Simpson model suggests that a reactivating vaccine may not necessarily be an undesirable trait, as it may offer protection in the later years of life through subclinical reactivation. The long-term effect on the incidence of zoster will take years to establish. Trials of the vaccine in adults and elderly patients may establish the importance of the boosting the immune response in the prevention of reactivated disease (5, 61-63).

5. ANIMAL MODELS OF VZV LATENCY

The predominant obstacle to a detailed analysis of the VZV latent state has been the lack of an animal model of the human diseases caused by VZV, and the inability to experimentally reactivate latent VZV by any means. VZV is quite restricted in its host range and no animal model mimics the two phases of human disease. While some VZV infected animals contain VZV DNA in their ganglia long after infection, failure to allow reactivation of VZV implies that such models must be viewed with caution. It is possible that the apparent establishment of latency in such animals may be a type of an abortive acute infection that is limited by host cell type-specific factors required to complete the VZV productive cycle.

The most established animal model for VZV in which latency may occur following infection is the guinea pig. Inoculation with VZV that has been adapted in guinea pig primary cell culture leads to a transient short-lived viremia lasting 3-6 days, during which time animals shed virus nasally and transmit virus horizontally (64-66). Seroconversion occurs reproducibly, and Arvin and colleagues have exploited this phenomenon to study VZV antigens involved in generating protective immunity (67, 68). VZV DNA can be found in some guinea pig ganglia long after inoculation (64, 67, 69). However, the guinea pig is not an easy model: adaption to infection requires isolation of primary guinea pig embryo cultures for virus adaption, infection does not occur in all animals and latency establishment rates seem to be highly variable. No skin lesions or clinical signs are seen in adult animals, although a very short term exanthematous rash can be observed in some VZV infected hairless weanling animals (66, 70). VZV has been shown to cause some ocular pathology and recent evidence shows it establishes a persistent infection following ocular inoculation, leading to a chronic uveitis (71, 72).

Other immunocompetent models that have been used to examine VZV latency and pathogenesis include the common marmoset (73), the rat (74-76), the mouse (77) and the rabbit (78). The common marmoset appears to be similar in many aspects to the guinea pig, in that infection leads to a short-lived viremia, shedding of virus and seroconversion. However, there are few clinical signs of pathology or disease. The ocular model in the rabbit eye generated considerable interest at one time as a potential model for herpes zoster ophthalmicus, but the model appears to have many highly variable parameters that have resulted in poor acceptance. The suggested establishment of latency in the adult rat (74-76), appears to occur without viral growth in peripheral tissue, drawing the caution

regarding abortive types of infection. A similar scenario might exist for the mouse, which is considered to be highly refractile to VZV infection. Ocular infection with VZV resulted in the expression of VZV RNA transcripts in a few neuronal and non-neuronal cells within the ganglia, but VZV is also found in other tissues long after infection (77). Recent developments in small animal models have focused upon the SCID-Hu mouse model, where infection is obtained in vascularized human tissue present in immunodeficient mice (79, 80). This animal model is likely to be very useful to test antivirals and identify factors affecting growth in specific cell types that can be implanted in SCID mice, such as T cells and skin. However, it has yet to be shown to be suitable for an examination of latency, as neural tissue implants are not yet available.

The closely related simian varicella virus (SiVV) of primates might be a model to which human VZV latency can be compared. SiVV shows many similarities to VZV in pathogenesis of disease and at the level of antigenic similarity, genome organization and DNA sequence (81-86). Antigenically, the two viruses share numerous common antigens, and VZV specific antibodies protect monkeys from SiVV challenge (83). Clinical SiVV primary disease appears more severe than human varicella, and symptomatic reactivated disease is rare. Initial studies have indicated that SiVV latency is established within the dorsal root ganglia of SiVV infected African green monkeys and limited transcription occurs (87, 88). It also seems likely that subclinical reactivation of SiVV occurs, as there are occasional outbreaks and epidemics of primary disease within isolated healthy monkey colonies which can only have come from reactivated virus from newly introduced animals. The molecular biology of SiVV is now rapidly developing because of the model's potential for the VZV latent state (81).

6. MOLECULAR ASPECTS OF VZV LATENCY

Because of the lack of an animal model, most VZV latency studies to date have relied upon the analysis of human cadaver ganglionic tissue, opening up a proverbial Pandora's box of problems and caveats. Human cadaver neuronal tissue undergoes rapid physical and physiological changes *post mortem*, and a critical issue in studies of human ganglia has been to minimize the time between death and specimen acquisition. The concern in such studies is that the changes in conditions may result in a partial reactivation of latent virus, even when there is no infectious virus obtained by co-cultivation. Thus, it could be argued that any gene transcription observed may be the result of a mortality- induced abortive infection. A second problem in examining human tissue samples for latent VZV is that, for the most part, extraordinary sensitive methods appear to be required to detect VZV in such tissues. Such sensitive methods are often themselves susceptible to misinterpretation and contamination, particularly where PCR-based methods are employed. There are often problems encountered with immunohistochemical staining procedures of neuronal tissues, which often give high backgrounds with certain antibodies and immunological reagents, leading to incorrect interpretation. Sampling also becomes an important issue if the frequency by which VZV establishes latency is very low or is preferential for particular ganglia. The higher incidence of zoster on the head and on the trunk point to the trigeminal and thoracic ganglia as being statistically the most likely sites of VZV latency, and it is these tissues which have been most frequently studied (41). VZV DNA can be detected in other sites such as the geniculate ganglia (31). However, it is clear that many ganglia do not yield detectable VZV signals, and latent VZV may be restricted to specific ganglia. As each ganglion may generate 20-100 sections or more, it becomes technically difficult if not impossible to completely analyze latent VZV in one individual.

Notwithstanding such limitations, studies of human autopsy material have yielded some interesting findings regarding the molecular biology of the VZV latent state. Current major issues which are most often addressed are the frequency with which VZV establishes latency, the tissue/cell type which may harbor latent VZV genomes, and the gene origin of RNA /protein transcripts expressed during latency.

6.1. Establishment of VZV latency frequency

The approaches used to study viral load within ganglia have included quantitative PCR amplification techniques, quantitative Southern blot analyses of complete ganglionic DNA, and extrapolation of positive signals from sections of complete ganglia analyzed by *in situ* methods. Complete ganglionic PCR enables section sampling errors to be reduced for possible geographic concentrations of positive signals, but yields little information on the specific cell types in which signals are distributed. There is one report of detection of VZV DNA in ganglia by Southern blotting methods (89), and some groups have reported failure (2, 41). The success of *in situ* methods is highly variable, and is subject to variations from local geographical concentrations of signal in specific ganglia and sub-ganglionic areas.

While earlier studies indicated up to 1 genome per cell in ganglia using quantitative Southern blot analysis (89), several studies suggested a far lower genomic load. A PCR amplification study of VZV ganglionic DNA by Mahalingham *et al* (34) reported positive VZV PCR signals from 13 of 15 subjects for trigeminal ganglia, and 9 of 17 subjects for thoracic ganglia, using PCR probes to two different regions of the viral genome. Using quantitative competitive PCR techniques, the reported frequency was very low, with only 6 to 31 copies of the genome being present in 100,000 ganglionic cell equivalents, or a rate of 0.006-0.031% (37). This level is 166 to 322 fold lower than a similar studies reported for HSV-1 (90). Estimations from *in situ* frequency by Croen *et al* (41) showed positive VZV signals in 0.01-0.15% of cells in ganglionic sections from 15 of 30 people. This was in agreement with levels reported in a previous study in which 0-0.3% of neurons showed positive signals by *in situ* methods (32), although the cellular site of latency conflicted between these two groups. For comparison, Croen *et al* found HSV-1 signals in 0.2 - 4.3% of neuronal cells in ganglia of 14 of 24 people, with a total signal from 37 of 61 individuals

showing hybridization (41). These authors concluded that the lower frequency of latency establishment was a contributing factor to the comparatively low incidence of zoster as compared to reactivated HSV-1.

New evidence has confounded the picture and has suggested that the number of latent VZV positive cells in ganglia may be much higher in some cases. Lungu *et al* (91) described the *in situ* detection of VZV signals in 5-30% of neurons and satellite cells from ganglia from two cadavers showing no signs of VZV disease at time of death. This number appears to be extraordinarily high and is not consistent with the apparent difficulty in detecting VZV DNA by Southern blotting and PCR amplification methods. Furthermore, the inability of Lungu *et al* to detect DNA from their latently infected ganglia by PCR amplification techniques puts some shadow of doubt over the high percentage reported. Even at the lower end of the spectrum, the value of 5% is one to three orders of magnitude higher than estimated by Croen *et al* and Mahalingham et al. However, a second recent study has also suggested a higher frequency in ganglia; Kennedy *et al* (92) reported the use of indirect *in situ* PCR amplification, standard PCR and *in situ* hybridization to assess latent VZV DNA in ganglia of 17 immunocompetent individuals and 11 AIDS compromised patients with no clinical sign of zoster at time of death. By PCR, 10 of 11 AIDS patients and 5 of 17 healthy patients showed positive amplification signals in ganglionic nuclei of 2-5% of cells. Once again, it is difficult to reconcile these high values with those reported by Mahalingham *et al* and Croen *et al*, and it is not consistent with the relative difficulty in detecting DNA by Southern blotting methods. Therefore, there is an inconsistent picture based upon the current reports.

6.2. Tissue/ cell type harboring latent VZV

While there is clear evidence that VZV enters and maintains latency in the ganglia, there is some controversy regarding the cells harboring latent VZV. Until recently, identification of the cell type harboring the latent VZV genome presented somewhat of a paradox, as it relied upon the detection of latency associated RNA transcripts for amplification of signals of latent genomes. Should latent VZV genomes be transcriptionally silent, they would not have been detected in these studies. Studies on HSV-1 have suggested that latently infected cells express highly variable levels of latency associated transcripts (93, 94) indicating that detection is highly dependent upon sensitivity of the particular assays used. I*n situ* hybridization procedures are also long and complex, and are subject to many variables that can be difficult to control. *In situ* hybridization methods are also subject to some artifacts, such as the neuronal nuclear accumulation of lipofuscin staining dyes pointed out by Croen *et al* (41).

There are currently two schools of thought on the cell type harboring latent VZV. The first is consistent with other alphaherpesviruses and proposes that VZV latency is established within the neuronal nuclei of the ganglia. This was originally proposed by work from the laboratories of R. Hyman (32) and D. Gilden (33, 89), and has been recently supported using *in situ* PCR amplification methods (36, 91, 92) and protein immunohistology methods (95, 96). Neuronal signals harboring latent VZV genomes have also been found in animal models such as the rat (74, 75), guinea pig (69) and mouse (77). However, while some studies have suggested that VZV is almost exclusively over the neuronal nuclei (36, 92), others have reported signals over both neuronal nuclei and satellite cells surrounding the nuclei (77, 91).

The second school of thought was proposed by the laboratory of Straus and coworkers from *in situ* hybridization data that suggested a non-neuronal site of VZV latency (2, 41, 48). Croen *et al* (41) compared *in situ* hybridized human autopsy tissues which had been serially sectioned and probed for HSV-1 or VZV, enabling a differential comparison of the latency of these viruses in the same subject. While latent HSV-1 was found predominantly in ganglionic nuclei, probes from three separate regions of the VZV genome identified RNA transcripts located exclusively within the non-neuronal cells. Specificity was demonstrated by showing that transcription was from limited regions of the VZV genome, was RNase but not DNase sensitive, and was only found in ganglia from seropositive adults. Subsequently, Meier *et al* (48) confirmed these studies and reported limited transcription from open reading frames 29 and 62 in ganglionic satellite cells. An interesting and attractive hypothesis was put forward that correlated their findings to clinical features of zoster (2, 41, 48, 97). It was proposed that VZV reactivation from satellite cells resulted in a productive ganglionic infection of additional satellite cells as well as neurons, enabling virus to spread to multiple neurons and cause the large geographical lesions characteristic of zoster. Inflammatory and immune responses to ganglionic replication contributed to the pain characteristic of post herpetic neuralgia. This is in contrast to HSV-1 reactivation from the neuronal nucleus, which does not absolutely require spread in the ganglia to gain access to the periphery. HSV reactivated disease is not typically associated with pain as is zoster, suggesting little neuronal damage or ganglionic inflammation. It was argued that a satellite cell harboring latent VZV would likely not be subject to the same neuronal triggers that can cause reactivation of HSV, which partly explained the differences between the reactivation phenotype of these two viruses.

It is clear that this issue is not yet resolved. If latent VZV is present in neuronal nuclei, it is consistent with a conserved site of latency for other alphaherpesviruses such as HSV, PRV and EHV-1. However, it is not consistent with the signals identified by Croen *et al* and Meier *et al*, particularly in light of their serial section analysis and use of HSV-1 to confirm its site of latency. As most authors have pointed out, procedural differences may account for the apparent discrepancies. One possibility is that the satellite cell signals represent a sub-population of latently infected cells in which progression to an abortive infection has occurred. It is also possible that the *in situ* PCR methods used by Kennedy *et al* are reporting the detection of latent VZV genomes in

which there is little or no gene expression. Unfortunately, it is currently impossible to determine if one cell type or another possess the capacity to reactivate VZV from the latent state, as reactivation cannot yet be induced experimentally.

6.3. VZV gene expression during latency

Considerable effort has been made to establish exactly what transcripts or proteins are made in neuronal tissue during latency. The identity of such transcripts is required to formulate a basis for possible mechanistic models of VZV latency. It is clear that, for the most part, the transcription of the VZV genome is largely suppressed, as for other alphaherpesviruses. It is also quite possible that some latent VZV genomes express no transcripts whatsoever, and the combined data of Croen *et al* and Kennedy *et al* indirectly supports this. By all current data, it seems likely that latent VZV does not express a positional equivalent to the HSV-1 latency associated transcript. If it existed, such a transcript would be predicted to be downstream and antisense to ORF61, the VZV equivalent of ICP0, and while there is a relatively large non-coding region of DNA sequence in the genome downstream of ORF61, a transcript mapping to this region has not been reported.

The first evidence of gene transcription during latency was reported by Vafai *et al* (98) who found expression of transcripts from VZV ORF63 using *in situ* hybridization of sectioned and explanted human ganglia. This was subsequently supported by studies in the rat model of VZV latency (74, 76), where ORF63 protein was found by immunohistochemical methods in dorsal root ganglia, as well as in neurons cultured *in vitro.* Of concern regarding that latter studies is the relatively high level of ganglionic cells showing positive signals; protein expression was detected for 50-80% of the neurons, which appears to be very inconsistent with the relatively low VZV genomic load during latency. As the ORF63 protein is also a possible immediate early protein (74), there is also concern that this represents an abortive infection in neurons in rat ganglia where later VZV cycle events are inhibited by non-permissiveness of the rat cell. However, studies of human ganglia (96) with the same antibodies indicate expression of the ORF63 protein during human VZV latency, albeit at much lower frequencies than that found in the rat ganglia studies. Frequency was estimated at 2-4 neurons per section and several ganglionic samples failed to give positive signals implying specificity of the antibodies for certain neurons. Interestingly, signals were found largely within the cytoplasm of neurons, despite this protein localizing predominantly to the nucleus in lytic VZV infected cells (99). These results raised the possibility of expression of a VZV protein during latency, but should be cautiously interpreted in light of immunohistochemical staining problems which are often encountered in examination of neurological tissues.

Evidence from other groups suggests that other VZV genes are transcribed during latency. Croen *et al* (41) and Meier *et al* (48) presented impressive data to suggest mRNA expression from ORFs 62, 29 and possibly ORF 4 during latency. Croen *et al* used strand-specific probes in *in situ* methods to show transcription from restricted regions including these three genes, and subsequent studies by Meier *et al* confirmed that *in situ* hybridization signals were specific for these ORFs. Meier *et al* also reported finding mRNA for ORFs 29 and 62 in pooled ganglia from 200 subjects by Northern blot analyses. Expression from multiple regions of the genome was not detected, including the region of the genome containing ORF63. As the ORF62 and ORF29 mRNAs are polyadenylated and likely complete, it is tempting to speculate that they encode functional proteins rather than possess intrinsic or enzymatic activities associated with RNA, such as are found for ribozymes. Recently, Lungu *et al* (95) have also reported expression of these proteins in ganglia, as detected using monospecific antibodies. As both VZV proteins from these genes have been shown to possess regulatory properties, it is tempting to speculate that these proteins cooperate in a neuronal environment to give rise to repression activities that maintain latency (see below). However, Lungu *et al*. have reported that these proteins are exclusively cytoplasmic and not nuclear, as would be found in lytic VZV infected cells.

Using a completely different approach, evidence has been described that suggests expression of ORF21 in VZV latently infected ganglia. Cohrs *et al* (100, 101) first used labeled ganglionic RNA and cDNA to probe Southern blots of VZV DNA, and identified expression from a region of the VZV genome which was subsequently mapped to gene 21. Human *post mortem* ganglia harboring latent VZV DNA were used to establish PCR amplified cDNA phage libraries (101, 102), from which ORF21 cDNAs were isolated. Interestingly, these authors reported that such transcripts were 3' truncated (102). It should be noted that the approach taken used several sequential PCR amplifications and PCR based selection procedures, and such procedures are well known to be very sensitive to possible procedural contamination. In subsequent work, an ORF21 specific RT-PCR amplification approach was used to demonstrate the presence of specific ORF 21 transcripts from RNA prepared from latently infected tissue (102). Probes for a second gene, ORF 40 encoding the major capsid protein, failed to amplify specific DNA. However, it should be noted again that nested set PCR approaches were required for detection of the latency transcripts, greatly increasing the concern of possible contamination errors inherent to nested PCR methods. These studies would be greatly enhanced by the confirmation of the existence of latent RNAs through *in situ* hybridization and non-PCR mediated analyses of human tissue.

Recent extension of studies from this group have reported the isolation of cDNA clones obtained from phage libraries of latent VZV harboring tissue which included genes 21, 63, 29 and 62 (103). All cDNAs were likely derived from mRNA, based on the mapping of the 3' termini and the demonstration of polyadenylation sequences in the clones. Such libraries were constructed from PCR amplified, hybrid-selected RNA obtained from ganglionic RNA, and as RNAs for ORFs 4, 10, 40 and 51 were not found in the libraries, it was concluded that the

isolation of cDNAs for 21, 63, 29 and 62 represented latency expressed transcripts.

Lungu *et al* (95) have recently presented evidence suggesting expression of several VZV proteins within the cytoplasm of neurons of latently infected ganglia. Using specific antibodies, gene products for ORFs 29, 4, 62 and 63 were identified in a high proportion of ganglionic neurons. These results await to be confirmed by others and must be interpreted with caution, but raise the novel possibility that VZV latency is very different from most alphaherpesviruses and is mediated through a nuclear exclusion mechanism of transcriptional regulatory proteins (95). Several issues have not yet been completely addressed, such as the mechanisms by which viral proteins are specifically excluded from the nucleus of latently infected neuronal cells. Furthermore, it is not yet clear why latently infected cells expressing VZV proteins not targeted by the immune response, particularly in healthy individuals with active and protective immunity.

6.4. Can a molecular model for VZV latency be proposed?

While evidence has accumulated to suggest the expression of several genes during latency, there has been little speculation on how the observed expressed genes might contribute to maintenance of the VZV latent state. Part of the problem is that we have only just begun to understand how these proteins function in the acutely infected cell, and it is likely that such functions may be quite different in a neuronal environment. Clearly, there must be a mechanism of transcriptional repression for the majority of VZV genes during latency. Two possible models to achieve this are through a direct repression of transcription by some of the latency expressed proteins and /or cellular proteins, or secondly, through an inability of viral transactivators to activate transcription. A repression of transcription may be consistent with expression of the ORF62 protein functions during latency, since while IE62 is powerful transactivator of most, if not all VZV gene promoters (104, 105), it demonstrates autorepression activities that are cell-type specific (105, 106). It is possible that such activities may extend to the majority of the VZV genome in the neuronal cell environment. Interestingly, IE62 may cooperate with the product of ORF29, which was proposed to be expressed in latency by Meier *et al* (48), and supported by work of Cohrs *et al* (103) and Lungu *et al* (95). In the acute VZV infected cell, ORF29 encodes a protein involved in the DNA replication complex, binding to and stabilizing single-stranded DNA (107). Recent evidence suggests that ORF29 protein may cooperate with IE62 to downregulate certain promoters (D. Boucaud, W. T. Ruyechan, and J. Hay, personal communication). An alternative strategy of latency, proposed by Lungu and coworkers (95) is that nuclear exclusion mechanisms prevent VZV regulatory proteins from acting as transcriptional activators. However, as it is largely considered that nuclear transport is by and large dependent upon the cellular localization machinery, such a model would require specific mechanisms to inhibit VZV proteins. While such a specific mechanism has been found for IE62, possibly dependent upon specific phosphorylation events (P. R. Kinchington and S.E. Turse, Manuscript in press), it does not extend to other VZV proteins.

There is not yet a model that fits the reported expression of ORFs 63 and 21. ORF63, proposed to be expressed during latency by four groups (74, 95, 96, 98) is transcribed on the opposite direction of ORF 62 and on the other side of the lytic origin of replication (10). ORF 63 protein has been suggested to affect transcription both positively and negatively in transfection studies (108), although recent evidence has not supported these earlier findings (109). Little else is known of the function of the VZV ORF63 protein. However, its HSV-1 homolog ICP22 has been shown to interact with transcription complexes and phosphorylate RNA polymerase (110, 111). It is possible that the VZV ORF63 protein may interact with RNA polymerase and ganglion cell-specific transcription complexes to inhibit VZV transcription. Regarding ORF21, very little is known about its functions. In acutely infected cells the protein is present in both the cytoplasm and the nucleus, and appears to be tightly associated with the nucleocapsid (112). Its HSV-1 homolog, UL37, is expressed as a late gene, and encodes a bifunctional protein that co-operates with the DNA binding protein from ICP8 on single-stranded DNA affinity columns (84). It also has an essential role in the structural integrity of infectious virus particles (113; P. Desai and S. Person, personal communication).

7. PERSPECTIVE

The ability of the herpesviruses to enter into the latent state is one of the most intriguing aspects of all viral pathogenesis, and yet has proved to be one of the most difficult to investigate and comprehend. The mechanisms underlying the establishment, maintenance and reactivation from latency remains far from resolution, not only for VZV, but for virtually all herpesviruses. However, for many herpesviruses, there are good animal models of entry, maintenance and reactivation from latency which closely resemble the human state, and these provide the means to dissect molecular events underlying this poorly understood phase of herpesvirus pathogenesis. In particular, the several models for the HSV-1 latent state should enable, through hard work and bright insight, a clearer picture to be established for the mechanisms underlying the HSV latent state.

This is currently not so for VZV. The lack of an animal or culture model in which reactivation from latency can be achieved is a huge obstacle confounding the analysis of the VZV latent state. Without the ability to reactivate latent VZV experimentally, the factors influencing and leading to reactivation *in vivo* cannot be determined. This also makes it difficult to determine whether current animal models of latency reflect a true latent state or an abortive type of infection restricted by the limited host range of VZV. In order to resolve many of the latency issues for VZV within the near future, the development of a reactivating animal model will be of enormous importance. It seems likely that the immune status is a critical determinant for reactivation in humans, and fruitful studies

may result from studies that focus upon immunosuppression in animal models, coupled with highly sensitive detection assays for subclinical reactions. In addition, studies of the SiVV in lower primates may give us clues and directions to pursue the VZV latent state. Several advances have been made towards understanding the molecular biology of SiVV, and knowledge is now to the point where its latent state can be studied in detail.

Given the limitations of current animal models, the probing of the VZV latent state will likely continue to rely heavily upon the analysis of human ganglionic tissue. Fortunately, methodologies are continually improving in sensitivity and specificity to the point where rare cells harboring latent VZV can be studied more easily and in more detail, so several issues should become better resolved within the near future. One of the most important to address is the VZV genomic load in human ganglia, which currently varies over three orders of magnitude. This can now be better addressed using improved quantitative PCR methodologies. In particular, the development of real time, quantitative PCR methods should enable a more accurate estimation of the levels of VZV in human ganglia. This author considers it important that the genomic load should be used as the groundwork on which *in situ* studies should be based. For example, by correlating genomic load per ganglion with *in situ* PCR hybridization, one should be able to determine the copies of the VZV genome per latently infected cell. Studies have suggested latent VZV DNA genomes are endless, either as concatemers or as circular molecules (114). While genomic load of VZV per ganglia may be much higher than positive *in situ* signals (e.g. due to multiple VZV genomes per latently infected cell), it simply does not make sense that a significant number of ganglionic cells express VZV proteins in the absence of any detectable viral DNA.

Regarding the cellular site of VZV latency, resolution will likely require improved *in situ* studies aimed at detecting viral DNA rather than latently expressed transcripts. It seems likely that this, too, will also rely heavily upon PCR methodologies and the application *in situ* to ganglionic tissue. By specifically detecting DNA and RNA separately, we may be able to resolve the basis for the conflicting observations of Croen *et al* and Meier *et al,* where latent transcripts were reported in satellite cells, with those of Kennedy *et al*. where latent genomes were detected predominantly in neuronal nuclei. As discussed in section 6.2, the caveat in examining RNA transcripts as signals for latency is that latent VZV genomes which do not express transcripts are not detected. Studies of HSV-1 latent genomes have strongly suggested that the expression of the HSV-1 LAT is highly variable, and it is quite possible that some latent VZV genomes are transcriptionally silent.

One of the most surprising features of VZV latency concerns the pattern of gene expression, which by all appearances is very different from all other alphaherpesviruses studied to date. Current data suggests that as many as five VZV genes are expressed during the latent state, some of which may be translated into proteins. The proteins expressed from these genes fall into two groups, those that may bind DNA (ORFs 62, 29 and a predicted DNA binding activity for ORF21 based on its HSV-1 homolog) and those that are involved in gene regulation (ORF62, 63, 4 and possibly 29). Clearly, these proteins will be under considerable scrutiny in the near future to identify their functions and their interactions. It may be much more difficult to address and identify functional interactions that are neuron- or ganglion-cell specific, but these will need to be considered in order to formulate the mechanisms by which these proteins act during latency. Furthermore, while it will be straightforward to determine how these genes are regulated in the acutely VZV infected cell; it will be much harder to address the neuronal-specific elements in their promoters which enable expression during latency. Last, considering that the alphaherpesviruses do show an overall similar affinity for neural tissue and ganglia as a site of latency, this author finds it perplexing that VZV has evolved such a unique pattern of gene expression during its latent state that differs from other viruses. Perhaps there are common mechanisms for all the neurotrophic herpesviruses which have yet to be identified.

All in all, the VZV latency will remains a perplexing state for many years to come.

8. ACKNOWLEDGMENTS

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