Molecular Studies in Varicella–Zoster Virus Ganglionic Latency

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Abstract

In this essay, which is to be read in conjunction with papers [1–9] included as the Appendix to this thesis, I aim to present the necessary background on the biology of Varicella–Zoster Virus (VZV), the clinical manifestations of VZV, and an up-to-date picture of the available knowledge on VZV latency. I situate the work done in Prof. P. G. E. Kennedy's laboratory in the current literature and describe my contribution to that work. In the last chapter I put forward a model of latency that is consistent with many experimental findings and suggest some possible directions for future research.

Declaration. I declare that the work presented in Chapter 5 has been carried out by me under the supervision of Prof. P. G. E. Kennedy and has not been presented elsewhere as a part of Ph. D. submission.

Signature: _____ (Esther Grinfeld) Date: 25/04/05

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Contents

Summary

In this thesis essay, which is to be read in conjunction with papers [1-9] contained in the Appendix, I describe work conducted in Prof. P.G.E. Kennedy's laboratory over the last eight years on the topic of Varicella–zoster virus (VZV) latency in the nervous system.

In the papers that comprise the present thesis, I studied various aspects of VZV latency in human PNS, a rat model and latent Simian Varicella Virus (SVV) in the PNS of African green monkeys. In particular, I investigated questions of latent virus localisation, viral gene expression during latency, and if VZV might be involved in Giant Cell Arteritis (GCA) of humans. All these topics have given rise to controversies in the literature and the key aim of our research was to attempt to resolve them by using sophisticated and reliable molecular biological techniques.

Our first paper on the subject of VZV latency [1] in which the polymerase chain reaction (PCR), in situ hybridisation (ISH) and in situ PCR amplification (ISPCR) were used on human trigeminal ganglia (TG), resolved a 10 year old controversy in the literature concerning the type of cells harbouring latent VZV. The results provided unambiguous evidence that latent VZV resides predominantly in the nuclei of neurons, occurring in 2–5% of neurons whereas it was found in only a vanishingly small fraction (less than 0.1%) of non-neuronal satellite cells.

Our DRG studies [2] confirmed this finding and indicated that more than one region of VZV genome was present. The co-localisation of 2 regions of the VZV genome in serial sections by (ISPCR) was shown and found both DNA and RNA in serial sections of the same tissue.

Since the functions of several genes expressed in latency may be known, this may help in un-

derstanding the mechanisms of latency and could identify genes and proteins which could act as therapeutic viral targets, for example, for anti-sense therapies, RNA silencing or vaccine development. This has been the second major direction of the work. In association with J. E. Bell, a study of VZV gene expression in latently infected and explanted TG and DRG of patients using ISH to detect gene products was conducted [3]. It was indicated that expression of genes 18, 21, 29, 62, and 63 in latently infected ganglia, with the proportion of HIV-positive patients expressing the genes being consistently higher as might be anticipated. Explanted ganglia (from HIV- negative patients only) expressed in addition genes 28 and 40, the differences in expression patterns between latently infected and explanted ganglia presumably reflecting virus reactivation, thus indicating that what is referred to as 'latency' is probably latency and not low-level persistent lytic activity. The latter cannot, however, be completely excluded at the present time.

To analyse further the processes involved in latency establishment, the mechanisms controlling the activity of gene 62 product, IE62 were investigated [4]. It is known that in latency this gene product is predominantly located in the cytoplasm, which may be the result of its phosphorylation by gene 66 product, the protein kinase 66-pk. Hence it is important to identify VZV gene 66 expression during latency. The hypothesis proved to be correct in that transcripts for VZV gene 66 were indeed detected in latently infected TG by RT-dependent nested PCR and ISH, while the 66-pk itself was detected by immunohistochemistry. In addition, the presence of proteins coded for by the VZV genes 21, 29, 62 and 63 has been confirmed by immunohistochemistry in human tissues shown to contain latent VZV [5].

VZV and other viruses have been implicated as contributing factors in many diseases, and screening tissue from patients with various pathological conditions for the presence of viruses is an important step in elucidating potential pathogenetic mechanisms. The presence of VZV genome in temporal artery biopsies of patients with Giant Cell Arteritis (GCA) was investigated. Viral involvement in GCA is plausible due to the presence in inflammatory cell infiltrates of multinucleated giant cells, which often occur in viral infections, including those caused by VZV. Studies conducted previously have been contradictory. It was found, using ISH and ISPCR, that

there was no evidence of the presence of VZV in arteries of GCA patients [6]. This strongly indicates non-involvement of VZV in the development of GCA.

A parallel study on DRG in a rat model of VZV latency was conducted [7]. The predominantly neuronal location of latent VZV DNA was confirmed, an important result in animal model validation. In addition, using ISH, it was confirmed that genes 21, 62 and 63 were most frequently expressed which is similar to human latency. However, viral transcripts were found in both a large number of non-neuronal cells in contrast with the predominantly neuronal localisation in humans. These studies have been extended using mutants in order to determine the importance of specific viral genes in establishing a latent infection [8]. Using VZV gene 14 and 67 deletion mutants, viral DNA was detected and genes 21, 29, 62 and 63 were still expressed. Therefore it was concluded that gene 14 and 67 products, glycoproteins gC and gI, are not necessary for the establishment of latency. It was also confirmed that the model represents true latency as peripheral tissues did not contain VZV DNA.

The animal model studies were extended to the SVV model in African green monkeys. In these monkeys SVV causes a varicella-like illness which has many similarities to its human counterpart. In naturally infected monkeys in which a latent SVV infection had been established, we used ISH and ISPCR to show that latent SVV resides in ganglionic neurons as demonstrated by the presence of DNA for SVV genes 63 and 21 [9]. In contrast to human ganglionic latency we found that SVV DNA was localised in the neuronal cytoplasm rather than the nucleus, although the reason for this difference is as yet unclear. This primate model is a promising development in the search for alternatives to working on human tissue.

In summary, a variety of molecular techniques have been used to make a number of important contributions to the difficult issues of latent ganglionic VZV nucleic acid localisation and latent viral gene expression in humans and animal models. We have also shown that this approach can be used in assessing possible VZV involvement in human CNS disease.

Chapter 1

Introduction

Varicella Zoster Virus (VZV) is an alphaherpesvirus that causes a primary infection known as varicella (chickenpox). The virus then becomes latent in the cranial, autonomic, dorsal root (DRG) and trigeminal (TG) ganglia and, after a variable period, it can be reactivated in later life, causing herpes zoster (shingles) which is a painful vesicular eruption usually limited to the distribution of one or more dermatomes. The most important complication of zoster is post-herpetic neuralgia (PHN), but VZV infections are also associated with a wide variety of serious neurological complications such as encephalitis, myelitis and vasculitis, especially in immunosuppressed individuals [42]. It has therefore been the object of much research work in the last two decades. Since VZV infections and their complications occur at the later stages of life, demographic changes will make understanding its biology and pathology even more important in the years to come; in fact, it has been dubbed the "re-emerging infection of the early XXIst century" [61].

The longterm goal of this research is to devise more effective therapies for, VZV reactivation in later life. A pre-requisite for this is to gain a full understanding of all aspects of VZV latency. Latency may be defined as **a dormant state of the virus characterised by the absence of isolatable infectious virus, with limited transcription but one where the virus retains the capacity to be reactivated.**

The following are key issues in VZV latency:

1. What cell types harbour latent VZV?
2. How is latency established and maintained? In particular, what genes are being transcribed and what proteins expressed during latency?
3. What signals trigger reactivation?

Answering these questions, which apply equally to HSV-I, is much harder for VZV, since VZV is cell-associated and is difficult to grow to high titre in culture. In addition, VZV replicates only in human and primate cells. Moreover, there is no universally accepted animal model of VZV latency, since all the available ones, such as the Liège group rat model used in [7, 8] do not allow reactivation (though there is an in vitro model using guinea pig enteric neurons that apparently does [22]). These factors explain why our knowledge of VZV latency has lagged behind that of HSV.

Conclusively answering the questions above could have significant implications for developing novel therapeutic strategies. For example, a highly restricted gene expression pattern in VZV latency raises the possibility of using anti-sense oligonucleotides or RNA silencing to block the function of these expressed viral genes. The demonstration of specific and limited protein expression during latency might also facilitate the development of passive immunisation or non-live vaccines to block viral functions in the immunocompromised individual, and potentially reduce or prevent the development of PHN (post-herpetic neuralgia), especially in the elderly population. Such vaccines would provide an alternative to the currently live attenuated vaccines.

Below I concentrate on questions 1 and 2, since answering them has been the basis of the research programme in Prof. P. G. E. Kennedy's laboratory in the last eight years. Some remarks on question 3 are also contained in Chapter 2.

Chapter 2

VZV biology

This chapter contains many important aspects of VZV biology that are relevant to the latency studies I have performed in Professor Kennedy's laboratory.

2.1 Introduction to VZV

VZV belongs to the subfamily Alphaherpesviridae of the family Herpesviridae of double stranded DNA viruses. Herpesviruses are characterised by an icosahedral capsid of diameter of about 125 nm with 162 capsomer spikes embedded in a tegument, an amorphous proteinaceous matrix. The virion is enveloped in a lipid membrane containing external glycoproteins; the overall diameter is about 215 nm [24].

One might assume that VZV is closely related to other alphaherpesviruses, such as HSV-1. This assertion is not always warranted and attempts to predict VZV gene product functions from the functions of homologous HSV-1 proteins may be problematic. In the modern classification, VZV belongs (together with the really closely related simian varicella virus (SVV) and the Equine HVs 1 and 4) to the $\alpha 2$ group, while HSV-1 belongs to the $\alpha 1$ group. In common with gammaherpesviruses, VZV genome is partially expressed in latency. Differences between

HSV-1 and VZV are noticeable already on the gross genomic level: VZV has a simpler type D genome (see below for description) while HSV-1 has a more complex type E genome typical of $\alpha 1$ viruses [69]. The genome of HSV is larger than that of VZV (153Kb as opposed to 125Kb) and codes for more proteins (at least 74 for HSV as opposed to about 70 for VZV). The most revealing fact about these viruses is contained in Figure 17.2 B of [69], from which it becomes clear that the branching of the phylogenetic tree that gave rise to the $\alpha 1$ and $\alpha 2$ groups occurred about 70 million years ago. This means that the two families of viruses evolved their strategies of confronting the host's (evolving) immune system independently. Therefore assuming that the two viruses should have arrived at the same solutions is not necessarily warranted.

In their illuminating discussion of the evolution of herpesviruses, McGeogh and Davison suggest a core 43 genes common to all these viruses and write (the italics are mine):

> ... most of the core gene complement of herpesviruses is concerned with specifying the icosahedral capsid structure, with synthesizing and packaging progeny DNA and with cell entry mechanisms, *while genes supplying all well-characterised control functions and also those for virus tegument and surface components are specific to subfamilies or to lineages within subfamilies.*

This point will be enlarged on in Chapter 6.

2.2 VZV genome organisation

The VZV genome is a linear double-stranded DNA molecule containing about 125 Kbp. It comprises a unique long region U_L, of about 105 Kbp, flanked by inverted repeat regions, terminal repeat long region (TRL) and internal repeat (IRL) region; these are very short, about 88 bp. The rest of the genome consists of the unique short region, 5.2 Kbp long, flanked by terminal repeat short (TRS) and inverted repeat short (IRS) regions, each about 7.4 Kbp long. The orientations are independent, so the VZV genome exists in 4 isomeric forms, two of which

predominate [24].

Though sequencing shows many minor amino acid differences, due to single nucleotide polymorphisms, between isolates of VZV [24, 105] (e.g. comparisons of VZV wild strain with the Oka vaccine strain show 15 differences in ORF62 alone), only a few distinct serotypes of VZV are known, such as the VZV-MSP virus (a gE mutant). It is thought that this is a distinctive VZV population that emerged about 50 years ago [103].

The following picture of the genome arises from recent studies (reviewed in [24, 29]): there are at least 70 unique VZV genes. ORFs 62, 63 and 64 are repeated (as ORFs 71, 70 and 69, respectively), but there are in addition VZV gene 33.5, 3'-coterminal with ORF 33, and VZV gene 9A, which maps inside ORF 8. Finally, there is a gene ORF(S/L) which is spliced in the 3' non-coding region. Eleven genes are thought to have overlapping reading frames. ORF42 and ORF45 are probably spliced segments of the same gene.

There are six genes (ORFs 1, 2, 13, 32, 57 and S/L) that have no homologs in the HSV genome. There is one gene, ORF13 which in fact has no homologs in the alphaherpesvirus family at all, but has a homolog among the gammaherpesviruses such as the Kaposi sarcoma virus.

The VZV genome is extremely stable but small numbers of single nucleotide polymorphisms can be used to distinguish three main genotypes; genotype A is common to Africa, Asia and the Far East, genotype C is the most common variant in The United Kingdom, and genotypes B and C in the United States and continental Europe [18].

2.3 Viral proteins

Throughout the years, a great deal of information has been collected concerning VZV gene products. There are a number of ways of organizing this data: by stage in the viral replication cycle in which they are synthesised (that is, immediate early (IE), early (E) and late (L); see below); by homology to HSV proteins (a practice can be somewhat misleading for reasons explained above); and by function. In this essay it was chosen to group VZV proteins by

function, an approach also followed in [24]. The information contained in [24, 92] is used in this section and supplemented with newer findings. Below the standard notation ORF*X*p for the protein coded by ORF*X* is used; for immediate early proteins in this thesis the notation IE*X* is used, since this is the norm in the literature. Thus ORF62p and IE62 refer to the same protein.

2.3.1 Transactivators

This group of VZV gene products includes mainly IE proteins, which are essential for the initiation of the viral replication cycle; some of them, as explained below, are transported with the nucleocapsid into the nucleus of the target cell. This group includes ORF4p, ORF10p, ORF61p, ORF62p and ORF63p. According to [92], all of these apart from ORF10p are IE proteins, while ORF10p is a L protein with ORF62 transactivational activity. Since ORF62 and ORF71 are the same, as are ORF63 and ORF70, one might refer to ORF63p as ORF70p (or to IE63 as IE70), but again, the standard notation is used. For details of transactivation activity of these proteins, the reader should consult Table 2 of [92], noting the very complex concentration-dependent interactions among these proteins and their activation/repression activity on E and L genes. Here ORF4p, ORF62p and ORF63p are concentrated on, as these are expressed in latency.

ORF4p is a 55-kD (452 amino acids) abundant tegument protein. Its localisation is mainly cytoplasmic but is influenced by ORF62p, which can cause its transport to the nucleus. It has been shown to transactivate promoters of IE, E, and L genes (by itself and in synergy with ORF62p) [35].

ORF62p is a 170-kD (1310 amino acids) abundant tegument protein. (Its molecular weight invites a comment. It is actually about 140-kD, but the protein is subject to a significant amount of post-translational modification such as phosphorylation by cellular and viral kinases.) During the viral replication cycle, it resides in the nucleus; its localisation at latency is cytoplasmic and influenced by ORF66p, a fact which is noted in Chapter 4 and in our paper [4]. This

protein influences intracellular localisation of other IE gene products, up- and down-regulates transcription of other transactivators, interacts with them in an intricate manner and is known to interact with a number of cellular factors, in particular with Sp1 and USF. Interaction with Sp1 is implicated in the regulation of expression of gE and gI, while USF influences expression of ORF28 and ORF29 [91].

ORF63p is a 45-kD tegument protein. It is found both in the nucleus and in the cytoplasm of infected cells [36]. The only transactivational activity demonstrated for it is repression of ORF62 expression in tandem with ORF4p.

Another protein that belongs in this section is ORF29p, an early gene product, which is the major DNA binding protein, a 130-kD protein localised in the nucleus of infected cells; it binds to single-stranded DNA and is known to modulate late gene promoter activity. It is expressed in latency.

2.3.2 Enzymes

These are mostly E gene products. Quite a number of them are "luxury" gene products and are non-essential for replication and/or latency establishment. These include ORF6p, a DNA helicase component; ORF8p, a dioxyuridine triphosphatase; ORF13p, a thymidylate synthetase; ORF16p, a unit of DNA polymerase; ORF18p and ORF19p, two components of a ribonucleotide reductase; ORF28p, a DNA polymerase; ORF33p, a protease; ORF33.5 encodes the virion assembly protein processed by ORF33p; ORF36p, a thymidine kinase; ORF47p, a protein kinase, implicated in T cell tropism [10]; ORF48p, a deoxyribonuclease, very important for viral DNA replication; ORF51p, an origin-binding protein; ORF52p and ORF55p, components of a DNA helicase/primase complex; ORF59p, a uracil-DNA glycosylase, and another important protein kinase, ORF66p, which has been implicated in ORF62p phosphorylation and cellular localisation and in skin tropism.

Since ORFs 18, 19 and ORFs 52 and 53 code for subunits of enzymatic complexes that have

no independent function separately, it would make sense always to look for expression of both ORFs 18 and 19 at the same time, as it would provide a valuable quality control mechanism for molecular biology procedures.

It may be of interest to speculate why VZV would need a ribonucleotide reductase. The explanation provided in [69] is that genes encoding DNA replication as well as nucleotide generating enzymes have been captured from host genomes and proved useful in a host, such as terminally differentiated neurons, that need de novo synthesis of nucleotides.

2.3.3 Glycoproteins

There are 8 glycoproteins: ORF5 codes for gK; ORF14 codes for gC, a glycoprotein involved in skin-specific virus/cell binding; ORF31 codes for gB, of major importance in non-cell type specific virus/target cell binding; ORF37 codes for gH, important in cell binding (often in conjunction with gL) and a major fusogen, responsible for syncytium formation; ORF50 codes for gM; ORF60 codes for gL, needed for gH expression; it also serves as a gH chaperone in the endoplasmic reticulum. ORF67 codes for gI, and finally ORF68 codes for gE; these two glycoproteins combine to form the viral Fc receptor. All the genes in this subsection are late genes. A detailed description of the biochemistry of the above glycoproteins is contained in [24].

2.3.4 Other proteins

Apart from capsid proteins, such as ORF40p and ORF21p, there are a number of VZV gene products with unclear function. Only a selected few are mentioned here. The HSV homolog of ORF17 is UL41, which codes for the host shut-off protein, an activity that has not been demonstrated in VZV. Recently, in [94] the authors characterised ORF2p, a membrane phosphoprotein. Finally ORF(S/L)p is a 21-kDa protein expressed during lytic growth; this protein is apparently involved in controlling viral cell adhesion properties [29].

2.4 The VZV replication cycle

Like HSV, the replication cycle of VZV can be divided into three phases, virus adsorption and entry, viral gene transcription, and viral assembly and exit.

2.4.1 Absorption and entry

As in the case of HSV, attachment of the VZV virion to a target cell requires a concerted action of its glycoproteins. Since heparin is known to inhibit absorption, it is likely that VZV attaches to heparan sulphate proteoglycans (HSPGs). The binding of VZV to heparin is direct and it is conjectured that the gB viral glycoprotein mediates this process. It is also thought that the process of attachment proceeds in two stages, the first, described above, being cell-type independent, with the result being the stabilisation of the virion on the cell surface which enables tighter, cell-specific binding to occur. It is plausible that the gC-mediated VZV skin tropism described in [79] is realised through the second, cell-specific binding step. There are also indications of gH and gH:gL complex involvement, and of putative sites of the secondary attachment being mannose-6-phosphate receptors on the cell surface. This is supported by the recent work of Chen *et al.* [23], who found that human cell lines deficient in cation-independent mannose-6-phosphate receptor resisted infection by cell-free VZV. Sadzot-Delvaux *et al.*[92] state:

> Obviously, eight glycoproteins is much more than needed for the above-described phenomenon. The additional glycoproteins could play identical roles as described above when the virus enters various cell types such as epithelial cells, neurons, satellite cells or lymphocytes.

The therapeutic implications of these statements are discussed in Chapter 6.

2.4.2 Virus replication

The result of these events is the fusion of the envelope of the virus and the target cell membrane which allows the release of tegument proteins and the capsid into the cell. By analogy with HSV, it is possible that VZV releases into the cytoplasm a host shut down protein (such a protein in HSV is encoded by the homologue of VZV ORF17, but no host shut-down activity has been demonstrated in VZV). The nucleocapsid travels to the nucleus, locates nuclear pores and releases through them the viral genome. The nucleocapsid is accompanied to the nucleus by a number of tegument proteins needed for the transcription of immediate early (IE) genes. Among these are ORF10p, and the IE proteins encoded by ORFs 4, 62 and 63.

The IE gene products brought into the nucleus are required to transactivate the expression of IE genes; this is most certainly true about IE62. Once IE gene products have been synthesised in the target cell and transported to the nucleus (it is known, for example, that IE62 causes nuclear localisation of IE4; for more details see Table 2 of [92]), expression of early (E) genes can proceed.

These are the main enzymes connected with DNA synthesis (ORF28p, ORF16p, ORF29p, ORF51p) and the "luxury" ORF36p, which is a non-essential thymidine kinase, ORF18p and ORF19p, subunits of ribonucleotide reductase, ORF13p, a thymidylate synthetase, as well as ORF47p and ORF66p, the protein kinases. These proteins are also transported into the nucleus to participate in VZV DNA replication.

VZV DNA replication is a four-stage process, involving (i) circularisation of the linear VZV DNA; (ii) replication and isomerisation by homologous recombination between the inverted repeats; (iii) more extensive replication by the 'rolling circle' mechanism; (iv) cleavage of concatemers to generate linear viral DNA. The origin of replication is located between ORFs 62 and 63 and contains at its centre an essential 45-bp palindromic sequence $(TA)_{17}$.

In the late phase, nucleocapsid and tegument proteins (ORF10p and ORF40p) and glycoproteins are synthesised in the cytoplasm while the nucleocapsid is assembled with the viral genome

wrapped inside it, in the nucleus of the infected cell.

2.4.3 Assembly and egress

Finally, by mechanisms that are not at all clear, the whole virion is assembled and released from the cell presumably by exocytosis. There are basically three hypotheses to explain how the naked nucleocapsid in the nucleus acquires its outer coating of tegument and membrane with the right assortment of glycoproteins:

(A) Nucleocapsids acquire their envelope by budding at the inner nuclear membranes; other parts of the outer coating of the virion accrue from vesicles formed at the outer nuclear membrane or the endoplasmic reticulum. This mechanism raises more questions than it answers; for example it is not clear how the glycoproteins would find their way to the nucleocapsid.

(B) The envelopment/disenvelopment/secondary envelopment theory postulates that the nucleocapsid, enveloped in the inner nuclear membrane, sheds it in the cytoplasm, and buds into large cytoplasmic vesicles, while glycoproteins are released from the trans-Golgi network (TGN) in microvesicles that fuse with the cytoplasmic vesicles containing the nucleocapsid. In this model the fate of tegument is not clear.

(C) This is similar to the basic envelopment/disenvelopment/secondary envelopment model (B), in that naked nucleocapsids are present in the cytoplasm, but they are subsequently wrapped in TGN cisternae containing viral glycoproteins which also bind the tegument.

2.5 VZV evasion of the host defense mechanisms

The success of VZV in maintaining itself to all intents and purposes indefinitely in humans means that it has evolved a number of effective strategies that allow it to propagate itself from host to host. Some of these aim to avoid the host immune defenses. Four broad mechanisms that human viruses use are mentioned in [50], viz., (a) antigenic mimicry; (b) rapid viral mutation (a

strategy favoured by the Retroviridae and the Orthomyxoviridae); (c) low antigenicity and (d) infecting the immune cells. The examples of (d) given in [50], of members of Togaviridae and of Retroviridae, do not include herpes viruses, but the indications are that both (c) and (d) are the modus operandi favoured by VZV.

In fact, the classification of [50] seems crude, and it is preferable to discuss the following facets of VZV–host immune system interaction:

1. subversion of immune system elements;
2. neutralisation of the innate immune response;
3. attenuation of the acquired immune response by decreased antigen presentation.

It is only the last of these three that is relatively well understood; somewhat misleadingly, this is what is commonly referred to as "immune evasion". Thus, the discussion below of items 1 and 2 is based on very imperfect knowledge obtained on viruses other than VZV. It stands to reason that similar mechanisms are at play in VZV infections.

2.5.1 Immune subversion

There is ample evidence [84] that the first contact of a mucosal pathogen, be it bacterial or viral, with the host immune system, is through various sentinel cells, most importantly dendritic cells (DCs). Dendritic cells become aware of the presence of the pathogen both directly, by recognising non-self antigenic patterns, or by reacting to danger signals from other cells. Recognition of pathogen, in a process that involves toll-like receptors [104][1], leads to phagocytosis of the pathogen by the DC, maturation of the DC and its migration to lymph nodes. Here it can present antigen to naïve T cells and elicit a T cell immune response.

[1]Toll-like receptors are primary trans-membrane proteins of immune cells that serve as a key part of the innate immune system. They are a group of pattern recognition receptors that bind to pathogen-associated molecular patterns. Stimulated by these pathogens, they convey signals to the inside of the cell, activating signal transduction pathways that result in transcriptional regulation and the stimulation of the appropriate immune response [34].

Transfer of virus from infected dendritic cells to T cells has been demonstrated [11]. This suggests that the virus utilises migratory capabilities of the dendritic cells to disseminate and initiate a viraemia. Additionally, VZV has been shown to infect DCs that are already matured and to modify their immune functions [81]. This is discussed later.

2.5.2 Neutralisation of the innate immune response

A relatively recent development is the realisation that many viruses [63, 82] can neutralise the complement system by expressing regulators of complement activation (RCA) proteins on the surface of infected cells. In the context of Herpesviridae, this has been documented for both HSV-1 and HSV-2 α-herpesviruses, which use the gC glycoprotein to bind and inactivate the C3 component of the complement system. Another well-known example is the Kaposi Sarcoma virus (KSHV), a γ-herpesvirus, which produces a protein, kaposica, homologous to a human RCA.

In [79], gC negative VZV mutants have been investigated; in that work it is mentioned that VZV gC does not bind C3a, however, the evidence from other herpesviruses makes it a strong possibility that VZV utilises some mechanism of complement neutralisation.

2.5.3 Attenuation of the acquired immune response

The best understood part of the interactions of VZV with the host immune system is the influence of the virus on T cell response [10, 15, 16]. The mechanisms used by VZV differ from those used by other viruses [10].

A major strategy is to downgrade MHC-I expression on VZV-infected T cells. This process does not require viral DNA synthesis, and is thought to be mediated by IE or E gene products. Compared with controls, the levels of MHC-I expression drop by about 75% in infected T cells. Similar findings were observed in human melanoma cells and fibroblasts. The putative mechanism by which expression of MHC-I molecules is inhibited is interference with the transport of

these molecules through the cell.

Another mechanism is the ability of VZV to block expression of genes coding for MHC class II molecules (and thus to evade CD4 T cell response). Many types of cell upregulate MHC-II expression in the presence of IFN-γ, but this upregulation is blocked in VZV–infected cells. In [12] it is reported that VZV infection interferes with the IFN-γ signal transduction via the JAK/STAT pathway. VZV infection inhibited the expression of STAT 1 alpha and JAK 2 proteins. For a fuller discussion of the mechanism of this suppression see [10, 15]. Therefore VZV is able to escape recognition by T cells by downregulation of expression of MHC class I and class II molecules on infected cells.

Another method of evading detection by VZV-specific T cells is downregulation of expression of intercellular adhesion molecule 1 (ICAM-1), which was observed, for example, in infected keratinocytes during zoster [85]; absence of ICAM-1 reduces the infected cells' antigen presentation to LFA-1 bearing T cells.

VZV is also able to modify the function of mature dendritic cells. These DCs downregulate cell-surface CD80 and CD86, activation markers involved in the activation of naïve T cells. The ability of these DCs to induce an allogenic T cell reaction was therefore significantly reduced.

Thus VZV has an intimate and far-reaching awareness of the immune capabilities of the host. I suggest that the virus is also able to monitor host immunocompetence and may use this ability in maintaining latency and in reactivation (see the model in Chapter 6 for details).

In this context, it seems that work on VZV in immunocompromised hosts (HIV-positive and AIDS patients, such as the work reported in [1, 3], is of particular interest.

2.6 Simian Varicella Virus

Since the work presented here also involves the Simian Varicella Virus (SVV), [9], it is appropriate to give a brief description of this virus [46].

SVV (Cercopithecine herpesvirus 7) causes chicken-pox like disease in Old World monkeys, symptoms being fever and vesicular rash on the face, torso and extremities. Like VZV, SVV can remain latent in the PNS of the host, localising to the neural ganglia. It reactivates in a similar manner to VZV.

Since (see [69]) the separation of the two viruses is relatively recent, of the order of 4-6 Mybp (million years before present), one can expect very many similarities. The SVV and the VZV genomes have the same (type D) genome structure; 69 SVV ORFs with homology to VZV ones are defined. Virions have similar morphology and protein content.

Monkeys immunised with VZV are protected against SVV challenge, which indicates very close antigenic relatedness of the two viruses. However, see remarks in section 4.5 concerning intracellular localisation of latent SVV in neurons.

Chapter 3

The clinical picture

In this chapter, I have two main aims. One is to describe the pathogenesis of chickenpox, the primary infection caused by VZV. Since the primary aim of this research is to understand and control latent VZV reactivation, it is important to understand the processes that lead from the entry of VZV into an organism to its establishment in the PNS. The presentation of these aspects of VZV function is mainly based on the work of Arvin and co-authors [10, 15, 16, 79, 80], whose papers should be consulted for references of original work.

The other aim is to briefly survey the various neurological complications that can arise as a result of latent VZV reactivation. VZV has a significant disease burden, especially in the elderly and immunodeficient subjects, therefore management of VZV-related diseases is of major importance in the developed world, with its aging population. In this survey I have been influenced in particular by references [42, 44, 53, 54].

3.1 Chickenpox

VZV primary infection manifests itself as varicella (chickenpox) , a common childhood disease. It is estimated [53] that about 4 million cases a year of varicella occur in the US .

Typically, mucosal surfaces are inoculated from respiratory secretions or skin lesions of carriers. The virus possesses sophisticated mechanisms [10] of subverting the host immune system allowing it to multiply unmolested for the incubation period of 10–21 days. Presumably, the virus enters lymphatic tissue close to the locus of inoculation which leads to primary viraemia by infecting peripheral mononuclear blood cells (PMBCs). This allows the virus to be transported to other sites of early replication, such as the reticuloendothelium of the liver. There is evidence that in common with other alphaherpesviruses, VZV causes apoptosis in PMBCs [59]. A secondary viraemia follows, which is the main mechanism of virus transport to the skin, where the characteristic lesions are formed.

The host immune response to the virus presence is initially restricted to NK killer cells and interferon-α, but the successful resolution of the infection is predicated on mounting a successful T-cell response, involving both CD4 and CD8 cells.

A contentious issue of major importance is the mechanism of establishment of virus latency in sensory ganglia. The consensus seems to be that the virus reaches the ganglia by retrograde transport from skin lesions. At the same time, there is evidence, both clinical and from animal models (see [10] for references) suggesting a haematogeneous route, e.g. by infected T–cells coursing through the ganglia. Additional support for the haematogeneous route is furnished by the work [74] on SVV, in which SVV DNA was detected in the ganglia of experimentally infected monkeys before the detection of rash; furthermore these authors also report cases in which there was no rash at all even though latency was established in the sensory ganglia. Though it appears that the case for the transport of virus to sensory ganglia by blood cells is convincingly made, the relative importance of the two routes is not clear at this stage. In [41], presence of VZV DNA in human nodose and celiac ganglia[1] is reported using PCR. On the face of it, this is evidence for the haematogeneous route. However, these authors also report the presence of HSV-I DNA, for which virus no such route exists. Hence it seems that this is

[1]The nodose ganglion is the sensory ganglion of the tenth (vagus) cranial nerve. It contains cell bodies whose axons are part of the autonomic nervous system that provides fibres for taste as well as cell bodies whose axons innervate the heart, bronchi and viscera. Celiac ganglia are part of the sympathetic nervous system that supply the spleen and multiple viscera.

a crucial open question, since clearly, if the retrograde transport of virus from skin lesions is important, local treatment with antiviral agents, or something less crude that would impede this transport would immediately (and cheaply) alleviate the infection burden. Some ideas how this could be done are contained in Chapter 6.

In the cotton rat model [95] it is shown that replication-deficient mutants can still establish latency. In this work ([8] and work in preparation), rats inoculated in the footpad express VZV genes in lumbar and thoracic ganglia. These findings indicate that in these animal models direct viraemia is an important mechanism of VZV dissemination.

3.2 Zoster

Following the primary infection, VZV can remain latent in the host, residing in cranial (trigeminal), dorsal root and autonomic ganglia (see the reviews [29, 53, 55] for references to the original work). It is estimated that 90% of normal individuals carry VZV DNA.

Latent VZV can reactivate. The incidence of reactivation grows with age of the host and is higher in immunocompromised individuals, such as those with cancer, organ transplant recipients and AIDS patients [29].

On reactivation, the virus produces primarily shingles (herpes zoster). The original observation that it is in fact the same virus which causes both chicken pox and shingles was made by Hope Simpson in 1954 when clinical observation indicated that varicella developed in children exposed to a relative with zoster, and experimentally by the inoculation of children with zoster vesicle fluids (reviewed by Brouer [21]). He hypothesised that if the viruses were the same the clinical behaviour should be indistinguishable, including incubation period, infectivity and protection against subsequent chickenpox but not other viruses. An outbreak of chicken pox on the remote island of Yell enabled him to establish that indeed these conditions were fulfilled. It is estimated that more than 500,000 cases of zoster occur in the US alone annually [44].

That it is the virus latent in the sensory ganglia, the reactivation of which causes zoster, and not

a *de novo* primary infection, has been demonstrated in the classical paper of Straus *et al.* [99].

Zoster is a painful eruption of skin, covering one or more dermatomes. It is characterised by a severe sharp pain. The sensation in the affected dermatomes is reduced, but the skin is extremely sensitive to touch. The progression of disease is usually from thoracic herpes followed by facial lesions. Other organs can be affected, such as eyes (zoster keratitis, which can lead to blindness, ophthalmophlegia, optic neuritis), parts of the head innervated by the maxillary and mandibular divisions of the trigeminal nerve (leading to osteonecrosis of the jaw and exfoliation of teeth), or the ear (zoster oticus). Facial muscle weakness is not uncommon (for example, the Ramsay–Hunt syndrome which expresses itself as zoster oticus combined with peripheral facial weakness). Cases of cervical zoster and of zoster–associated arm weakness (zoster paresis) have been also reported. The usual treatment for zoster involves a combination of acyclovir and analgesics.

3.3 Complications of zoster

These are numerous and can be painful and sometimes life-threatening. In addition to the ones described in some detail below, VZV has been implicated in the Gillain Barré syndrome [54] and genital herpes [19].

3.3.1 Postherpetic Neuralgia

Postherpetic neuralgia (PHN) is the most important complication of zoster infection and has been defined as dermatomal pain persisting for more than 4–6 weeks after the rash. However there is no universal consensus as to the exact time definition of PHN with some definitions varying between 1–6 months. PHN is characterised by the development of chronic hyperalgesia (increased response to noxious stimuli) and allodynia (pain in response to innocuous stimuli) accompanied by spontaneous pain of a burning or aching nature. Ninety five percent of PHN cases are in those aged over 60. Over 40% of patients with zoster develop PHN. The incidence

of PHN is apparently slightly greater in women and following trigeminal distribution zoster. No clear understanding of the aetiology of this debilitating condition exists. The detection of VZV-specific DNA in the mononuclear cells (MNCs) of patients with PHN is suggestive of the persistence of VZV. One study ([71]) has indicated that the abundance of VZV DNA in ganglia is higher in PHN patients than in zoster patients without PHN. Therefore it is possible that MNCs trafficking through these ganglia may engulf virus whose DNA is then available for extraction and amplification by PCR. A greater virus burden or a productive infection found in these ganglia in PHN compared to latency [73], would provide a rationale for aggressive treatment with antivirals. The existence of ganglionitis without rash has been supported by the presence of radicular pain up to 100 days preceding zoster, pre-herpetic neuralgia [40]. Further, a report [38] has described patients with acute trigeminal distribution zoster who, after years free from pain, developed severe trigeminal PHN. It should be appreciated that permanent abnormalities of neuronal electrophysiological activity may also be important in the pathogenesis of PHN, irrespective of any persistent virus infection [53].

3.3.2 VZV vasculopathy

On reactivation, VZV can spread to arteries in the brain and the spinal cord. Both large and small vessels can be affected. Large vessel vasculopathy is more characteristic of elderly immunocompetent patients. Large vessel encephalitis is fatal in up to 25% of cases. Angiography reveals focal constriction and segmental narrowing. Small vessel vasculopathy is more often seen in immunocompromised individuals and is accompanied by headaches, fever and mental state changes [42].

3.3.3 VZV myelitis

This is another serious (in immunocompromised patients) zoster complication the mechanism of which is unknown. It can be acute, chronic or recurrent. In severe cases, spinal cord necrosis,

inflammation and parenchymal VZV invasion are observed.

3.3.4 Zoster sine herpete

This term refers to zoster-associated pain without antecedent rash [48]. Involvement of VZV in these conditions was verified by detection of VZV DNA and antibody in cerebrospinal fluid of patients (see [44] for references to the clinical work). Similar findings, of VZV genomes without dermatological evidence of reactivation, were made in cases of unilateral facial palsies, meningitis and meningoencephalitis.

3.4 VZV reactivation following HCT

There is frequent VZV reactivation following hematopoietic cell transfer (HCT) [15]. Clearly, HCT recipients are immunodeficient, which makes reactivation more likely even without HCT (from the presentation of [15] it is not clear how frequent reactivation is in patients requiring HCT before treatment); this would explain the prevalence of herpes zoster in such patients. However, neuropathic pain and disseminated VZV infection without skin lesions indicate that cells pick up the virus while trafficking through infected ganglia.

3.5 VZV and GCA

It is well documented that, in the process of cell to cell spread, VZV has the ability to fuse cells into syncytia, the mechanism of which seems to involve glycoproteins such as gE [57]. In addition, cumulative evidence suggests that VZV can infect the walls of CNS arteries. It is hypothesised that VZV spreads from ganglionic reactivation sites to the arterial wall by neural pathways [75]. Hence it is reasonable to suspect VZV involvement in the aetiology of diseases that present syncytia in inflammatory infiltrates. One such disease is giant cell arteritis (GCA).

VZV involvement in this disease has been a matter of some debate. Helweg-Larsen *et al.* [47] looked at 13 GCA-positive biopsies and 17 controls and found no evidence of VZV by PCR. Mitchell and Font [78] looked at 35 GCA and 29 normal biopsies and found that 9 (26%) were positive by PCR. In [86] more sensitive methods were used and in none of the 10 GCA biopsies was any evidence of VZV DNA or antigen found. Thus the evidence for VZV involvement in GCA is contradictory and in need of clarification.

Chapter 4

VZV latency

This chapter contains a discussion of the the cellular localisation of latent VZV, patterns of gene expression in latency and factors influencing reactivation.

4.1 Cellular site of latent VZV

It is well established [92] that the virus establishes latency in trigeminal (TG) and dorsal root ganglia (DRG) of the PNS (where like HSV-I it is found in an episomal state). A priori there could be two reasons for that. One is that in the cells of the PNS the virus is well-protected from the cell-mediated components of the host immune system, while the other is that the cells harbouring it cannot support a productive lytic cycle. The evidence of reactivation suggests that only the first of these is true, i. e. that the virus hides in a compartment that will allow it to weather the period of extreme immunosurveillance. If this is the case, there must be information traffic between viral control components and the outside of the harbouring cell. This may be the (tacit) paradigm of VZV latency. Neurons are good candidates for a hiding place for a latent virus as they express almost no MHC class I molecules and so the cell is protected from CD8 T cell elimination. Also HSV-1 resides in neurons [33] so their homology and other similarities might suggest that there is a common site of latency for both.

The exact localisation of latent VZV in TG and DRG has been a topic of much debate. A good review of the history of this controversy and the relevant references are contained in [53]. Briefly, historically there have been two schools of thought, one suggesting that VZV is latent in neurons exclusively, and the other, that it resides as exclusively in non-neuronal (such as perineuronal satellite) cells. The earliest paper to use ISH to localise VZV [49] reported VZV RNA in neuronal cytoplasm in latently infected TGs. This was confirmed in 1987 by the same group [100] and independently in [43]. However, a further study using labeled riboprobes indicated the presence of latent VZV exclusively in perineuronal satellite cells [33], which was confirmed in 1993 in a further study from the same laboratory [77]. A study using the sensitive technique of *in situ* PCR hybridisation [37] found latent virus DNA exclusively in the cytoplasm of neurons. An important paper on this subject is the study by Lungu *et al.* [65] who found VZV DNA in both types of cells though in higher numbers than expected from quantitative studies [89, 73, 30].

One of the major goals of this research was to resolve this controversy to the satisfaction of all the relevant researchers in the field and, as will be shown below, we accomplished that goal.

4.2 VZV gene expression during latency

While in HSV-1 the only transcripts found to be expressed during latency are the latency-associated transcripts that are partially overlapping and complementary to the transcriptional activator ICP0, the general consensus is that during VZV latency, VZV genes 21, 29, 62 and 63 are expressed, with some evidence of expression of genes 4, 18 and 66 as well (see, for example, [32]). The original studies of transcription [33] used ISH and Northern blotting which detected transcripts from genes 29 and 62 but not 28 or 61. Subsequently VZV transcripts for VZV gene 21 were identified in TG and DRG [28]. The expression of genes 21, 29, 62 and 63 has been confirmed using cDNA libraries [27, 31] and *in situ* hybridisation [77].

In vitro and animal model studies indicate that many of the genes expressed in latency, such as ORF21 [106], and others such as ORFs 47 and 66 [95], ORF2 [94] and ORF61 [96], are not necessary for the establishment of latency.

4.3 Protein expression in latency

Unlike latency in HSV-1, where no proteins are expressed, latent VZV expresses a number of gene products. There have been several reports of expression of VZV gene 63 encoded protein [36, 72]. Lungu *at al.* [66] in 1998 were the first to report the presence of proteins encoded by VZV genes 4, 21, 29, 62 and 63. These proteins have been found in the cytoplasm of the latently infected neurons, leading the authors to propose that the restriction of these regulatory proteins from the nucleus interrupts the cascade of events leading to a productive infection and results in latency.

4.4 Reactivation

There is very little information on what causes reactivation of latent VZV. In [107] a correlation between the amount of UV irradiation and frequency is established; in [76] a case study with astronauts implicates stress as another possible factor. Since it is well known that reactivation is enhanced in the immunocompromised, such as HIV positive, patients, one needs to understand the immune response raised by the host to VZV; furthermore, since immune dysregulation is a common result of stress, and since stress is implicated in reactivation, it makes sense to connect the dysregulation caused by stress and VZV reactivation.

Both MHC-I restricted and MHC-II restricted cells are sensitised during primary infection with VZV [10]. The T cell response is predominantly Th1 type and is characterised by a high level of IFN-γ. VZV-specific CD4 cells also exhibit MHC-II cytotoxicity against infected cells. Loss of VZV-specific T cells is thought to increase the risk of reactivation [10].

This scenario meshes well with what is known about immune dysregulation following stress. For details the reader is referred to [88]. The relevant facts are that stress-induced release of catecholamines can result in the suppression of IL-12 production and the increase of IL-10 production, a shift in the phenotype of CD4 T cells from Th1 to Th2 and decrease in IFN-γ production.

I shall use the above data to produce a rudimentary latency maintenance and reactivation model below.

In [60], in a 4 year follow-up study it was claimed that vaccination with the Oka strain leads to frequent asymptomatic reactivation. Thus it is possible that the virus reactivates spontaneously much more frequently than indicated by the occurrence of zoster, but is dealt with efficiently by an immunocompetent host. The explanation given in [60] for their findings have been hotly disputed, with some researchers such as [97] claiming that the increase in titre in vaccines was due to exogeneous boosting.

4.5 Animal studies

There have been several reports in the literature (reviewed in Myers and Connelly [83]) of animal models in primates where infection induced seroconversion but no disease was produced or virus recovered. Weanling guinea pigs have been used to evaluate humoral and cell-mediated response as they undergo a limited viraemic infection. However, the model is inconsistent. Rabbit eye models have also been investigated and no clinical illness observed. A scid-hu mouse model where human thymus, liver or skin implants were engrafted into male CB-17 scid/scid mice has proved useful in assessing contributions of gene products to VZV replication in intact human tissues containing differentiated skin and T cells (see, e.g. [17]). There have also been isolated reports on neonatal rat models and mouse models.

Major limitations of animal models are that they are critically inoculum dependent and VZV is difficult to grow to high titre, and the fact that no *in vivo* animal model has shown reactivation

of VZV, though recently [22] there has been a report of an *in vitro* guinea pig enteric ganglion model which may prove useful as it appears to mimic some of the properties of latency, including reactivation of virus when non-neuronal cells are added to the neuronal cell populations. In the Liège group rat model (e.g. [7, 93]), inoculation of VZV-infected Vero cells in both footpads leads to seroconversion but no symptoms of disease; latency was characterised by virus genome persistence in the DRG for many months, selected viral transcription and at least one viral protein. Reactivation has not been obtained in vivo, but has occurred ex vivo after repeated stresses. Many similarities with VZV latency in humans were found, making this model useful for vaccine and antiviral studies.

Finally, SVV in primates has been investigated as a putative VZV model as it has many similarities to VZV in humans. Initially, experiments were performed on intratracheally inoculated African green monkeys [74], but it was found that virus DNA was still detected in ganglia, liver and blood MNCs, primarily in CD4+ and CD8+ cells, months to years post infection. Therefore this is a model of persistence rather than latency. More recently, naturally infected monkeys, that is SVV-seronegative monkeys which have been exposed to SVV by being placed in cages with SVV-intratracheally infected monkeys, have been described [70, 9]. These monkeys developed mild varicella 10-14 days post infection and SVV DNA persisted at multiple levels of the neuraxis but not in lung or liver 6–8 weeks later, thus providing a possible model of latency. This model may have more relevance to the human situation [70, 9].

Chapter 5

Results

This chapter describes the contribution of the work I have carried out in Professor Kennedy's laboratory to VZV latency studies.

5.1 Studies of VZV latency in humans

5.1.1 Cell localisation of latent VZV

Given the controversy about the site of latent VZV in the human PNS as described in the previous chapter, paper [1] addressed this issue using trigeminal ganglia (TG) samples from a number of different laboratories, run by people who took opposite sides in the localisation debate. This study used a large number of subjects (30; 2 neonates, 11 individuals with HIV infection and 17 immunocompetent individuals) with the results being confirmed by multiple techniques. VZV DNA in ganglia was detected in 15 individuals by using PCR alone, and in 12 individuals (6 normal non-HIV and 6 positive HIV individuals, but not neonatal ganglia) by using ISPCR. When ISH alone was used, 5 HIV-positive individuals and only 1 non-HIV individual showed VZV nucleic acid signals in ganglia. Oligonucleotide probes and primers to VZV genes 18, 21, 29 and 63 were used. *In situ* PCR enabled the detection of VZV DNA in an additional 5 im-

munocompetent and 1 HIV-infected individual compared to in situ hybridisation (ISH). Using ISH and ISPCR in a blind study where the source of the ganglia was unknown, it was shown conclusively that latent VZV is largely concentrated in the nuclei of (2%–5%) neurons with only a negligible number of satellite (glial) cells (< 0.1%) infected.

An extended study on DRG using ISH and ISPCR was conducted on 12 individuals (3 normal and 9 HIV-infected) [2], with comparable results, i.e. 2–5% of neuronal nuclei and only occasional non-neuronal cells were positive for VZV DNA and two regions of the genome (gene 4 and 40) were present in serial sections.

5.1.2 Studies of gene transcription

I have also conducted studies of VZV gene expression in latency. Evidence of the transcription of VZV genes 4, 21, 29 and 63 in the DRG [2] was found by ISH, consistent with the results of others. In addition the co-localisation of DNA and RNA for gene 29 was shown in serial sections. In paper [3], ISH was used to determine the frequency of RNA expression for 9 VZV genes in TGs of 35 human subjects, 18 of whom were HIV positive. RNA for VZV gene 21 was detected in 7/11 normal and 6/10 HIV positive subjects, gene 29 in 5/14 normal and 11/11 HIV positive subjects, gene 62 was detected in 4/10 normal and 6/9 HIV positive subjects and gene 63 in 8/17 normal and 12/15 HIV positive subjects. RNA for VZV gene 4 was detected in 2/13 normal and 4/9 HIV positive subjects and gene 18 4/15 normal and 5/15 HIV positive subjects. RNAs for VZV genes 28, 40, and 61 were rarely or never detected. In addition VZV gene 63 encoded protein was detected mostly in the cytoplasm of 25% of TG from HIV-infected and normal cases.

I also participated in a study of expression of VZV ORF66 [4] in association with R. Cohrs and D. H. Gilden in Denver. This is an important gene to investigate, as its product, ORF66p, (a protein kinase) has been shown to be responsible for the phosphorylation of ORF62p and its cytoplasmic localisation [58]. VZV ORF66 transcripts were detected by RT-dependent, nested PCR and ISH. Twenty seven ganglia from 22 individuals were analysed by ISH for transcription

of VZV genes 66 and 62. In serial sections from 5 ganglia, transcripts from both genes were detected in neuronal nuclei. In one case VZV gene 62 was detected in the absence of VZV gene 66 RNA. In addition VZV ORF66p was located by in situ histochemistry in the neuronal cytoplasm of 3 TG of 11 studied (from 7 subjects).

5.1.3 Detection of VZV-encoded proteins in latency

In paper [5] we detected and thereby confirmed the presence of ORF21p, ORF29p, ORF21p, ORF62p, ORF63p in the neuronal cytoplasm. A total of 10/22 ganglia were positive for VZV 21p, 9/23 for VZV gene 29p 5/5 for gene62p and 10/10 for gene 63p. Positive labelling was predominantly in the neuronal cytoplasm, but not in non-neuronal cells. In 2 samples there was also neuronal nuclear staining. Approximately 5-10% of neurons were positive. In addition, as described above, ORF66p has been detected in neuronal cytoplasm [4].

5.1.4 Disease study: GCA

In order to study VZV involvement in GCA [6], in situ PCR for VZV genes 37 and 62 and ISH using DIG-labelled probes for VZV genes 37 and 62 were used in an attempt to detect VZV DNA in arteries from 15 histologically proven GCA and 7 normal controls. No VZV DNA was detected in artery tissue.

5.2 Studies of animal models of VZV latency

5.2.1 Studies of the Liège rat model

In paper [7], an ISH study in a rat model was conducted in a total of 23 rat DRG from 16 animals, and it was demonstrated that VZV DNA was found almost exclusively in (5%–10% of) rat DRG neurons. Gene expression in ganglionic tissue was also studied at multiple time

intervals (1 week, 1 month and 18 months) after footpad inoculation with VZV. As in humans, expression of VZV genes 21, 62 and 63 was found.

In addition in situ PCR and ISH were used to detect VZV DNA and ISH to detect VZV RNA were used in an investigation of whether the deletion of gC and gI were required for the establishment of latency in the rat model [8]. Transcripts corresponding to the three classes of genes ORF 4, 62 and 63 (IE) ORF 18, 21 28 and 29 (E) and ORF 40 (L) were studied in 27 DRG from 20 rats that had received footpad inoculation with different mutant VZV at 1 week, 1 month and 18–24 months post inoculation. VZV DNA and RNA were detected in neuronal and non-neuronal cells with transcripts for VZV genes 62 and 63 being the most frequent at 18–24 months for both mutants. Transcripts for VZV gene 40 were not detected. .

5.2.2 SVV gene expression in latency

Using the natural model of SVV infection described in section 4.5, two months after natural infection with SVV, African green monkey ganglia (18 ganglia from 2 animals) were analysed using DNA ISH and ISPCR for SVV genes 21 and 63. Seven ganglia of 9 tested from one animal and 4/9 from another were positive by ISH and a further 5 ganglia were positive by ISPCR. In addition, it was found that neuronal cytoplasm was the predominant site of SVV genome localisation [9].

Chapter 6

Discussion

It can be concluded from initial detailed studies that the neuron is the predominant site of latent VZV in human trigeminal ganglia. The localisation results contained in [1], as well as confirming the initial observations in the Gilden laboratory, have been confirmed by an ISH study in TG [102], and by studies such as [62, 64], in which ganglia were dissociated into pure populations of neuronal and non-neuronal cells and then PCRs for VZV DNA were performed. In addition, our work was confirmed in the laboratory of S. E. Straus, where the original work claiming that VZV was latent in the peri-neuronal satellite cells was performed (see [89], and Proc. International Herpesvirus Workshop, Reno Nevada, July 2004). Thus it is now universally accepted that this work has resolved the controversy of the localisation of latent VZV. A related question is that of VZV burden, that is, of the number of copies of VZV genome per infected cell. My results of 2–5% of neurons containing VZV DNA are consistent with approximately 8 copies per infected neuron [53], which is comparable to the results obtained for HSV-1 which have been calculated in other studies such as [73, 89]. Consistent with this are the results of [62] and [64] described above, where viral DNA was found to be present in about 1.5% of neurons, but in no non-neuronal cells; their computation of VZV burden gives 2–9 copies of viral genomes per infected cell. In the paper of Cohrs *et al.* [30] the use of real time PCR for the detection of VZV nucleic acids showed for the first time the variable abundance of VZV.

The work presented in [1] also compared the burden of immunocompetent patients to that of HIV-positive ones and found it to be larger in the immunocompromised population (for non-AIDS tissue the presence of viral DNA was usually detectable only by in situ PCR, whereas for HIV-positive tissue, ISH was sufficient for detection).

The initial studies in our laboratory on localisation and expression of VZV in DRG [2] confirmed that genes 4, 21 29 and 63 were expressed and in addition occasionally VZV gene 18 was expressed. This, along with the fact that 2 disparate regions of the genome (genes 4 and 40) were detected in the same region of tissue on serial sections indicate that the entire genome was present. DNA and RNA for VZV gene 29 were also present on serial sections in the same region of the DRG. Ideally one would show both in the same cell by double labelling but this proved to be technically difficult.

The frequency of RNA expression for 9 VZV genes in TGs of 35 human subjects, 18 of whom were HIV positive [3] was studied. Genes 21, 29, 62 and 63 were detected with high frequency, confirming the results of previous studies, and RNA for genes 4 and 18 with lower frequency. RNA for genes 28, 40 and 61 was not detected, suggesting that they are unlikely to be latency-associated genes. In all cases, the immunocompromised subjects showed higher rates of gene expression but no significant expression of additional genes.

It has been estimated that 87% of human TGs from seropositive individuals contain VZV DNA. Unfortunately, since post-mortem tissue must be used, the theoretical possibility that viral reactivation may have occurred after death or on ganglion removal, exists. Therefore any study of localisation or transcription of VZV during latency must be interpreted with caution. To address this problem, explanted fresh TG and DRG were cultured for up to 11 days and the pattern of gene expression in explanted tissue was investigated. Interestingly, in addition to frequent expression of genes 29 and 63 (which is now considered the hallmark of latency), we found high levels of genes 18, 28 (both E genes), and 40. This indicates that what is seen in latency studies is true latency and not the result of viral reactivation upon excision of tissue. The detection of ORF40 requires special comment. ORF40p is a nucleocapsid protein, and ORF40 is a late gene.

In at least one sample ORF40 RNA was detected only after 168 hours of explantation, without being detected earlier. It is very probable that this is the most complete portion of a productive lytic cycle from a tissue explanted post-mortem. It seems to us that many more studies of this sort are required (for example, checking co-expression of ORF18 and ORF19).

In addition this paper contained the first demonstration of positive staining with a monoclonal antibody to VZV 63p. Approximately 25% of samples investigated showed positive cytoplasmic staining in neurons, with very occasional staining in neuronal nuclei or non-neuronal cells. These results confirm the importance of VZV gene 63 in latency and support the previous studies indicating significant and restricted gene expression in latency.

I also participated in a study of expression of VZV ORF66 [4]. This is an important gene to investigate, as its product, ORF66p, (a protein kinase) has been shown to be responsible for the phosphorylation of ORF62p and its cytoplasmic localisation [58]. VZV ORF66 transcripts were detected by RT-dependent, nested PCR and ISH. Our group performed the ISH experiments and located VZV ORF66p in neuronal cytoplasm by *in situ* histochemistry. This was the first demonstration of VZV gene 66 expression in latency; it raises the possibility that the co-expression of these genes and the location of ORF66p may play a role in latency establishment. Since the activation of VZV gene transcription takes place in the nucleus, the phosphorylation of ORF62p by ORF66p, a protein kinase, leading to cytoplasmic localisation of ORF62p, may modulate its ability to transactivate, which in turn may play an important role in the establishment of latency.

In the study of the translation of VZV genes during latency [5] the results of one other study [66] were confirmed in a larger sample size and using one monoclonal antibody. Unfortunately, these results could not be confirmed by Western blotting (experiments carried out by R. Cohrs and P. G. E. Kennedy), indicating a probable low abundance of VZV proteins; however it does lend support to the finding that these proteins, which localise to the nucleus of cells during lytic infection, are detected in the cytoplasm of latently infected neurons. The restriction of regulatory proteins from the nucleus of latently infected neurons might interrupt the cascade of

virus gene expression that leads to a productive infection.

In order to study VZV involvement in GCA [6], ISPCR and ISH were used to try to detect VZV DNA in arteries from histologically proven GCA and of normal controls. No VZV DNA was detected in artery tissue, a result which should strengthen the case for the non-involvement of VZV in GCA and direct the search towards other syncytia forming viruses or other possible aetiologies. Recently a PCR based study involving 147 GCA biopsies found no evidence of VZV, confirming our results [90]. It should be noted, however, that the absence of VZV does not preclude a possible role for VZV in the initiation (not the propagation) of the disease.

In these studies, we have also extended the understanding and utility of the rat model of VZV latency establishment using both wild type and mutant viruses [7, 8]. In [7], using ISH for 8 different genes representing the 3 classes of infection, the pattern of viral gene expression at 1 week after infection (where many genes were expressed) was different from that observed after 1 and 18 months. The results obtained, of limited gene transcription similar to the human situation, even 18 months post inoculation goes some way towards validating the rat model as a viable model of VZV latency. PCR in situ amplification was also used to determine the cell specificity of latent VZV DNA. Obviously, the results do not perfectly mirror the situation in human tissue as larger numbers of cells are infected and in particular, a larger number of non-neuronal cells have both VZV DNA and RNA (at an approximate ratio of 3 : 1). Immunohistochemical studies using monoclonal antibodies established the presence of ORF63p in both nuclei and cytoplasm of the infected cells. This study also confirms the importance of VZV gene 63 expression and translation in VZV latency.

In a later study of the same model [8], deletion mutants of glycoproteins gC and gI (encoded by ORFs 14 and 67, respectively) were used to see whether these gene products are needed for the establishment of latency. Using the expression of genes 21, 29, 62 and 63 as indicating latency, it was concluded that these two genes are dispensable for the establishment of latency in rat DRGs. This study complements the mutant work done in vitro and in the cotton rat model of Cohen [94, 95, 96, 106] in which infected melanoma cells are injected intramuscularly along

both sides of the thoracic and lumbar spine. In these studies DNA and RNA are extracted from the ganglia after one month and PCR is performed. Presence of ORF 63 transcripts and the absence of ORF 40 transcripts is taken to signify the establishment of latency

Using DNA ISH and DNA in situ PCR for simian varicella virus (SVV) genes 21 and 63, it was found that neuronal cytoplasm was the predominant site of SVV genome localisation [9]. It is possible that SVV has diffused from the nucleus during the harsh procedures of ISH and ISPCR, or there may indeed be significant biological differences in the latency process due to differences in viral and/or host factors. This supports the use of natural infection with SVV as a model for VZV latency.

Of all the results concerning deletion mutants, the result of [95] concerning ORF66 and my own study of ORF14 [8], which show that ORFs 66 and 14 are dispensable for the establishment of latency, are the most interesting for a number of reasons. First, since ORF66p is instrumental in localising ORF62p in the cytoplasm, and ORF62p seems a likely candidate for a latency maintaining protein (LMP), this result, if confirmed in other systems, should indicate that latency establishment and maintenance are different processes. It would be particularly interesting to know whether ORF66 deficient mutants reactivate with the same efficiency as the wild strain. Concerning ORF14p, gC, recall that in the SCID-hu mouse model [79] gC was responsible for skin tropism. It is possible that the establishment of latency in the rat model by gC-negative mutants is an indication that in that model the haematogenic route for latency establishment is the dominant one, but whether this has any implications for the situation in vivo is not clear.

Methodologically, the study of VZV latency is in a very unsatisfactory state. There do not seem to be any competing hypotheses to explain any of the known facts of viral gene expression. (The only relevant model I have come across is the one of Cohrs and Gilden [29]: "... during latent infection sequence-specific binding of 21p/29p complex inhibits IE62 transactivation of ORFs 20 and 28." Clearly something more encompassing is called for.) The usual strategy, especially using deletion mutants, is of the "try and see" type.

First of all, the following question has to be confronted. If the maintenance of the VZV latent

state is somehow related to the gene products expressed in the latent state, which seems likely (to argue otherwise would seem to be a violation of parsimony principles but it is conceivable that the sequences are being transcribed by accident, because they are able to be transcribed because of histone patterns or are an epiphenomenon), why does HSV express nothing but the latency-associated transcript (LAT). There are two possible answers to this question. First, a less elegant solution, is as follows. Since the morphology is more or less conserved through the alphaherpesvirus families, it is very possible that HSV brings in its nucleocapsid sufficient amounts of LMPs not to need to synthesise them *de novo*. Since all proteins have finite life-span, there must be also a mechanism to make LMPs invisible to the cell proteolytic machinery. The second answer is that HSV is more sophisticated virus than VZV and that HSV latency, once established, is maintained by subverting host control elements. Very recent findings, published after this thesis was submitted, indicate that ORF63p is critical for the establishment of latency in a rodent model [25, 26]. I conclude this section by outlining a possible model for VZV latency. It postulates the virus being capable of interpreting information concerning the state of the host's immunocompetence. The model assumes that establishment of latency is not crucially dependent on the host's immunocompetence, though this is not difficult to take into account by remembering that the regulatory proteins such as ORF62p are tegument proteins and so can change conformation, e. g. by contact with various interleukins and bring this information into the host cell thus deciding on the immediate fate of the virion. In fact it is known [10] that T cells induced to proliferate and secrete cytokines during primary infection recognise ORF62p. This model is based on the following assumptions:

1. The interior of the neuron, more precisely the neuronal cytoplasm gives a faithful representation of the host's generalised immunocompetence. For example, the amount of extracellular IFN-γ or ILs 2 or 12 could be communicated to the interior of the neuron. That, at least in fibroblasts this is indeed true in the case of IFN-γ, and that VZV can interfere with this particular signal transduction pathway, is known from the work of Abendroth and Arvin [10, 12]; see section 2.5.3 for details.

2. This information, which correlates with the host ability to mount an anti-VZV cell-mediated

response, is available to VZV "sentinel proteins", the best candidate for which now is ORF62p, IE62. This picture is consistent with its cytoplasmic localisation in latency. It is crucially important to be able to characterise ORF62p interactions with ILs and with cellular factors. If this picture is correct, I would predict that ORF62 mutants would not be as sensitive in reactivation.

3. When there is some evidence of immune dysregulation (see section 4.4), for example, if IL-12 levels are depressed and IL-10 levels are raised, this information is passed on to the cellular sites where the VZV genome is sequestered and the lytic cycle is started. This view is consistent also with the high level of co-occurrence of VZV reactivation and autoimmune disease episodes and in fact with the folklore view of the immune system, viz. that boosting the immune system "in general" helps prevent specific disease.

Some researchers (see e.g. [10]) explicitly state that risk of reactivation is inversely proportional to the numbers of circulating VZV specific T cells. However, this does not contradict the view presented here, since these numbers would depend on the general level of host immunocompetence. Furthermore, I would like to quote from a paper by Arvin [15]:

> Despite the correlation between loss of VZV-specific memory cells and the risk of VZV recurrence, the absence of detectable responses for prolonged periods appears to be a necessary but not a sufficient condition for VZV reactivation from latency. This observation suggests that non-specific antiviral immunity helps to restrict the symptoms of VZV reactivation.

(... and perhaps not just "the symptoms?") In the absence of a satisfactory animal model of reactivation, the type of explantation experiments carried out in [3] may provide a good platform for falsifying the above model. This could be done by incubating explanted tissue with IFN-γ or IL-12.

6.1 Final comments

It is clear that the work of characterising latency is still in its infancy; only the presence of about 13% of VZV genes has been probed in published literature. Work in progress using microarray techniques [56] seeks to remedy this defect in order to obtain a fuller picture of VZV and host genome activity during latency. Clearly, once such information is available, the focus will switch to characterising protein-protein interactions between different VZV gene products, between VZV gene products and host cell proteins, and, something that has been neglected so far, direct RNA-protein interactions. All this will surely aid in achieving the therapeutic goals described in the Introduction. Furthermore, at this stage the search is still on for an unequivocal hallmark of latency. Expression of VZV gene 63 is considered a good indication of latency, yet our results in [3] indicated that gene 63 activity is not unique to it. More studies on explanted tissue on the pattern of [3] are needed to differentiate fully between latency and post- excision reactivation. The following quote from [29] can not be improved upon: "Our results highlight the dearth of knowledge concerning VZV gene expression during latent infection."

There is also a dearth of understanding of the nature of communication between the latent virus and its host cell, and by implication, with the organism as a whole. These communication processes, surely, are the key to understanding and thus controlling of the processes of reactivation of the latent virus: the virus has a way of gauging the host's immunocompetence vis à vis itself and reactivates when the host is deemed sufficiently immunocompromised. Of course this does not rule out the possibility of continual sub-clinical reactivation. Thus an interesting venue of research would focus on host cells themselves and establish their ways of communicating, via receptors for ILs and TNFa, with the body's immune system. Once these receptors are well characterised, one should investigate the interaction of viral proteins, such as IE62, and others yet to be discovered, with these receptors and other constituents of the signal transduction pathway.

6.2 Suggestions for future research

6.2.1 Latency establishment inhibition

As discussed in Chapter 3, there are two ways for the virus to reach sensory ganglia, either by retrograde transport from skin lesions, or by the haematogenic route, from e.g. infected T cells. Establishing the relative importance of these two mechanisms should be a priority. ORF47 mutants which do not seem to infect T cells [80] are an obvious candidate for such a study in an animal model. Another possibility is to compare the abundance of latent VZV genomes in DRGs and in ganglia which do not innervate the skin, both coming from the same source; preferential infection of DRGs will indicate that the retrograde transport route is operational. Of course the fact that zoster is the preferential way of latent virus reactivation already indicates that this route is important. If the retrograde transport mechanism is an important way of virus delivery to sensory ganglia, an obvious strategy of controlling the establishment of latency seems to present itself. It makes sense to assume that the initiation of the axonal transport uses the same mechanism as virion absorption to a target cell in a productive lytic cycle. Thus it is not clear that local administration of heparin to skin lesions would not inhibit (simply by kinetic effects) the penetration of the virus into neurons. Additionally, a closer study of major fusogens such as gH will reveal the structure of their active sites and the nature of the cell-type specific receptors they bind to. Hence once neuron-specific glycoprotein is identified and characterised, administering the ligand it binds to to skin lesions will perforce lower the probability of latency establishment.

6.2.2 Microarray analysis of VZV transcription

Using a chip containing 75bp oligomers to each VZV ORF will enable the study of variations in the entire transcriptome to variations in host cell type, virus strain and the analysis of different clinical samples and their relation to disease phenotype. In addition host microarrays can be used to determine differences in cellular gene regulation on infection with VZV. These studies

may provide background information for experiments to link these various response phenotypes with mechanisms of VZV pathogenesis that are important for the natural course of human infection [52].

6.2.3 Novel vaccination procedures

Many complications of zoster seem to involve a common mechanism: on reactivation, the virus is disseminated by infected T cells and it is its seeding (in arteries, brain, etc.) that causes the pathology. Once it is conclusively shown what gene product is responsible for T cell tropism (e.g. the ORF47 protein as suggested in [10]), it makes sense, on first manifestation with zoster, to use passive vaccination with anti ORF47 protein antibodies to decrease the probability of T cell virus transport to target organs. Alternatively, immunisation with polyvalent antibodies to several latency associated proteins could be administered to those most at risk.

6.2.4 RNA silencing

An emerging technology that can have implications for latency research is the use of small interfering RNAs (siRNAs) [45, 68, 98]. These are small molecules of double stranded RNA with overhangs, which have the property that in many systems they initiate the degradation of RNA molecule they bind to. In theory it is possible to direct siRNA against mRNA of any gene and thus cause its timed and time-dependent inhibition; see [68] for references of such work on the respiratory syntycial virus. There are many practical problems related to the delivery of siRNA to target organs; [68] also contains reports of siRNA delivery in the mouse. If the technical problems were to be resolved, a possible procedure to deliver siRNA directed against mRNA of the IE genes coding for proteins expressed in latency at different times after inoculation (for example, in the Liège rat system) in order to see expression of which viral genes is necessary for latency establishment and maintenance. This is in theory a very flexible technique which allows using combinations of siRNAs directed against different mRNAs and a

complete freedom in scheduling delivery.

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