Concluding overview: looking back, looking forward

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The γ -herpesviruses are a group of related agents which share the same broad strategy for infection of and persistence within the lymphoid tissues of their hosts. Yet in evolutionary terms these agents are sufficiently diverse to display multiple different molecular mechanisms whereby that strategy can be achieved. Attempts are made to relate the different *in vitro* growth transforming capacities of the γ_1 -herpesviruses, the T-lymphotropic γ_2 -herpesviruses and the B-lymphotropic γ_2 -herpesviruses to what is known about the biology of these virus infections in their natural or in experimental hosts. The review then summarizes the evidence linking γ -herpesviruses with oncogenesis and proposes that the diverse spectrum of Epstein–Barr virus and human herpesvirus 8-associated human tumours falls into three pathogenetically distinct categories. Many questions remain unanswered in the areas of γ -herpesvirus biology and disease pathogenesis: resolving these questions will require a broadening of our experimental approaches and a willingness to relinquish ‘single-model’ panaceas.

Keywords: γ -herpesviruses; transformation; persistence; oncogenesis

1. INTRODUCTION

The motivations for this issue were twofold: first, the increasing numbers of newly discovered γ -herpesviruses, particularly viruses of primate origin, and the rapidity with which their genomic sequences are becoming available for phylogenetic analysis; second, the realization that many of these agents are associated with hyperproliferative or frankly neoplastic disease, begging the question whether these diverse associations might be accommodated into some broader conceptual framework. The preceding papers presented overviews of those γ -herpesviruses which are the principal foci of current research, namely the mouse γ_2 agent MHV-68, the γ_1 and γ_2 agents of both New and Old World primates, and the prototypic γ_1 and γ_2 agents of man, Epstein–Barr virus (EBV) and the Kaposi sarcoma-associated human herpesvirus 8 (HHV-8). This selection, omitting several other γ_2 -herpesviruses of non-primate origin, may be viewed by some as too catholic. It is readily acknowledged that in the future these other agents have the potential to inform questions of virus biology just as they are currently informing the study of γ -herpesvirus evolution. For the moment, however, most of the available biological data comes from the viral systems given special attention in this issue and, even confining ourselves to these systems, the picture is already sufficiently diverse as to emphasize how interesting but complex γ -herpesvirus–host relationships can be.

2. EVOLUTIONARY RELATIONSHIPS: CONSERVATION AND DIVERGENCE

As McGeoch (this issue) tells us, genomic sequencing of the γ -herpesvirus subfamily has provided important

insights into the evolution of these agents as well as raising a number of questions about the origin, and in some cases accelerated diversification, of γ -specific genes. Sequence relationships are now the accepted criterion for herpesvirus classification and, to emphasize the importance of this advance, it is worth recalling that for several years after its discovery MHV-68 was thought likely to be an α -herpesvirus by virtue of its ability to replicate in cells from various species *in vitro*, and then possibly a γ_1 -herpesvirus by virtue of its apparent B lymphotropism *in vivo*. It was only later, through genomic sequencing, that MHV-68 was correctly identified as a γ_2 -herpesvirus.

Sequence comparisons, based on eight out of the 42 core virus lytic-cycle genes present in all mammalian herpesvirus genomes, confirm the γ -subfamily as a distinct branch of herpesvirus evolution. However, a central point emerging from this analysis is the antiquity of the split between the γ_1 (lymphocryptovirus) and γ_2 (rhadinovirus) genera, making it clear that the γ_1 and γ_2 herpesviruses of primates have each been separately co-evolving with their primate host species for millions of years. This long evolutionary separation is further reflected by their acquisition of largely distinct sets of γ -specific genes. In the γ_1 -herpesviruses (at least as exemplified by EBV and its Old World primate relatives) most of the acquired genes are unique to the γ_1 genus, have no obvious cellular homologues and are associated with latent rather than lytic infections; these viruses have also acquired a small number of genes with obvious cellular homologues (*FGARAT*, *Bcl2*, *IL-10*) but these are lytic-cycle genes that are also present in some if not all γ_2 -herpesviruses. The γ_2 -herpesviruses each have their own complement of unique γ_2 -specific genes plus a much broader range of genes with obvious cellular homologues

host	virus/host	natural host			experimental host	
		transformation <i>in vitro</i> /target	disease <i>in vivo</i>	conditions for disease	transformation <i>in vitro</i> /target	disease <i>in vivo</i>
New World primates	Cal-HV3/tamarin	+ / B cells	BLPD/lymphoma	? immune impairment	n.t.	n.t.
Old World primates	LCV macs/monkeys	+ / B cells	BLPD/lymphoma	SIV infection	+ / B cells	n.t.
	LCV papio/baboon	+ / B cells	BLPD/lymphoma	? immune impairment	+ / B cells	n.t.
	LCV pongo/orang-utan	+ / B cells	n.t.		n.t.	n.t.
	LCV gor/gorilla	+ / B cells	n.t.		n.t.	n.t.
	LCV pan/chimpanzee	+ / B cells	n.t.		n.t.	n.t.
man	EBV/man	+ / B cells	BLPD/lymphoma	T-cell suppression, HIV infection	+ / B cells	BLPD/lymphoma in New World monkeys
			Burkitt's lymphoma	holoendemic malaria, HIV infection		
			Hodgkin's disease			
			leiomyosarcoma	T-cell suppression; HIV infection		
		? / T cells	T/NK cell lymphomas			
		? / gastric epithelium	nasopharyngeal and gastric carcinomas			

Figure 1. γ_1 -Herpesviruses and neoplastic disease. (BLPD, B-lymphoproliferative disease; n.t., not tested.)

whose identities imply a potential to affect the growth and/or survival of infected cells or the accessibility of the virus to host immune responses—the classification of these genes into latent- and lytic-cycle groupings is as yet less clearly defined.

There is a broad strategic conservation between the γ_1 -herpesvirus and γ_2 -herpesvirus genera in that both sets of viruses appear to target lymphocytes as their principal reservoir for persistence *in vivo* and (based largely on evidence from EBV and MHV-68) appear to establish that reservoir through a transient phase of lymphoproliferation that is associated with virus latent gene expression in infected cells. However, there is functional divergence between the two sets of viruses, and between individual members of the γ_2 genus, in terms of the molecular mechanisms whereby this is achieved. As best exemplified by EBV and HHV-8, individual γ -herpesviruses may infect the same host species and even share the same main target cell lineage, and yet, through their separate evolutionary histories, establish balanced agreements with their hosts that differ significantly in detail.

3. VIRUS INFECTIONS *IN VITRO* AND THE IDENTIFICATION OF TRANSFORMING FUNCTIONS

One important difference between the currently known γ_1 - and γ_2 -herpesviruses is their growth transforming capacity *in vitro* when assayed on appropriate target cells of the natural host.

(a) γ_1 -herpesviruses

As summarized in figure 1 and as first shown for EBV, all γ_1 -herpesviruses thus far identified have the ability to infect mature resting B cells of their natural host *in vitro* and to induce the proliferation of such cells into permanently growing lymphoblastoid cell lines (LCLs) in which every cell carries episomal copies of the viral genome. This is a highly efficient system, which is independent of T-cell help and where upward of 10% of infected cells stably acquire the transformed phenotype. Using EBV as the prototype, transformation is associated with the co-ordinate expression of two non-coding small nuclear RNA species (EBER1 and EBER2), six nuclear antigens (EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C and EBNA-LP), three latent membrane proteins (LMP-1, LMP-2A and LMP-2B) and one or more putative polypeptides encoded by spliced transcripts from the BamHI A region of the genome. Although all known γ_1 -herpesvirus isolates from Old World primates contain this full complement of latent-cycle genes, only EBNA-1, EBNA-2, EBNA-3A, EBNA-3C and LMP-1 are absolutely essential for transformation *in vitro*, while EBNA-LP and possibly LMP-2A are required for optimal transforming efficiency; this clearly implies that the other genes have important functions that are not manifest in the *in vitro* transformation assay. In this context LMP-2A has been shown to interact with the same Src family tyrosine kinases in B cells as the signalling components of the surface immunoglobulin (Ig)/B-cell receptor (BCR) complex. The interaction has at least two biological effects detected in different assay systems: one is to block signal transduction events induced by BCR ligation, in particular the anti-Ig-induced switch from latency into

lytic cycle in EBV-positive B-cell lines *in vitro*; the other is the ability of LMP-2A to act as a surrogate BCR when expressed as a transgene in mouse pro-B cells, and thereby allow surface Ig-negative cells to enter the mature B-cell pool.

This engagement of BCR-like signalling pathways by LMP-2A, while apparently not required for virus-induced transformation *in vitro*, is likely to be critically important for virus biology *in vivo*. At the moment, however, this is a supposition and it is worth noting that the first New World primate γ_1 -herpesvirus to be identified and sequenced appears to have a less extensive range of transformation-associated genes, with no detectable LMP-2 homologue and just one copy of an EBNA-3-like sequence in place of the EBNA-3A, EBNA-3B, EBNA-3C gene block.

Considerable progress has been made, particularly over the past five years, in understanding the functions of the transforming proteins. As discussed by Bornkamm & Hammerschmidt (this issue), while EBNA-1 is critical for maintenance of the viral episomes by ensuring their equal segregation at each cell division, EBNA-2 is a key transactivator of gene expression through its capacity to bind to and activate RBP-J κ , a cellular regulator normally activated by the Notch family of cell signal transducers. EBNA-LP appears to serve as a co-activator of EBNA-2 function, whereas the EBNA-3A, EBNA-3B, EBNA-3C proteins are thought to be competitive regulators of the EBNA-2/RBP-J κ interaction. EBNA-2 directly activates expression of the LMP genes as well as a number of cellular genes, the best described as yet being *c-myc*. The appearance of LMP-1 complements EBNA-2's role in the transformation process by activating a range of cellular transcription factor complexes, most notably NF- κ B and AP-1. These effects are achieved through LMP-1's ability to activate signal transduction pathways normally linked to CD40, a B-cell surface molecule whose ligation is a critical step in cognate T-cell-dependent B-cell activation. The combination of these two pleiotropic effectors, EBNA-2 and LMP-1, initiates the same programme of cellular gene expression, including cell-cycle entry genes, as is normally induced in B cells by specific antigen binding and the receipt of cognate T-cell help. Classical γ_1 -herpesvirus-induced transformation therefore operates through a small number of interventions at key gatekeeper points governing B-cell activation and proliferation. These points lie upstream of the cyclin-dependent kinase and retinoblastoma (RB)–E2F protein interactions, which are often targeted by small DNA viruses in their transformation of non-lymphoid cell lineages. Importantly, γ_1 -herpesviruses have evolved such that their growth transforming programme of gene expression is only initiated in a B-cell environment, that is the cell type in which growth transformation is required as an integral part of the viral life cycle *in vivo*.

(b) γ_2 -herpesviruses

In contrast to the above system, there are no corresponding examples of direct *in vitro* transformation of the lymphocytes of the natural host by γ_2 -herpesviruses. It is of course formally possible that cells at an appropriate stage of differentiation have not been present within the target populations tested *in vitro*, but this appears to be an

increasingly unlikely explanation. Rather one has to consider either that virus-driven lymphoproliferation is not central to the biology of some γ_2 -herpesviruses or that such proliferation does occur but is dependent upon a complex set of cell–cell interactions that are not reproduced *in vitro*.

Despite these caveats, both of the T-lymphotropic γ_2 -herpesviruses of New World primates, *Herpesvirus saimiri* (HVS) from squirrel monkeys and *Herpesvirus ateles* (HVA) from spider monkeys, are capable of transforming T cells from certain other species of New World primate *in vitro*. The T-cell lines that grow out carry episomal copies of the viral genome and are latently infected, but with an unusually high number of cells showing spontaneous activation of lytic-cycle genes. Interestingly, as Fickenscher & Fleckenstein (this issue) describe, viruses of HVS subtype C (HVS-C) can also transform human T cells and the non-permissive nature of the transformants has allowed unequivocal identification of true latent-cycle genes. These are positional homologues of the LMP-1 gene and encode membrane proteins, StP-C and Tip, which collectively mediate interactions with a number of cell-signalling pathways. These include the NF- κ B pathway (through StP-C acting in a manner similar to LMP-1), the microtubule-associated protein kinase pathway (through StP-C association with Ras) and the lck pathway (through Tip acting in a manner similar to LMP-2A). It is interesting that StP-C and Tip, in some respect functional relatives of the EBV LMPs, should be capable of inducing T-cell transformation in the apparent absence of any virus-coded nuclear proteins (i.e. EBNA surrogates). However, in rare circumstances EBV-induced transformation of human T cells *in vitro* has been reported, and in these cells viral latent protein expression was limited to EBNA-1 (the virus genome maintenance protein, for which no HVS functional homologue has yet been identified) and LMP-1 plus LMP-2A. It is also worth noting that the other HVS subtypes and HVA, which are more species-restricted in their T-cell transforming function, each encode just one transformation-associated protein from a gene in the same region of the genome—these proteins, StP-A in the case of HVS sub-type A and Tio in the case of HVA, in each case reproduce some of the signalling interactions shown collectively by StP-C and Tip.

Such genetic divergence combined with functional conservation within the StP/Tip/Tio gene family of HVS and HVA is reminiscent of a similar phenomenon observed among some of the B-lymphotropic γ -herpesviruses. Thus among the very closely related γ_1 -herpesviruses of Old World primates, there is an unusually marked sequence divergence within the LMP-1- and LMP-2A-homologous genes but retention of the key sequence motifs that mediate LMP interactions with cell signalling pathways. The same is true of the *K1/RI* and *K15/RI5* genes of HHV-8 and its Old World primate relatives; these are positional homologues of LMP-1 and LMP-2A, respectively, and also encode membrane proteins whose functional domains, in this case interaction sites with Src family tyrosine kinases, are conserved. Given the positional homology between the StP/Tip/Tio, LMP-1 and *K1/RI* gene blocks (described by

McGeoch, this issue) and the fact that all encode membrane proteins, it is tempting to speculate that these proteins perform broadly equivalent functions in the viral latent cycle. In fact, the available evidence suggests that *K1/RI* are lytic- rather than latent-cycle genes—conceivably an ancestral membrane protein-coding gene acquired early in primate γ -herpesvirus evolution has diversified over time and been selected for different uses in different viruses.

The most closely studied B-lymphotropic γ_2 -herpesviruses, namely MHV-68 and HHV-8, show no obvious B-cell transforming function *in vitro* on cells of their natural hosts. Consequently, as Moore & Chang (this issue) describe, many of the more interesting γ_2 viral genes have been tested individually for activity in standard rodent cell transformation assays. This approach is best developed for HHV-8 where several genes have shown activity in the 3T3 mouse fibroblast assay, including the LMP1 positional homologue *K1*, the as yet poorly characterized *K12* gene and the viral homologues of the interferon regulated factor (vIRF) and of the IL-8 receptor (vIL-8R). The physiological relevance of these activities remains to be determined since most if not all of these genes are silent in HHV-8-associated tumours. Interestingly, however, one of the genes constitutively expressed in these tumours encodes the nuclear antigen LANA-1—besides performing an EBNA-1-like viral genome maintenance function, LANA-1 also binds RB and p53 and, like the RB- and p53-binding proteins of small DNA tumour viruses, can complement cellular oncogenes in the *in vitro* transformation of primary rodent fibroblasts. Several other potentially interesting HHV-8 genes, such as the viral homologues of cyclin D (v-cyclin), IL-6 (vIL-6) and CC chemokines (v-MIPs), appear to be inactive in the rodent cell transformation assays yet may have important roles in deregulating B-cell growth.

Whatever the details, it seems likely that any γ_2 -herpesvirus-associated expansion of the infected B-cell pool *in vivo* will be more dependent upon paracrine factors and/or collaboration with other cell types than is the case for γ_1 -herpesviruses. More speculatively, the mechanism of γ_2 -herpesvirus-induced growth deregulation seems more likely to involve multiple interceptions at downstream positions in several cellular pathways, rather than the γ_1 -herpesvirus model of interception at a few key gatekeeper points. We also need to remember that HHV-8 is associated not just with B-lymphoproliferative disease but also with Kaposi's sarcoma (KS), a tumour of endothelial cell origin. Candidate viral transforming proteins therefore also need to be assayed in endothelial cell culture systems, such as the one in which HHV-8 infection itself is reported to be capable of providing a subtle growth advantage.

4. VIRAL INFECTIONS *IN VIVO* AND VIRAL STRATEGIES FOR PERSISTENCE

We still know relatively little about γ -herpesvirus–host interactions *in vivo*. In the context of the γ_1 -herpesviruses, since these agents are restricted to primates any analysis of experimental virus infection requires transmission either to the naive natural host or to a closely related primate species. As Wang (this issue) describes, EBV

transmission to certain species of New World primate allows one to study acute EBV-associated lymphomagenesis but not the classical events of primary infection and subsequent establishment of the virus carrier state. Consequently, most of what we know about γ_1 -herpesvirus biology *in vivo* comes from the considerable literature of EBV infections in the human, a literature that is essentially descriptive and where hypothesis testing is severely limited. Importantly, a recently developed model in which rhesus macaques are experimentally infected with rhesus γ_1 -herpesvirus appears to reproduce several aspects of the EBV–host interaction. This should allow individual viral gene functions to be analysed *in vivo* using engineered viral mutants.

A similar situation is to be found with respect to HHV-8 and its close relatives in Old World primates. Again, one can learn only so much from the study of HHV-8 infections in man. As Damania & Desrosiers (this issue) describe, experimental infections of rhesus macaques with rhesus γ_2 -herpesviruses are now possible and, particularly when combined with simian immunodeficiency virus (SIV) infection to impair host T-cell responses, can reproduce at least some aspects of γ_2 -herpesvirus-associated disease pathology.

Turning to the T-lymphotropic γ_2 -herpesviruses, very little work has been carried out looking at HVS or HVA infections in their natural host though, as with EBV, these viruses are acutely lymphomagenic on transmission to certain other New World primates. Again, while such a model has been important in dissecting the oncogenic potential of these viruses, it tells us little about the real biology of HVS and HVA infection. This leaves the murine γ_2 -herpesvirus MHV-68 as the exemplar for the analysis of γ_2 -herpesvirus biology *in vivo*, and it is interesting to consider the MHV-68 literature against what is known from the analysis of EBV and HHV-8 infections in man.

(a) *Lessons from EBV and HHV-8*

Comparative studies of the biology of EBV and HHV-8 infections *in vivo* have considerable instructive value since both viruses are believed to target B cells as their principal reservoir for persistence. At the moment much more is known from the EBV system, largely because EBV research is in its fourth decade and HHV-8 research is still in its first. However, EBV's much higher prevalence in the human population is another advantage and, in addition, the association seen in Western countries between delayed EBV transmission and a self-limiting lymphoproliferative disease, infectious mononucleosis (IM), provides invaluable access to the events of primary EBV infection.

As described by Crawford (this issue), and by Boshoff & Weiss (this issue), respectively, both EBV and HHV-8 are transmitted by the oral route. Yet in both instances we do not know the identity of the cells that first encounter orally transmitted virus or support local virus replication, information that remains crucial to a proper understanding of virus biology. In the more intensely studied EBV system, the balance of current evidence favours direct entry into B cells in oropharyngeal lymphoid tissue, although the possibility of initial virus replication in an (as yet undefined) epithelial site within the oropharynx cannot be entirely discounted. Thereafter the

virus induces a transient phase of B-cell proliferation, associated with expression of the full range of latent-cycle genes, apparent both in the blood and in extrafollicular areas of lymphoid tissues. Arguably the naive host provides the virus with a window of opportunity to expand its pool of latently infected cells before immune controls develop. Subsequently, in at least some of these proliferating cells, the growth transforming programme is downregulated and viral antigen expression is either largely or completely switched off as the cells return to the resting phase with a cell surface phenotype (IgD⁺, CD27⁺) characteristic of the long-lived memory B-cell subset. As Bornkamm & Hammerschmidt (this issue) discuss, it is not yet clear whether acquisition of this phenotype is directly imposed by the viral infection *per se* or requires the virus-infected cells to undergo the natural pathway of selection into memory, i.e. by transit through a germinal centre. It will be interesting to see whether HHV-8 likewise becomes segregated into the circulating memory B-cell compartment of its host or has evolved another means of achieving a stable reservoir in the (otherwise predominantly labile) human B-cell system. Perhaps the most important lesson to come from recent work on EBV infection *in vivo* is that cells at different stages of B-cell differentiation can adopt different patterns of viral gene expression, moving first from latent antigen-driven growth transformation to a 'silent' infection in the resting B-cell pool and later, possibly in association with antigen-driven plasmacytoid differentiation, to reactivation into lytic cycle. It seems inherently likely that HHV-8 will also be responsive to differentiation-dependent cellular controls in its colonization of the B-cell system, although the long evolutionary separation of HHV-8 and EBV almost certainly means that the details of the virus–B-lineage interaction will be different.

However well developed the above interactions might be, they alone are not enough to achieve asymptomatic virus carriage *in vivo*. The fact that T-cell-immunocompromised individuals are at increased risk of EBV- and HHV-8-associated disease strongly suggests an important role for the host cellular immune response in stabilizing the virus–host balance. Several, though by no means all, facets of the host T-cell response to EBV infection have been the subject of detailed study over many years and are described by Moss *et al.* (this issue). The study of IM patients has been particularly instructive in showing that a large proportion of the atypical lymphocytosis typifying this disease is made up of activated CD8⁺ T cells that are specific for EBV antigens and not, as had been anticipated, cells with irrelevant specificities that had been co-opted either by 'bystander activation' or by a virus-coded or virus-induced superantigen. Interestingly the response to lytic-cycle antigens is roughly tenfold larger than that to latent-cycle antigens, perhaps reflecting the influence of antigen load on the extent of T-cell amplification. However, it is the total size of the virus-specific response that is most surprising and the realization that much of the response is focused on a few immunodominant epitopes drawn principally from immediate early and/or early proteins of the lytic cycle and from the EBNA-3A, EBNA-3B, EBNA-3C subset of latent-cycle proteins. The implication is that the large lytic antigen-induced response is important in bringing foci of virus replication

under control—however, this is by no means proven and the possibility needs to be borne in mind that (as with certain other herpesvirus systems, including apparently HHV-8) lytically infected cells are partially protected from immune recognition. On the other hand, the evidence from adoptive T-cell transfers with LCL-stimulated T-cell populations strongly suggests that latent antigen-specific effectors are indeed capable of eliminating EBV-transformed B cells *in vivo*. Such T cells are likely to offer the host long-term protection against the re-emergence of lymphoproliferative lesions. Even so these same T cells cannot prevent the establishment and maintenance of a reservoir of EBV-positive resting B cells which, as long as endogenous viral antigen expression is switched off, remain essentially invisible to the immune system. It seems reasonable to propose that active HHV-8 infections are subject to similar T-cell surveillance, though research in this area is just beginning and the identity of the immunodominant antigens is awaited with interest. In particular, does the capacity of the HHV-8 proteins K3 and K5 to inhibit antigen processing in lytically infected cells render the lytic infection much less immunogenic to CD8⁺ T cells than is the case for EBV? And how strong are responses to the HHV-8 latent-cycle proteins expressed in virus-positive malignancies?

Finally, the link between EBV and IM has been important in another context, by demonstrating how the finely balanced, largely asymptomatic, relationship between a γ -herpesvirus and its host can be disturbed by a simple change in the epidemiology of virus infection. Clearly EBV has evolved in a host species where intrafamilial transmission in infancy is the norm, and IM is a novel disease of adolescence or young adulthood arising as a result of a change in this norm. The relationship between the cellular events of IM and those occurring during asymptomatic primary infection remains to be determined. However, one possibility is that the symptoms of IM are essentially immunopathological by-products of the need to expand T-cell responses in order to deal with a larger viral challenge than that usually delivered via oral transmission in infancy. Whether such immunopathology arises via cross-reactive self-recognition by virus-specific CD8⁺ T cells, as Moss *et al.* (this issue) discuss, or via T-cell-secreted cytokines such as TNF- α is not known. However, a role for T-cell-mediated pathology is implicit in the striking parallels observed between classical IM and two rare but frequently fatal complications of primary EBV infection in infancy where hyperacute IM-like symptoms accompany atypical T-cell expansions. These are the familial X-linked lymphoproliferative syndrome (XLP) and the non-familial virus-associated haemophagocytic syndrome (VAHS). In both situations the symptoms of 'fatal IM' appear to be the result of a failure to downregulate T-cell proliferation. In the case of XLP, a feedback pathway that normally regulates the extent of immune CD8⁺ T-cell expansion is not operational, whereas, in the case of VAHS, EBV has itself accessed the T-cell compartment and is driving oligoclonal T-cell proliferation.

(b) *Lessons from MHV-68*

Many of the key questions about γ -herpesvirus infections *in vivo* can be addressed with greater precision in the

MHV-68 model, as the chapters by Nash *et al.* (this issue), and by Doherty *et al.* (this issue), on the biology and immunology of the system, respectively, demonstrate. How far one can extrapolate from this murine model to human γ -herpesvirus infections is a moot point, not least because MHV-68, though clearly a γ_2 -herpesvirus, has its own complement of γ -specific genes only some of which are shared with HHV-8. In particular, it lacks obvious functional homologues of the K1/R1, K15/R15, StP/Tip/Tio proteins encoded by other γ_2 -herpesviruses but does carry four unique genes (*M1–M4*), which are positional homologues of K1/R1 and StP/Tip/Tio.

One particular advantage of a small animal model is the ability to study the early events of primary infection before the immune response develops. Experimental MHV-68 infection of mice via the respiratory route leads to an initial focus of lytic virus replication in lung epithelium and this in turn elicits a marked CD8⁺ T-cell infiltration into the lung. Once again, the bulk of this response is made up of cells specific for one or two immunodominant virus lytic-cycle epitopes, essentially paralleling the situation seen in IM. Priming the host T-cell system with epitope-based vaccines before viral challenge leads to a severe dampening of initial virus replication in the lung. This strongly suggests that these T cells are biologically effective and capable of recognizing lytically infected cells, even though MHV-68 carries an immune evasion gene that is expressed in lytic cycle and is functionally homologous to HHV-8 *K3*. Interestingly a different MHV-68 gene also expressed at this time, *M3*, encodes a multispecific chemokine receptor. Work with *M3* deletion mutants indicates that this secreted protein serves to dampen initial inflammation in the lung and thereby prolongs the period of primary virus replication. MHV-68 has therefore independently acquired at least two immune evasion genes which may well function incrementally, favouring virus replication in primary foci early in the infection and, perhaps more importantly, prolonging subsequent reactivations into lytic cycle in the immune host.

Following this initial MHV-68 replication in the lung, the infection generalizes to the B-cell system either directly or via infection of dendritic cells or macrophages, which carry the virus to lymphoid tissues. There then follows a transient lymphoproliferative phase during which the load of virus-infected B cells in spleen and lymph nodes rapidly increases, then falls to a steady-state level. Though this lymphoproliferative phase is ostensibly similar to the situation in IM, there are also important differences. Thus the expansion is associated with a B-cell-specific programme of viral gene expression, in particular involving the *M2* gene, but it is not solely virus-driven. Studies with gene-knockout animals clearly show that proliferation is dependent upon the presence of CD4⁺ T cells (which are also expanding at this time) and on the integrity of the CD40 pathway mediating B–T-cell signalling. As in IM, this phase is also associated with expansion of the CD8⁺ T-cell pool. However, while the CD8⁺ expansion includes a virus latent antigen (*M2*)-specific component, it is predominantly made up of V β 4-positive T cells that are not demonstrably virus specific and that appear to be induced by some indirect (super-antigen-like?) mechanism that is again dependent upon

host	virus/host	natural host		experimental host		
		transformation <i>in vitro</i> /target	disease <i>in vivo</i>	conditions for disease	transformation <i>in vitro</i> /target	disease <i>in vivo</i>
non-primates	MHV-68/mouse	—	?B lymphoma	?immune impairment	n.t.	n.t.
New World primates	HVS/squirrel monkey	A / —	—		+ / T cells	T lymphoma in
		B / —	—		+ / T cells	New World
		C / —	—		+ / T cells	monkeys
	HVA/spider monkey	—	—		+ / T cells	
Old World primates	RV1 macs/monkeys	—	?retroperitoneal fibromatosis		n.t.	n.t.
	RV2 macs/monkeys	—	B cell hyperplasia arteriopathy	SIV infection SIV infection	n.t.	n.t.
man	HHV-8/man	? / endothelial cells	Kaposi's sarcoma	T-cell suppression, HIV infection	n.t.	n.t.
			primary effusion lymphoma	T cell suppression, HIV infection		
			multicentric Castleman's disease	T-cell suppression, HIV infection		

Figure 2. γ_2 -Herpesviruses and neoplastic or associated diseases. (n.t., not tested.)

the presence of both B cells and CD4⁺ T cells. The implication is that MHV-68, which itself lacks direct B-cell growth transforming function, has evolved mechanisms for colonizing the B-cell system that are T-cell dependent. Furthermore, as tracking of latently infected B cells has shown, this process of colonization appears to involve transit through germinal centres. Long-term latency is established in splenic B cells (and probably also macrophages) though the details of this virus–cell interaction and the identity of the B-cell subset involved remain to be determined.

One important lesson from the MHV-68 system is the apparent dissociation between virus replication in the lung, the lymphoproliferative (IM-like) phase of viral dissemination and the subsequent establishment of latency in the spleen. Latency can be established in circumstances where vaccine responses have greatly reduced lung replication and where the absence of CD4⁺ T cells has precluded the initial expansion of infected B-cell numbers in the spleen. Indeed there even appear to be (naturally occurring?) murine γ_2 -herpesvirus strains that are close relatives of MHV-68 but which lack the unique *MI–M4* gene complement, including *M2*, and can establish persistence without involving a lymphoproliferative phase. By contrast, examples of wild-type EBV isolates that lack the transforming function are extremely rare and their transmissibility is still in question, while no HHV-8 isolates with equivalent deletion of a γ -specific gene complement have as yet been identified. Furthermore, all known strains of HVS, a virus that like MHV-68 can be isolated from naturally infected host animals by growth in a permissive cell culture system, appear to retain the StP locus as well as the lymphocyte transforming function; this implies that this function is important for the survival of HVS in the wild.

5. γ -HERPESVIRUSES AS ONCOGENIC AGENTS IN ANIMAL MODELS

The possibility that MHV-68 is B lymphomagenic in its natural host has excited considerable interest, particularly since the viral genome shares many of the cell-homologous genes that are also present in HHV-8 as well as a series of unique genes positionally homologous to *K1* and *LANA-1*. However, as Nash *et al.* (this issue) describe, the oncogenic potential of the virus remains *sub judice*. In some studies lymphoma incidence was increased in MHV-68-infected animals, especially following T-cell suppression, but there is as yet no consistent evidence for the presence of the viral genome in all tumour cells. The isolation of a MHV-68 genome-positive B-lymphoma cell line, S11, from one such animal has provided an important *in vitro* model of virus latency in B cells but could conceivably have been derived from a tumour cell that had become coincidentally infected *in vivo*.

By contrast, the T-lymphotropic γ_2 -herpesviruses of New World primates, HVS and HVA, are clearly lymphomagenic but, as far as is known, not in their natural hosts. Tumours are only induced in certain species of New World and (for HVS-C) Old World primates for which these viruses also display T-cell transforming activity *in vitro* (table 2). The rapid appearance of these acute peripheral T-cell lymphomas and their presentation as

pleiomorphic lesions at multiple sites all suggest that the processes of *in vitro* transformation and *in vivo* lymphomagenesis are essentially analogous.

A similar conclusion can be drawn with respect to the γ_1 -herpesviruses of primates, except that here the viruses have *in vitro* transforming activity for B cells of their natural host and, where tested, can cause B-lymphoproliferative disease in that host under conditions either likely or known to have been associated with T-cell impairment. As described by Epstein (this issue) and by Wang *et al.* (this issue), the γ_1 -herpesviruses of baboons (LCV papio) and of tamarins (Cal-HV3) were both initially isolated from B-cell lymphomas that arose spontaneously in primate colonies, whereas the γ_1 -herpesvirus of one macaque species was first isolated from B-cell lymphomas arising in animals following SIV-induced immunosuppression (figure 1). The multifocal presentation of these tumours closely resembles that of the B-lymphoproliferative lesions induced in certain New World primate species by experimental infection with high doses of EBV. Again in these experimental EBV infections there was a high correlation between lymphomagenicity *in vivo* and the ability of the virus to transform cells of the susceptible species *in vitro*.

The development of rhesus macaque colonies that are free of their endogenous γ -herpesviruses opens up new possibilities for studying the pathogenesis of virus-induced disease. Infection with the rhesus γ_1 -herpesvirus has already been shown to reproduce many of the events of primary EBV infection, whereas infection with the rhesus γ_2 -herpesvirus, combined with SIV-induced T-cell suppression, induces arteriopathy and B-cell hyperplastic lesions similar to those seen in multicentric Castleman's disease (MCD) (figure 2).

6. γ -HERPESVIRUSES AS ONCOGENIC AGENTS IN MAN

The one advantage that the study of human viruses in their natural host has over animal models is the size of the infected host population, hence the ability to identify virus-associated diseases too rare to be reproduced experimentally. The present list of EBV- and HHV-8-associated tumours, described by Crawford (this issue), and by Boshoff & Weiss (this issue), respectively, is indeed surprisingly varied (figures 1 and 2) but is classifiable into three pathogenically distinct tumour groups.

(a) *B-lymphoproliferative lesions evolving to malignancy*

In circumstances where host T-cell surveillance is impaired, both EBV and HHV-8 infections of the B-cell system can induce lymphoproliferative foci, which histologically may resemble preneoplastic lesions but which can progress further to frank malignancy. In the case of EBV, these lesions are most frequently seen in post-transplant recipients during the period of maximal T-cell suppression and in end-stage AIDS patients. They arise as oligoclonal or monoclonal foci of EBV-transformed B cells in which the proliferating cell compartment expresses the full spectrum of EBV latent proteins, although there may be some intraclonal heterogeneity in latency patterns at the single cell level associated with different degrees of

lymphoblastoid and/or plasmacytoid differentiation. In their initial form these tumours are diploid with no obvious cellular genetic changes, though such changes (including *c-myc* translocations) may be detectable in later onset post-transplant tumours; the latter, also EBV-positive, tend to be monomorphic in appearance, occasionally with some downregulation of viral gene expression. The implication is that the initial virus-driven lesions have the potential for further clonal evolution and/or selection, though there are very few examples where the process has been documented in an individual patient over time. Interestingly such tumours are very rarely if ever observed in the absence of immune impairment, indicating the efficiency with which the T-cell system normally controls a resident EBV infection.

The relationship between HHV-8 and MCD is somewhat similar in that MCD typically presents as a virus-positive lesion in T-cell-immunocompromised individuals, most commonly in HIV-infected male homosexual cohorts where HHV-8 prevalence is unusually high. However, a histologically similar lesion is seen in individuals without overt immunosuppression and here up to 50% of the cases are also HHV-8-positive. All HHV-8-associated cases of MCD contain a population of virus-infected plasmablasts that, by Ig gene rearrangement, are initially polyclonal and derived from naive (pregerminal centre) IgM κ -bearing B cells. Virus gene expression in these cells is still not clearly defined but often includes expression of the viral IL-6 homologue, raising the possible role of cytokine growth stimulation in MCD development. These lesions can also occasionally progress to frank HHV-8-positive plasmablastic lymphoma of monoclonal origin, though the associated cellular genetic changes involved in clonal selection are not yet defined.

(b) *Other, more complex, forms of B-cell lymphoma*

Both EBV and HHV-8 are associated with other lymphomas of B-cell origin whose pathogenesis is distinct from the lesions described above (§6(a)).

In the case of EBV, the clearest example is Burkitt's lymphoma (BL), a tumour of germinal centre B-cell origin with a characteristic chromosomal translocation involving transposition of the *c-myc* oncogene into an Ig gene locus. Although HIV infection is a risk factor for BL, interestingly this tumour does not arise in the setting of gross immune impairment; rather the two principal cofactors (holoendemic malaria for endemic BL and HIV-induced lymphadenopathy for AIDS-BL) are both associated with a hyperstimulation of germinal centre activity. Essentially all endemic BL and 30–40% cases of AIDS-BL are EBV genome-positive and display a form of latency in which only a single latent cycle protein, EBNA-1, is detectably expressed. The role of EBV in Burkitt's lymphomagenesis remains unknown, although the consistency with which the virus is observed (especially in endemic BL) is strong circumstantial evidence for a causative link.

There are parallels between the EBV-BL association and that between HHV-8 and primary effusion lymphoma (PEL), an extremely rare tumour of post-germinal centre B-cell origin with a distinct phenotype, mode of presentation and association with intense immunosuppression. PEL cells consistently carry the

HHV-8 genome and in at least some cases are co-infected with EBV; the derivation of HHV-8-positive, EBV-negative PEL cell lines has provided a valuable *in vitro* model of latent HHV-8 infection in which a number of viral genes (including v-cyclin and LANA-1) are constitutively expressed. The role played by HHV-8 in tumour pathogenesis, and the importance of immune impairment in that context, are not known—conceivably the impairment may allow HHV-8 to access a B-cell population from which it is normally excluded.

Another aetiologically complex tumour of B-cell origin, Hodgkin's disease (HD), is linked to EBV in *ca.* 40% of cases arising in the immunocompetent Caucasian population and in virtually all cases of AIDS-associated HD. Here the malignant cell population is of post-germinal centre cell origin, carrying hypermutated Ig genes often with stop codons, which, in normal B-cell differentiation, would have led to counter-selection and cell death. Survival of the tumour progenitor cell beyond the germinal centre stage may, in EBV-positive cases, be rendered possible by the dual actions of LMP-1 and LMP-2A. Both proteins are highly expressed in the malignant cell clone and together can substitute for signals (from CD40 ligation and antigen-bound surface Ig) normally required for positive selection into memory. An effector role for LMP-1 in particular is strongly supported by the finding that in several EBV-negative cases of HD the tumour cells have acquired an inactivating mutation of the *I κ B- α* gene, the effect of which is to induce constitutive activation of the NF- κ B pathway, just as LMP-1 expression does. At present, therefore, scenarios of virus-associated lymphomagenesis are easier to construct for HD than for either BL or PEL.

(c) *Tumours of non-B-cell origin: accidents of viral access to atypical target cells?*

The association between HHV-8 and Kaposi's sarcoma, a tumour proposed by Boshoff & Weiss (this issue) to be of lymphatic endothelial origin, is extremely strong and extends to all three (classical, endemic and HIV-associated) forms of the disease. Though the point remains controversial, it is possible that HHV-8 infection of endothelial cells or their precursors is a cul-de-sac that the virus accidentally enters under conditions of impaired immunosurveillance *in vivo*. This would place KS in the same category as several EBV-associated tumours of non-B-cell origin, i.e. as accidents of viral access to atypical target cells. Importantly, KS lesions also show a progression to frank malignancy through different histological stages and so it may be possible to follow viral gene expression initially within the subset of virus-infected spindle cells through to that seen in virus-positive monoclonal lesions. This may uncover flexible patterns of expression, since several of the HHV-8 genes conventionally considered as lytic-cycle genes (for example v-MIPs) encode angiogenic factors that could influence the early development of KS lesions, whereas expression may be more restricted at later stages. Again the key contributions made to KS cell growth by the virus remain to be determined, but potential effectors of cellular change are LANA-1, the virus-coded cyclin homologue and the virus-coded inhibitor of apoptosis v-FLIP. The observation that KS lesions may regress with a recovery of host

T-cell competence suggests that at least some of these viral antigens could be targets for the CD8⁺ T-cell response.

There is a range of EBV-associated malignancies that likewise may arise through the virus accessing atypical target cells. The most clear-cut, and to some extent the closest parallel to KS, is the leiomyosarcoma to which heavily immunocompromised patients (especially children) are prone and which are EBV-positive in every case yet examined. How the virus contributes to neoplasia in this unusual target cell type is completely unknown at present, although there is a histological progression from benign to malignant stages of the disease that needs to be characterized virologically. Other examples of a similar principle, it could be argued, include EBV's associations with a range of tumours of T or NK cell origin as well as with undifferentiated carcinomas of the nasopharynx (NPC) and of gastric mucosa. None of these malignancies is associated with immune impairment *per se* and so the factors that predispose to virus infection of the relevant target cell population remain to be determined. In each tumour type, viral antigen expression is limited to EBNA-1 with varying levels of LMP-2A and/or LMP-1 transcription. The pathogenesis of these malignancies is a subject of intense scrutiny at present, following recent results suggesting that under particular *in vitro* conditions EBV can induce proliferation of T cells and also extend the doubling capacity of gastric epithelial cells through forms of infection that do not require the B-cell-specific pattern of full latent gene expression. Arguably the controls that so closely link virus latent gene transcription to cell differentiation in the B-cell lineage are no longer operative when the virus accesses atypical cell types and, in particular, expression of the LMPs may be released from its usual constraints. The consequences are a markedly increased oncogenic risk. Indeed the risk can be dramatically high as, for instance, in the case of those children who present with primary infection manifesting as VAHS—this condition is known to be linked to atypical virus entry into the T-cell pool and, of those children who survive the acute disease, a high proportion develop EBV-positive T-cell lymphoma within a further one to two years.

7. CONCLUSIONS

The γ -herpesvirus subfamily offers significant challenges to scientists working in a number of interrelated disciplines: virus evolution; the virus–cell interactions involved in persistence; immune responses to viral infect-

ion; and the pathogenesis of virus-associated malignancies. As the number of agents available for study increases, we need to be mindful of the danger of seeking 'single-model' solutions to each of the above issues. Rather we should be aware of the depth of evolutionary time over which the γ_1 and γ_2 genera have been separated and recognize that only quite closely related viruses, such as the γ_1 -herpesviruses of Old World hosts or the T-lymphotropic γ_2 -herpesviruses of New World hosts, are likely to have struck the same detailed arrangements with their hosts. We should also strive to make a virtue of the diversity that exists even among these close relatives. For instance, what is driving the accelerated diversification of latent genes in γ_1 -herpesviruses, or of the *K1/R1*, *K15/R15* and *StP/Tip/Tio* genes in γ_2 -herpesviruses? And what is the origin of the allelic polymorphism at loci such as *EBNA-2* in EBV and *K15* in HHV-8?

An important priority must be to look again at the interaction of γ_2 -herpesviruses with their natural hosts. While we might provisionally accept that virus-driven lymphoproliferation is a key feature of γ_1 -herpesvirus infection, is this necessarily a requirement for γ_2 -herpesviruses? It is still possible to argue that the T-cell transforming functions of HVS and HVA are experimental artefacts and that these viruses have evolved to put the StP/Tip/Tio proteins to alternative use in their natural host, perhaps as lytic-cycle genes. The true significance of unique γ -specific genes or of viral homologues of cellular genes can only be determined in infections in the natural host, a fact recognized both by Wang (this issue) and by Damania & Desrosiers (this issue) in their recent work with γ_1 and γ_2 primate viruses, respectively. In this context the MHV-68 system has the clearest potential to dissect viral gene functions *in vivo* and the analysis of gene-deleted viruses is eagerly awaited.

Where then does this leave the γ -herpesviruses as oncogenic agents? I would argue, from the evidence of EBV and HHV-8's association with diverse human tumours, that most if not all members of this subfamily will have some oncogenic potential. Whether the precise experimental conditions can be found to reveal that potential is another question, and to search for such conditions should not be allowed to become an end in itself. A more productive strategy might be to integrate studies of γ -herpesvirus more closely with work on Marek's disease virus, an α -herpesvirus by genomic criteria, yet one which, atypically, has acquired transforming genes and is lymphomagenic. Exceptions to the rules have a habit of improving our understanding of what the rules really are.