Review

Epstein–Barr virus in Hodgkin’s disease

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Summary

Epstein–Barr virus (EBV), a B-lymphotropic herpesvirus widespread in human populations, is carried by most individuals as an asymptomatic lifelong infection. Much progress has been made in our understanding of virus infection/persistence, and in the role of the cytotoxic T lymphocyte response in control of that infection. This same virus is linked to several malignancies, including endemic Burkitt’s lymphoma, post-transplant lymphoproliferative disease and to many cases of Hodgkin’s disease (HD). Recent evidence showing that HD, like the other EBV-associated lymphomas, is of B-cell origin suggests that the pathogenesis of these malignancies may share more common ground than previously thought. The biology and cytotoxic T-cell control of primary and persistent EBV infection, and the links between EBV and all three lymphomas are reviewed. The expression of viral antigens in EBV-positive HD raises the possibility of developing tumour immunotherapy, using relevant components of the EBV-specific T-cell response; progress to date, and future prospects for immune control of EBV-positive HD are discussed.

Key words: Burkitt’s lymphoma, cytotoxic T-lymphocyte response, Epstein-Barr virus, Hodgkin’s disease, immunotherapy, post-transplant lymphoproliferative disease, viral pathogenesis

Introduction

Epstein–Barr Virus (EBV) is the best known of a subfamily of herpesviruses, the so-called lymphocryptoviruses, that are only found among Old World primates, are genetically highly related to one another and share many common biological features. In particular these viruses (i) are highly B lymphotropic, targeting B cells through a specific interaction between their major envelope glycoprotein and a B-cell-associated complement receptor molecule CR2, and (ii) have potent growth transforming ability for target B cells in vitro, yet (iii) are able to persist within the B-cell system in vivo as a largely asymptomatic infection. This highly stable virus: host relationship, the product of millions of years of virus: host co-evolution, would be purely of academic interest were it not for the finding that a range of different human malignancies are EBV genome-positive.

Arguably one can now begin to classify these malignancies into two broad categories: those that are of B-cell origin and therefore arise as rare accidents of the normal process of virus persistence within the B-cell system; and those that are of non-B-cell origin and arise as a result of the virus accessing an unusual target cell type. In the first category would be EBV-positive tumours such as Burkitt’s lymphoma and immunoblastic lymphoma of the immunosuppressed; in the second category would be nasopharyngeal carcinoma, gastric carcinoma, T-cell/natural killer (NK) cell lymphoma and leiomyosarcoma of the immunosuppressed. Very important in the present context therefore are the recent results of immunoglobulin (Ig) gene analysis in Hodgkin/Reed–Sternberg (HRS) cells which clearly show that classical Hodgkin’s Disease (HD) is a tumour of B-cell origin [1]. HD therefore belongs in the first category of EBV-associated malignancies and, if we are to understand its pathogenesis, then we need to focus first on the normal interactions of EBV with the B-cell system.

EBV: B-cell interactions

Lytic (virus replicative) infection

EBV is normally transmitted via oropharyngeal secretions, infectious virus being detectable at low titres in the throat washings of most asymptomatic virus carriers and at high titres in the throat washings of infectious mononucleosis (IM) patients undergoing primary EBV infection [2]. The oropharyngeal cells that sustain virus replication have not yet been unequivocally identified. However, the original notion that these are of epithelial origin has been difficult to reconcile with subsequent work on tonsillar tissues from IM patients where evidence of viral infection (both lytic and latent) is entirely restricted to lymphoid cells [3]. It is possible, therefore, that in most circumstances the initial target cell for orally transmitted EBV is a locally infiltrating B lymphocyte and that, in this particular local environment, these cells can sustain a lytic infection. Certainly B lymphocytes are capable of replicating EBV, albeit in-
efficiently, following experimental infection in vitro and, importantly, cells naturally infected in vivo are triggered into lytic cycle when explanted into culture [4]; this suggests that they could similarly be responsive to physiological induction signals in vivo.

The events of the EBV replicative cycle are essentially similar to those seen for all herpesviruses in that there are three broad phases of gene expression involving sequentially the immediate early, early and late viral genes. Many of the early genes (encoding key enzymes involved in viral DNA replication) and late genes (encoding virion structural proteins such as the virus capsid antigen complex, VCA) have recognisable homologues throughout the herpesvirus family; interestingly, however, EBV carries a number of immediate early and first wave early genes such as BZLF1, BRFL1 and BMLF1 that are unique to lymphocryptoviruses and encode transcriptional transactivators important in the early progress of the lytic cycle.

Latent infections

Though the very early events of primary infection are probably localised to the oropharynx, by the time the clinical symptoms of IM appear the infection has also generalised throughout the B-cell system. This appears to be achieved through a latent growth transformation in which the virus drives the rapid expansion of B-cell clones in a manner similar to that seen on the experimental infection of resting B cells in vitro to form lymphoblastoid cell lines (LCLs) [5]. Certain the same sets of viral latent gene transcripts are detectable in infected B cells in the two situations [6, 7], and immunohistochemical analysis of tonsillar tissue from IM patients reveals the presence of large numbers of EBV-infected B lymphoblasts, usually in extrafollicular areas [3]. Arguably the acquisition by lymphocryptoviruses of this unique set of ‘growth transforming’ genes reflects the importance of the initial virus-induced B-cell expansion to the successful establishment of virus latency/persistence within the lymphoid system.

The identity of these genes, and the functions of their protein products, is key to any understanding of EBV-associated tumourigenesis. They are briefly described here in the context of virus-induced B-cell transformation as seen in vitro [reviewed in 8]. Viral transcription is initiated from the BamHI W promoter, leading to the co-expression of two Epstein–Barr nuclear antigens EBNA2 and EBNA-leader protein (EBNA-LP). EBNA2 is a transcriptional transactivator with specificity for a number of viral and cellular genes, and operates at least in part through binding to a ubiquitous cellular regulator of transcription, RBP-Jk [9]; EBNA-LP co-operates with EBNA2 to optimise transcriptional activation but through an unknown mechanism. A subsequent switch from the BamHI W to the EBNA2-dependent BamHI C promoter is coincident with a broadening of EBNA expression to include the EBNA3 family of proteins (EBNAs 3A, 3B, 3C) and EBNA1 [10, 11]. The EBNA3 proteins appear to regulate EBNA2 activity by competitive binding of RBP-Jk [12], whereas EBNA1 binds to the origin of plasmid replication (ori-p) on the viral genome and is essential for subsequent maintenance/segregation of the viral episome in proliferating cells [13]. A second target for EBNA2-mediated transactivation are the genes encoding the latent membrane proteins LMPs 1 and 2. Of these LMP1 is the major effector, along with EBNA2, of virus-induced cellular change. LMP1 acts as a constitutively active (i.e., ligand-independent) receptor at the cell membrane in a manner analogous to the (ligand-dependent) tumour necrosis factor (TNF) receptor family of cell surface receptors [14]. These transmit signals through binding/oligomerisation of TNF receptor associated factors (TRAFs), leading to a variety of downstream effects that include the activation of at least two sets of broad-spectrum cellular transcription factors, NFκ-B and AP-1 [reviewed 15]. Many of the effects of LMP1 on the resting B-cell phenotype, such as growth promotion, upregulation of cell surface adhesion molecules and the enhancement of cell survival capacity, are in fact transiently seen following ligation of CD40, a member of the TNF receptor family that is preferentially (but not exclusively) expressed on B cells [16]. The second membrane protein, LMP2, also appears to be important, though not essential, for the in vitro transformation process [17]; its mechanism is only partly understood, but it is known to bind members of the src tyrosine kinase family via an ITAM motif in the LMP2A molecule’s intracytosolic N-terminal domain. One consequence of this is an ability to interfere with signaling via B-cell surface receptors (such as immunoglobulin) that transmit via src tyrosine kinases; possibly this could ‘energise’ the latently infected B cell to physiological signals in vivo; in particular it might prevent inappropriate activation into lytic cycle [18]. In addition, recent work with transgenic mice suggests that LMP2 may be able to substitute for B-cell receptor signaling, such that B cells lacking a receptor, and which therefore would not normally survive, can nevertheless continue development and populate the spleen [19]. Finally, there are in addition another family of highly spliced viral transcripts expressed from the BamHI A region of the genome that are detectable in latently-infected growth transformed cells; they have the capacity, through alternative splicing, to encode a number of small polypeptides, but the existence and/or functional importance of such products remains to be determined.

One important aspect of virus latency in vivo, which to date has not been reproducible in any conventional in vitro system, is the switch in viral gene expression that seems to accompany the transition from primary to persistent infection [6]. This is characterised by down-regulation of the BamHI W and C promoters such that none of the above EBNA transcripts are expressed; the true nature of this form of latency is not understood, but it appears to be associated with the continued detectability in peripheral blood B cells of the LMP2A and...
BamHI A transcripts, and in some cases with trace detection of an EBNA1 transcript from an alternative promoter in the BamHI Q region of the genome. Interestingly, by this stage the virus-infected B cells are in the resting state, and are selectively harboured within the IgD-ve component, a marker of the long-lived memory cell population [20]. This is consistent with the notion that EBV in some way mimics the signal delivered to a normal B cell by coincident exposure to its cognate antigen (via surface Ig) and to an antigen-specific T helper cell (via a CD40/CD40 ligand interaction), thereby delivering the infected B cell into the 'antigen-exposed' memory B-cell pool. That transition normally requires the movement of an antigen-stimulated B cell through a germinal centre and its subsequent selection based on improved affinity for antigen; whether EBV-driven selection into memory necessarily involves the trafficking of the infected cell through the germinal centre is unknown, but is an important question to resolve vis-à-vis EBV's potential contribution to HD pathogenesis.

These qualitative changes associated with the transition from primary to persistent infection occur in parallel with quantitative changes in viral load. Thus the number of latently-infected B cells detectable in the circulation falls from its peak (up to 10% in rare cases [21], 0.1%-1% in a typical case [22, 23]) to much lower values [24]. At the same time, the shedding of infectious virus into the throat is also reduced in titre. However, the virus infection is never completely cleared, and convalescent IM patients continue to harbour detectable numbers of latently-infected B cells in the blood, and to shed low levels of infectious virus in the oropharynx [2]. This reflects the situation that is found in all asymptomatic virus carriers, where virus persistence occurs in the face of on-going humoral and cell-mediated immune responses [25]; how such persistence is achieved is of fundamental interest since the persistent state is the one from which virus-positive malignancies such as HD arise. To understand this we must first look at those facets of the virus-induced immune response which are thought to be important in controlling both primary and persistent infection.

Cytotoxic T-lymphocyte control of EBV infection

In symptomatic IM there is a huge expansion of activated T lymphocytes, mainly of CD8-positive type. It had been speculated that this is largely a non-antigen-specific polyclonal expansion of the CD8-positive T-cell system, or an expansion of a particular Vβ subset induced by a virus-coded superantigen. More recent work has shown that this is not the case; rather the expansion shows all the hallmarks of a virus-driven, antigen-specific response [26]. Thus particular Vβ subsets are expanded, but different subsets are involved in different patients; furthermore the expansions are associated with markedly oligoclonal (rather than polyclonal) T-cell response repertoires [27]. Most importantly, some of the most dramatic expansions have been functionally characterised and shown to be specific for particular EBV epitope peptides presented in the context of particular major histocompatibility complex (MHC) alleles.

Surprisingly the most immunogenic peptides are derived from immediate early and early lytic cycle proteins, for example the human leukocyte antigen (HLA) B8-restricted RAKFKQLL epitope from BZLF1, and the HLA A2-restricted GLCTLVAML epitope from BMLF1 [28]. Callan et al. [29] used tetramer MHC-peptide complexes to directly measure frequencies of cytotoxic T lymphocytes (CTLs) specific for some of these epitopes in patients with IM, and found surprisingly high frequencies, with up to 44% of CD8-positive T cells specific for the BZLF1 RAKFKQLL epitope in HLA B8 individuals, and up to 6.6% specific for the BMLF1 GLCTLVAML epitope in HLA A2-positive donors.

This work with IM patients was the first clear demonstration that lytic antigens do elicit a strong CTL response. Even though these reactivities are also represented in CTL memory, their presence had previously been undetected because most in vitro protocols examining the content of memory used autologous LCL cells as the stimulus, and very few such LCL cells express lytic cycle antigens. What is now clear, however, is that virus replicative lesions are subject to CTL control in both primary and persistent phases of the infection.

Latent EBV infections also elicit CTL responses, and generally these are better characterised in terms of antigen specificity and immunodominance than responses to lytic cycle antigens. This reflects the availability of LCL cells as a source of latently-infected stimulators for in vitro studies, and the efficiency with which they induce the reactivation of CTLs from memory. CTL responses to latent antigens can be found in both primary infection and in memory, although they are five to ten fold less frequent than responses to lytic epitopes when measured using tetrameric MHC-peptide complexes [30].

Whilst for any one individual peptide epitope choice is determined by the HLA type, there nevertheless appears to be a hierarchy of immunodominance amongst the EBV latent proteins, which is seen across a wide range of HLA backgrounds, and in both primary and memory responses. Thus in most donors, the response to LCL stimulation in vitro is dominated by T-cell clones reactive to epitopes from the EBNA 3A, 3B, 3C family [31, 32]. Reactivities to some of the other latent antigens such as EBNA2, Lp and LMP1 and 2 are subdominant, and are generally only seen when cloning or selective stimulation techniques are used. Examples of some strongly immunodominant, and of subdominant, epitopes and their HLA restricting alleles are given in Table 1.

The molecular basis of this pattern of immunodominance is not clear, but may represent the ease with which a particular latent protein enters the antigen processing pathway. An extreme example of this is seen with EBNA1. Until recently, no T-cell responses to EBNA1
EBV and B-cell lymphomas

The frequent development of EBV-positive B-cell lymphomas in immunocompromised patients who have recently undergone bone marrow transplantation, or who are taking immunosuppressive drugs following solid organ transplantation, in itself illustrates the importance of the T-cell system in controlling EBV infection. These tumours are largely if not completely EBV-driven, and although generally oligoclonal in their early stages as shown by immunoglobulin markers, tend to progress to monoclonality in many cases [35], perhaps reflecting outgrowth of the fastest growing clone. Disease risk is influenced not only by the duration and degree of immunosuppression, but also by the patient’s EBV status at the time of transplantation, the risk being increased by the occurrence of primary EBV infection while immunosuppressed [36, 37]. The tumour cells invariably show an LCL-like phenotype, expressing the full complement of EBV latent antigens [38, 39]. If immunosuppression is reduced they often regress, suggesting that they are susceptible to a restoration of T-cell control [40].

To date two studies have investigated the therapeutic efficacy of CTL therapy for immunoblastic lymphomas arising in bone marrow transplant recipients, where the tumours arise in B cells of donor origin. In 1994 Papadopoulos et al. [41] treated five such immunoblastic lymphoma patients with peripheral blood mononuclear cells (PBMCs) from the bone marrow donor. This achieved tumour regression in all patients, but was accompanied by severe graft-versus-host disease (GVHD) in most cases. In the second study [42], two bone marrow recipients with high EBV loads indicative of elevated lymphoma risk [37, 43], and one recipient who had already developed lymphoma, were given EBV-specific polyclonal CTL preparations that had been reactivated in vitro from the bone marrow donor. All showed a reduction in EBV DNA levels, and in the patient with lymphoma, the tumour regressed. A further seven patients also received prophylactic CTLs, and none of these developed EBV reactivation or lymphoma over the ten month follow-up period. There were few problems with GVHD with this regime. The infused cells were tagged with the neomycin resistance gene, and were directly detectable in the peripheral blood for at least 10 weeks by PCR amplification for the marker gene. Subsequently, persistence of these marked cells could be detected by in vitro reactivation for longer periods, and in one case their reappearance to levels directly detectable in peripheral blood was observed in response to a rise in the EBV load in vivo [44].

Three factors probably contribute to the success of T-cell therapy for PTLD in bone marrow transplant patients. Firstly, tumour cells express the full range of EBV latent antigens, including the immunodominant EBNA3 group, and appear to have good antigen presenting function. Secondly, because the tumour is of donor origin, it is relatively easy to obtain the appropriate autologous EBV-specific CTLs because they can be made from the immunocompetent bone marrow donor. The final, and perhaps most important, point is that infused CTLs are entering an environment permissive for their growth, where the recipient has an 'empty' bone

had been detected, since this protein is protected from intracellular processing by the presence of an internal glycine-alanine repeat (GAR) region [33]. CTL clones specific for targets expressing a GAR-deleted EBNA1 molecule have recently been discovered in several donors, and the sequences of two epitopes within EBNA1 published [34]. These responses may actually be primed in vivo by EBNA1 released from infected cells and reprocessed by dendritic cells; to what extent responses to other EBV proteins are also induced by such 'cross-priming' remains to be determined.

Thus there is now a large body of evidence suggesting that the cytotoxic T-cell response to EBV is effective in controlling physiological infection. Since some of the EBV latent antigens are expressed in EBV-positive malignancies, could this EBV-specific response be used in their treatment? The aim would be to boost CTL responses to viral antigens expressed by tumour cells. This could be done in one of two ways: either by transferring back to the patient autologous in vitro-expanded CTLs specific for one or more of the expressed EBV antigens, so-called adoptive transfer; or by boosting the patient’s own immune response in vivo by vaccination with relevant T-cell epitopes from one or more of these EBV antigens. To explore this question, we shall first look at the two best known EBV-positive B-cell malignancies, post-transplant lymphoproliferative disease (PTLD) and Burkitt’s lymphoma, in relation to their susceptibility to T-cell control, before examining the possibility of CTL therapy for EBV-positive HD in more detail.

### Table 1 Examples of well-defined EBV-encoded latent CTL epitopes restricted through HLA alleles that are relatively common in Caucasian populations.

<table>
<thead>
<tr>
<th>EBV antigen</th>
<th>Epitope location</th>
<th>Epitope sequence</th>
<th>HLA restriction</th>
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</thead>
<tbody>
<tr>
<td><strong>Dominant epitopes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBNA3A</td>
<td>158–166</td>
<td>QAKWRQLQTL</td>
<td>B8</td>
</tr>
<tr>
<td>EBNA3A</td>
<td>325–333</td>
<td>FLRGRAYGL</td>
<td>B8</td>
</tr>
<tr>
<td>EBNA3B</td>
<td>399–408</td>
<td>AFDKSDAK</td>
<td>A1</td>
</tr>
<tr>
<td>EBNA3B</td>
<td>416–424</td>
<td>IVTDASFV</td>
<td>A11</td>
</tr>
<tr>
<td>EBNA3C</td>
<td>258–266</td>
<td>RRIYDIEL</td>
<td>B27</td>
</tr>
<tr>
<td>EBNA3C</td>
<td>281–290</td>
<td>EENLIDVFR</td>
<td>B44.02</td>
</tr>
<tr>
<td><strong>Subdominant epitopes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EBNA1</td>
<td>574–582</td>
<td>VLFDAIKDL</td>
<td>A2.03</td>
</tr>
<tr>
<td>EBNA1</td>
<td>407–417</td>
<td>HPVGEADEFYEY</td>
<td>B35.01</td>
</tr>
<tr>
<td>LMP1</td>
<td>125–133</td>
<td>YLLEMLWR</td>
<td>A2.01</td>
</tr>
<tr>
<td>LMP1</td>
<td>159–167</td>
<td>YLQQNWWT</td>
<td>A2.01</td>
</tr>
<tr>
<td>LMP2</td>
<td>426–434</td>
<td>CILGGILTMV</td>
<td>A2.01</td>
</tr>
<tr>
<td>LMP2</td>
<td>329–337</td>
<td>LLWTLLVL</td>
<td>A2.01</td>
</tr>
<tr>
<td>LMP2</td>
<td>356–364</td>
<td>FLYALALL</td>
<td>A2.01</td>
</tr>
<tr>
<td>LMP2</td>
<td>200–208</td>
<td>IEDPPFNSL</td>
<td>B40</td>
</tr>
<tr>
<td>LMP2</td>
<td>419–427</td>
<td>TYGVPVMCL</td>
<td>A24</td>
</tr>
<tr>
<td>LMP2</td>
<td>340–350</td>
<td>SSCSSCPLSK</td>
<td>A11</td>
</tr>
</tbody>
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This could be done in one of two ways: either by transferring back to the patient autologous in vitro-expanded CTLs specific for one or more of the expressed EBV antigens, so-called adoptive transfer; or by boosting the patient’s own immune response in vivo by vaccination with relevant T-cell epitopes from one or more of these EBV antigens. To explore this question, we shall first look at the two best known EBV-positive B-cell malignancies, post-transplant lymphoproliferative disease (PTLD) and Burkitt’s lymphoma, in relation to their susceptibility to T-cell control, before examining the possibility of CTL therapy for EBV-positive HD in more detail.
marrow, with no possibility of rejection of foreign CTLs, a rich growth-promoting cytokine milieu and chronic antigenic stimulation by a high EBV load. This allows the administration of relatively few cells which then expand more than 1000 fold; this may explain their clinical effectiveness as well as their prolonged persistence in vivo. However, when we consider PTLD in recipients of solid organ transplants, only one of these three factors, the first, applies. Although it has been shown that CTLs can be generated from stored pre-transplant PBMCs from transplant patients, and that these CTLs reduce EBV load when infused post-transplant [45], there have been no studies of their antitumour effect, and it will be interesting to see whether CTL therapy is as successful for PTLD in this setting.

In contrast, endemic Burkitt’s lymphoma, another B-cell tumour, has a more complex pathogenesis, an important step being the acquisition of a c-myc/Ig locus translocation [46, 47], resulting in activation of the c-myc oncogene [48]. The presence of ongoing Ig variable region hypermutation in the malignant clone [49], and cell markers such as CD10 and CD 77 [50] identify the malignant cell population as having arisen from germinal centre B cells, since germinal centres are known to be sites where somatic mutation of Ig variable regions occurs during affinity maturation of the antibody response [51]. It may be that continued targeting of the translocated c-myc gene upstream of the Ig locus by the somatic mutation machinery contributes to myc activation. Burkitt’s lymphoma is particularly prevalent in areas of malaria endemicity, and chronic malaria infection may act as a risk factor for Burkitt’s lymphoma by increasing germinal centre cell proliferation.

In endemic Burkitt’s lymphoma, as opposed to the sporadic form, EBV is invariably present [52], and thus it is likely that EBV plays a role in pathogenesis. However, the precise nature of this role remains unclear, particularly since EBNA1 is the only viral latent antigen detectably expressed in tumour cells [53]. The virus could act early in tumourigenesis by expanding the population of B cells at risk from chromosomal translocations; alternatively EBV infection of a B cell after it has acquired a chromosomal translocation may allow such atypical cells to survive. In both of these situations, latent genes other than EBNA1 would be required for cell expansion and enhanced survival. In addition, EBV may still continue to play a role at a later stage of tumour growth, with EBNA1 itself contributing to the malignant phenotype, a possibility raised by recent studies in EBNA1 transgenic mice [54].

The limitation of viral antigen expression, coupled with the downregulation of cellular adhesion molecules such as ICAM I and LFA3 [55], and reduced expression both of the TAP transporter proteins [56] and MHC class I molecules [57], reduces the susceptibility of the tumour cells to CTL-mediated lysis, making the tumour ‘immunologically silent’ even in the immunocompetent host. Unless this system can be manipulated in some way, development of CTL therapy for Burkitt’s lymphoma is unlikely ever to be a feasible option. There are several possible strategies. Firstly, the expression of immunodominant EBV latent antigens might be induced using 5’-azacytidine as a demethylating agent [58], and/or defects in the class I antigen processing and presentation pathway reversed by CD40 ligation [59]. Finally, one could attempt to target MHC class II epitopes in EBNA1; CD4-positive MHC class II-restricted CTL to EBNA1 have been described, but again, however, do not see endogenously expressed antigen [60].

These two tumours illustrate the requirements for CTL therapy: presence of immunogenic viral antigens which can be processed and presented in combination with MHC molecules; accessory cellular adhesion molecules; and the presence of a strong virus-specific CTL response within the patient. Bearing these in mind, let us now turn our attention to EBV-associated Hodgkin’s disease.

**EBV and Hodgkin’s disease**

*Evidence for a link*

Hodgkin’s disease (HD) has long been suspected to have an infectious aetiology. It presents with many clinical features of an infectious disease, such as fever and sweats, lymphadenopathy and hepatosplenomegaly, leukocytosis and eosinophilia. Many epidemiological features of HD suggest that it can arise as an uncommon complication of a common infection, for example the bimodal age distribution, geographic distribution, case clustering and relation to social class [reviewed 61]. A link with EBV was first postulated because EBV infection shares many of these features, and a past history of IM is associated with a fourfold increased risk of developing Hodgkin’s disease, particularly within three years of IM [62]. Further circumstantial evidence for an association between HD and EBV came from serological studies. Patients with HD have higher antibody titres to EBV capsid antigen (VCA) than controls [63]. A retrospective study of banked serum samples showed that patients with HD had significantly elevated anti-VCA antibody titres at least three years prior to the onset of HD, suggesting that the host-EBV balance had altered prior to the development of the tumour [64]. However the definitive evidence for the involvement of EBV in HD came with the detection of the EBV genome in Hodgkin/Reed–Sternberg (HRS) cells by in situ hybridisation [65, 66]. Subsequently the expression of EBV latent antigens by HRS cells has been demonstrated [67, 68], and EBV-encoded RNA detected [69, 70].

*Patterns of EBV positivity*

The most sensitive current test for the presence of EBV within HRS cells is in situ hybridisation for the EBERs, EBV-encoded small nonpolyadenylated RNA species which are abundant in latently infected cells. The availability of this test has allowed an accurate assess-
ment of the EBV association with HD in its several histopathologically distinct forms. In the West, EBV-positivity tends to be linked to particular subtypes, though not exclusively. Around 70% of mixed cellularity (MC) cases are EBV-positive, and lymphocyte-depleted (LD), although a rare subtype, is invariably positive. Nodular sclerosing (NS) type tumours are less frequently EBV-positive (10%-40%), while the non-classical lymphocyte-predominant (LP) subtype is almost always EBV-negative [68]. Overall up to 50% of Caucasian HD cases are EBV-positive. There is also an age effect. Patients at the extremes of life are more likely to have EBV-positive tumours, whereas, surprisingly perhaps, adolescents who develop HD in the years following IM are less likely to have EBV-positive tumours. This reflects the high proportion of the NS subtype in this age group.

The geographical variation of EBV-positivity has also been studied, and there is increasing evidence that a higher proportion of tumours in developing countries are EBV-positive. Allied to this is an absence of the 'young adult' peak of NS disease. In Kenya 92% of tumours are positive across all subtypes compared to 48% of tumours examined in Italy as a comparison [71]. In Oriental populations, around 65% of HD cases are EBV-positive, with the same subtype distribution as the West [72].

An aetiological role for EBV

Thus in a significant proportion of cases of Hodgkin's disease, EBV is present within HRS cells. The question now arises as to whether EBV is in some way involved in the aetiology of the tumour, or is merely an 'innocent bystander'. In order to answer this question, we need to consider what is known of the origin of the HRS cell. It seemed counter-intuitive that the only histological subset of HD that is consistently EBV-negative is the subset which is definitely of B-cell origin by cell phenotype markers and surface Ig expression [73, 74]. Now it is clear that other subtypes of HD are also B cells by genotype. In the majority of cases of classical HD (i.e., NS, MC and LD HD) one can detect immunoglobulin (Ig) gene rearrangements within HRS cells, suggesting a B-cell origin. These can also be detected within the HRS cells from LP HD. Furthermore, the Ig gene rearrangements within single cells taken from the same patient are identical, suggesting that HRS cells represent a clonal population of cells derived from a single B cell [75, 1].

More detailed study at the single cell level has revealed that HRS cells from both classical and LP HD also show somatic hypermutation of the rearranged Ig genes, indicating that the B cells from which they are derived must have passed through germinal centres [1]. In LP HD, HRS cells are capable of producing Ig and have ongoing somatic mutations, indicating that they are derived from antigen-selected germinal centre B cells, exactly like the malignant cells in Burkitt's lymphoma. By contrast, in classical HD, there is no on-going somatic mutation, and, in fact, in many cases the mutations which have been acquired prevent translation of the Ig gene, so-called crippling mutations. Normally such defective B cells would be programmed to self-destruct by apoptosis; therefore in classical HD the tumour progenitor cell must have been rescued by some transforming event which allowed its continued survival [76].

So what is the role of EBV in these events? In EBV-positive cases of classical HD, the EBV genome is present in all HRS cells, and is monoclonal [77], indicating that EBV infection occurred prior to clonal expansion of the malignant cell population. EBV produces three latent antigens in EBV-positive HRS cells: EBNA1, LMP1 and LMP2 [78]. As discussed earlier, there is circumstantial evidence that EBNA1 may contribute to the malignant phenotype [54], and recent work in transgenic mice has suggested that LMP2 might be important in promoting survival of atypical B cells, although further work is needed to confirm this [19]. In addition, LMP1 is expressed in such large amounts in HRS cells, and has such profound effects on cell phenotype in other settings that it is unlikely to be 'neutral' in HD. It is likely that LMP1 is contributing to HD cell growth and survival, perhaps by providing the signal which rescues B cells with crippling mutations from apoptosis, and/or by promoting clonal expansion, thereby facilitating the acquisition of further potentially oncogenic mutations. Thus where EBV is present, it is probably playing a significant role in lymphomagenesis, and the contribution of each of the expressed latent antigens remains to be determined.

In EBV-negative cases of classical HD, other events such as infection by another (as yet unidentified) transforming virus or cellular oncogene mutations may be important in allowing survival of a germinal centre cell with crippling mutations. Alternatively, it has been postulated that EBV may again be implicated through some 'hit and run' mechanism: in this context, Razzouk et al. [79] have reported the detection of chromosomally integrated fragments of the EBV genome in some cases of sporadic EBV-negative Burkitt's lymphoma, and it is possible that EBV may have been present in the early stages of tumour growth, but have been lost at a later stage. It is interesting to speculate as to whether such a mechanism occurs in the large peak of EBV-negative cases of HD occurring in adolescents within a few years of IM. However, a percentage of patients with EBV-negative HD are also EBV sero-negative (Chapman, unpublished); this is very strong evidence that EBV is not essential for tumourigenesis.

EBV strain variation and pathogenicity

There are two subtypes of EBV, type 1 and type 2, which are classified on the basis of allelic polymorphism of EBNA2 and the EBNA3 group [80, 81]. The geographic distribution of these two strains differs substantially: type 1 is the dominant type in the West, where type 2 makes up less than 10%; in contrast type 2 is more
prevalent in Africa and Papua New Guinea, forming over 25% of the EB virus strains in circulation. In the West, the majority of cases of EBV-positive HD are associated with type 1 virus, although there is some suggestion that type 2 virus is preferentially associated with HD occurring in the setting of immunocompromise [82, 83]. However, this may merely reflect the increased frequency of type 2 infections in patients with defects in cell-mediated immunity, for example patients with human immunodeficiency virus (HIV) infection [84].

In addition to the broad distinction between type 1 and type 2 isolates, minor genetic polymorphisms exist, and there has long been debate over whether any of these polymorphisms may be markers of greater oncogenic potential, and/or are particularly associated with EBV-positive Hodgkin’s tumours. LMP1 is of particular interest in the context of HD. Several LMP1 sequence variants have been described, the best characterised having a 30-base pair deletion in the carboxy terminal region of the molecule [85, 86]. This variant is common in Chinese populations, and in LMP1 transfection experiments in human epithelial cells confers a more malignant phenotype as evidenced by tumour induction in SCID mice [87]. Similar studies of LMP1 transfectants have suggested that the truncated LMP1 is less immunogenic than the full length form in a mouse system [88]. In one study of HD cases, presence of the deletion appeared to be associated with histologically more aggressive tumours, with higher numbers of HRS cells and necrotic areas, but there was no correlation with clinical outcome [89]. However, Dolcetti et al. [90] reported similar frequencies of the LMP1 deletion variant in non-AIDS-associated HD as in strains from healthy virus carriers from the same geographic region, suggesting that the variant does not have increased tumorigenicity. The reported increase in frequency of the variant virus in AIDS-associated HD [90] may be explained by an increased incidence of type 2 EBV in this population, since the Caucasian type 2 virus strains frequently carry the LMP1 deletion [91]. Overall, therefore, data on frequencies of the LMP1 deletion mutant within HD cases and the normal population from the same geographical area suggest that the occurrence of EBV isolates carrying the LMP1 deletion in HD reflects the prevalence of this polymorphism in EBV strains infecting the general population [90, 91].

Polymorphisms have also been described in the EBNA1 molecule, and some of these have been reported to be preferentially associated with endemic Burkitt’s lymphoma [92]. However, in Caucasians there is currently no evidence for an association between HD and any particular EBNA1 polymorphism [93].

Cytotoxic T-cell therapy for Hodgkin’s disease

Although Hodgkin’s disease has a good prognosis in the early stages, once the disease is advanced or has relapsed post initial chemotherapy, the prognosis is poor and further conventional treatments ineffective. Thus research in this field has been dominated by the need for alternative therapeutic strategies, and one obvious option is to target EBV antigens by immunotherapy. There are two possible approaches: the adoptive transfer of autologous in vitro-expanded cytotoxic T cells specific for antigens expressed in HD cells, or vaccination to induce these particular responses.

We must first ask how an EBV-positive malignancy expressing immunogenic EBV antigens can develop in an ‘EBV-immune’ patient. To address this point, we shall consider three questions.

Are EBV-specific CTL responses detectable in the blood of HD patients?

It is well known that patients with untreated Hodgkin’s disease have a general suppression of immunity which persists after successful treatment [reviewed 94]. General T-cell function is impaired, as measured by mitogen- or antigen-induced proliferation, and by delayed type hypersensitivity skin testing. Recent evidence suggests that this may be at least partly due to down-regulation of the zeta chain of the T-cell receptor [95], a component which is involved in intracellular signaling/cell activation after interaction of the receptor with its epitope ligand. Zeta chain down-regulation is found in CTL from patients with a number of other tumours, for example colorectal carcinoma [96] and melanoma [97], and may represent a general mechanism of immune impairment in cancer patients. In Hodgkin’s disease, the extent of the reduction in zeta chain levels seems to correlate with disease severity [98]. Zeta chain down-regulation can be reversed in vitro by a number of methods, including combined CD3/CD28 stimulation for 48 hours [95], and culturing T cells in the presence of interleukin-2 for 10 days [98], restoring T-cell function to near normal levels. However, zeta chain down-regulation may be more difficult to reverse in vivo. Despite the general immunosuppression seen in HD patients, two groups have recently published results demonstrating that it is possible to grow EBV-specific polyclonal CTL populations from peripheral blood lymphocytes of patients with EBV-positive Hodgkin’s disease, although these grow much more slowly than CTL from normal donors [98, 99]. Our more recent results also show that EBV-specific CTL clones can be generated from the blood of patients with both EBV-positive and -negative HD; the frequency and specificities of these responses for the EBV latent antigens are similar to those seen in healthy long-term virus carriers. Clones specific for LMP2 have been isolated from several donors with EBV-positive tumours, although as yet no responses to LMP1 have been found (Chapman, unpublished). This work confirms that patients with HD do make cytotoxic T-cell responses to EBV latent antigens, suggesting that other factors must be important in allowing EBV-positive tumours to evade detection by EBV-specific CTL.
**What is the antigen-presenting function of the HRS cell?**

EBV latent antigens are recognised by HLA class I-restricted CTL. The class I pathway involves the cleavage of endogenously synthesized proteins by the proteasome complex into peptide fragments, which are then transported into the endoplasmic reticulum by the heterodimer transporter associated with peptide processing (TAPs 1 and 2). Here the peptides compete for binding to HLA class I molecules, and the most stable peptide: HLA class I complexes are transported to the cell surface for recognition by specific CTLs [100]. Defects in this pathway have been identified in a number of human malignancies including, as already described, Burkitt’s lymphoma [57]. In HD it was initially reported that class I molecules were down-regulated on HRS cells, implying defective antigen presentation [101]. More recent studies [102, 103] have demonstrated that most EBV-positive Hodgkin’s tumours in fact have high levels of expression of class I molecules, and of TAPs 1 and 2. In *vitro* work on available HD cell lines (all of which are EBV-negative) has shown that, in those lines which do express surface HLA class I molecules, vector-introduced EBV antigens can be processed and presented to specific HLA class I-restricted CTL with resultant killing of the HRS cell [99, 103].

**What is the effect of the local environment within the tumour?**

Dolcetti et al. [104] reported in 1995 that EBV-specific CTL could be found within the tumour infiltrating lymphocyte (TIL) population of one case of EBV-negative HD. This work was extended by Frisan et al. [105] who cultured TIL from EBV-positive and -negative Hodgkin’s biopsies, and tested them for EBV-specific cytotoxicity against a panel of HLA-matched and -mismatched target cells. They found that while the polyclonal CTL populations derived from EBV-negative tumours showed EBV-specific, HLA class I-restricted responses, CTL derived from EBV-positive tumours showed either no, or only nonspecific, killing. In one patient with an EBV-positive tumour, they were able to generate EBV-specific CTLs from a blood sample but not from TILs, suggesting that EBV-specific CTL responses were being suppressed locally within the tumour. In contrast to this, using autologous LCL as stimulator cells we have been able to generate EBV-specific specific CTL clones from the tumour-infiltrating lymphocyte population of EBV-positive tumours in three cases. These clones are infrequent, and of those analysed to date, all are specific for epitopes within the immunodominant EBNA3 group of viral latent antigens. No clones specific for the latent antigens expressed in HRS cells have been found at this site, but this work continues. In two of these cases, similar reactivities were also seen in pre-treatment blood samples from the same patients [Chapman: unpublished]. The discrepancy between our results and those of Frisan et al. may be due to differences in the CTL reactivation protocols used. As our clones have all been cultured *in vitro* for several weeks prior to being tested for EBV-specific cytotoxicity, these results tell us nothing about the activation status of tumour-infiltrating lymphocytes *in vivo*; they merely indicate that EBV-specific memory CTLs are present at this site.

EBV-positive HRS cells produce a number of potentially immunosuppressive cytokines such as interleukin-10 (IL-10) [106] and transforming growth factor-β (TGF-β). In this context, Potters et al. [107] investigated the effect of the supernatant from a Hodgkin’s cell line, L428, on CD3/CD28-induced T-cell activation, and showed firstly that the supernatant inhibited T-cell activation, and secondly that TGF-β was the active inhibitory component. In other settings IL-10 is a potent inhibitor of T-cell activation [108, 109], and may also play a role in Hodgkin’s disease, although Lee et al. found no effect of human IL-10 on CTL recognition of HRS cell lines [103]. It is important to clarify to what extent the cytokine milieu affects CTL activation and/or effector function in HD, as well as identifying the source of these local cytokines. In this context, HRS cells make up less than 1% of the total cell population in Hodgkin’s disease lesions, and the local infiltration of non-malignant cells could clearly contribute substantially to cytokine synthesis. Also, HRS cells are frequently surrounded by a rosette of activated CD4-positive cells [110, 111] which may also prevent cytotoxic T cells from reaching the HRS cells by steric hindrance.

**Prospects for CTL-based therapy**

Despite these theoretical problems, adoptive CTL therapy for EBV-positive Hodgkin’s Disease remains an interesting and feasible option, and some progress has already been made. Roskrow et al. [98] recently published results of their studies of adoptive transfer of EBV-specific polyclonal CTL in three patients with multiply relapsed Hodgkin’s disease. Each patient was given two doses of 20 million CTL per m², at two-week intervals. Using gene-marked CTL, they demonstrated that infused cells persisted for more than 10 weeks in all three patients, and that this persistence was associated with an increase in EBV-specific cytotoxic activity. EBV DNA levels in PBMCs fell in two of the three patients, and all three experienced improvement in stage B symptoms such as fever, sweats and reduced appetite. Assessment of long term clinical effect was difficult as two of the three patients received chemotherapy six weeks after infusion of T cells; the third received no further chemotherapy and remained well with stable disease four months after CTL infusion.

These early results are encouraging, as they suggest that adoptive transfer of CTL to patients with EBV-positive Hodgkin’s disease will be a useful therapeutic tool in the future, despite the theoretical problems. We now need to build on this start, increasing the number of patients undergoing CTL therapy, and ideally conduct-
ing a randomised controlled trial of this novel treatment. The method employed could be further refined in several ways.

Firstly, it is possible to selectively expand in vitro CTLs specific for the particular viral antigens expressed in HRS cells, that is, LMP1 and LMP2 [99], and deliver these to the patient. This may improve efficacy and reduce side-effects. Taking this train of thought to its extreme, one could selectively reactivate CTL clones specific for just one peptide epitope within one of these two antigens, restricted through an HLA class I allele common in Caucasian populations, such as HLA A2.01. Epitopes restricted through this HLA allele have been described in both LMP1 and LMP2 (see Table 1) [112–114]. At least one of these, the CLGGLLTMV epitope in LMP2 has been shown to be antigenically conserved in a small group of EBV-associated HLA A0201-positive Hodgkin's tumours [115], and thus would be a potential target for CTL generated in vitro using the synthetic peptide as a stimulus. CTL populations specific for individual EBV epitopes can be reactivated successfully using peptide-pulsed autologous dendritic cells as stimulator cells, and the polyclonal effector populations thus induced shown to be capable of recognising the relevant endogenously expressed EBV antigen [116]. In conjunction with this strategy, the idea of adjuvant therapy with cytokines or their blockers needs to be explored. If cytokines such as IL-10 or TGF-β are implicated in the suppression of T-cell responses within the tumour environment, it may be possible to deliver specific cytokine antagonists at the same time as infusing T cells, to boost the activity of the transferred CTL in vivo.

Secondly, another method of selectively boosting CTL responses to a particular EBV epitope would be to reactivates cytotoxic T cells in vivo by vaccination with that epitope, either administered alone or loaded onto autologous dendritic cells. The dendritic cell approach has proved successful in other tumour systems [117, 118], and a trial using this approach in another EBV-positive malignancy, nasopharyngeal carcinoma, is currently underway. Even more interesting is the possibility of vaccination using recombinant viral vectors which express the whole antigen (LMP1 and/or LMP2) or a defined polypeptide construct.

Conclusions

Some progress is now being made in our understanding of the pathogenesis of EBV-associated Hodgkin's disease; in particular, the confirmation of its 'post-germinal centre' B-cell origin has highlighted the importance of examining the fate of EBV-infected B cells within and beyond the germinal centre. Importantly we now recognise that all three EBV-associated lymphomas, PTLD, Burkitt's lymphoma and HD, may share more in common than previously thought. Certainly all three reflect different aberrations of EBV persistence within the B-cell system, culminating in malignancy in cells at different stages of B-cell differentiation. The immune control of persistent EBV infection is now well characterised, particularly CTL-mediated control, and recent work has shown that at least one the above tumours, PTLD, can be successfully treated by CTL therapy. This encourages us to consider possibilities for CTL-based therapy for EBV-positive HD; over the next few years it is to be expected that this type of therapy will be developed as a useful adjunct to conventional treatment, and in time may supersede chemotherapy and radiotherapy as the standard treatment for patients with EBV-positive Hodgkin's disease.

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