

Epstein–Barr Virus and Hodgkin's Lymphoma

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KEY WORDS

■ EPSTEIN–BARR VIRUS ■ HODGKIN'S LYMPHOMA
■ IMMUNOPATHOLOGY ■ DIAGNOSIS ■ TREATMENT

SUMMARY

Hodgkin's lymphoma (HL) is a lymphoproliferative disorder of B-lymphocytes. Epstein–Barr virus (EBV) antigens can be detected in tumours in up to 40% of all HL cases. Patients with EBV-associated HL also show increased levels of EBV-infected B-lymphocytes in blood compared with normal individuals and non-EBV-associated HL cases. A peculiar pattern of restricted EBV-antigen expression, dominated by latent membrane protein-1 (LMP-1), LMP-2 and EBV nuclear antigen-1, is the characteristic feature of tumour-specific Hodgkin/Reed–Sternberg cells. This knowledge has generated studies examining adoptive immunotherapy of autologous or allogeneic cytotoxic T-cells for the treatment of refractory EBV-positive HL cases. Whether aborted EBV or another infectious aetiology is involved in non-EBV-associated HL cases remains an open question.

Introduction

THOMAS HODGKIN WAS the most prominent British pathologist of his time and a pioneer in preventative medicine. Hodgkin described the disease that bears his name in 1832, in a paper entitled, 'On some morbid appearances of the absorbent glands and spleen'.¹

Hodgkin's lymphoma (HL) had long been postulated to have an infectious aetiology. Evidence that supports this theory includes clinical symptoms such as persistent fever, night sweats, weight loss and lymphadenopathy,² together with the abundance of reactive lymphocytes surrounding the sparse, malignant Hodgkin/Reed–Sternberg (HRS) cells that are found in HL tumours.³ The disease has an unusual bimodal age–incidence curve, peaking in early childhood and late adulthood.^{4,5} Previous epidemiological studies have identified the delayed occurrence of infections common in childhood to be a significant risk factor for HL development in young and middle-aged adults.⁶

Histological/Phenotypical Features of HL

One of the characteristic features of HL is the presence of a small population of bizarre-looking large mono- or multinucleated HRS cells within affected tissue (Figure 1).⁷ These cells, classified as B-lymphocytes, often only comprise 1–2% of the total tumour burden and regularly express CD15, CD30, and CD40 (co-stimulatory molecules involved in the activation of normal B-cells) on their surfaces.⁸ HRS-like cells classically occur in tumour infiltrates, which can be characterized by some degree of an inflammatory background and are composed of cells with different phenotypes. Depending on the disease subtype and the point in the disease process, the composition of this mixed-cell phenotype is varied, although upregulation of anti-apoptotic transcription factors that are part of the Bcl-2 family – Bcl-2 and Bcl-xL – have been observed in most HL cases.⁸

There is also a statistically significant inverse relationship between Bcl-xL and apoptosis, suggesting that Bcl-xL plays an important role in HRS cell survival ($P < 0.01$). Increased expression of Ki67 (a marker of cell proliferation) by HRS cells indicates that these cells are undergoing cell division, is associated with advanced disease stage, and may indicate aggressive disease. Furthermore, HRS cells normally show activation of nuclear factor (NF)- κ B,⁹ which is involved in the transcriptional activation of the genes that regulate different cellular processes.

Cytogenetic investigations of immunoglobulin (Ig)-gene arrangements amplified from single micromanipulated HRS cells show that these cells largely represent clonal populations of germinal centre B-cells.¹⁰ Ig-gene rearrangements are found in HRS cells in most HL patients, indicating that B-lymphocytes are the precursors of HRS cells in many, if not all, cases.¹⁰ In addition, somatic mutations within the rearranged Ig genes show that HRS cells in both classical (i.e. nodular sclerosis, mixed cellularity and lymphocyte-depleted HL) and lymphocyte-predominant (LP) HL originate from germinal centre B-lymphocytes.¹⁰ HRS cells differ from most other B-cell-lineage lymphomas in that although they carry Ig genes which have undergone rearrangements that should allow them to produce immunoglobulins, they do not express these proteins.¹¹ Hence, any infectious agent associated with HL must activate an intrinsic pathway to block this expression. This suggests that HRS cells are derived from a compartment of germinal centre B-cells that were destined to die but escaped apoptosis by some transforming event.¹¹ One such candidate for cell transformation is Epstein–Barr virus (EBV) infection.

Associations between EBV and HL

Epstein–Barr virus DNA and its gene products can be detected in 25–50% of HL biopsy samples, localized within the HRS cells.^{12–14} EBV is a ubiquitous human herpesvirus that infects over 90% of the world's population, usually during childhood. Although the virus establishes lifelong latency,¹⁵ there is persistent viral replication in the oropharyngeal cavity and the presence of a few EBV-DNA-positive B-cells in circulation in all EBV serum-positive individuals. EBV can replicate in a small number of B-cells via cellular DNA polymerases, adopting a mode of viral replication that is capable of evading the immune system and permitting the continuous persistence of a few infected B-lymphocytes. Furthermore, continuous viral replication in oropharyngeal epithelial cells occurs in all EBV-seropositive individuals.¹⁶

When primary infection is postponed until adolescence, EBV causes infectious mononucleosis (IM) in up to 50% of patients. IM is associated with an at least threefold greater risk for subsequent HL.^{17–19} Further evidence that links EBV to HL includes altered

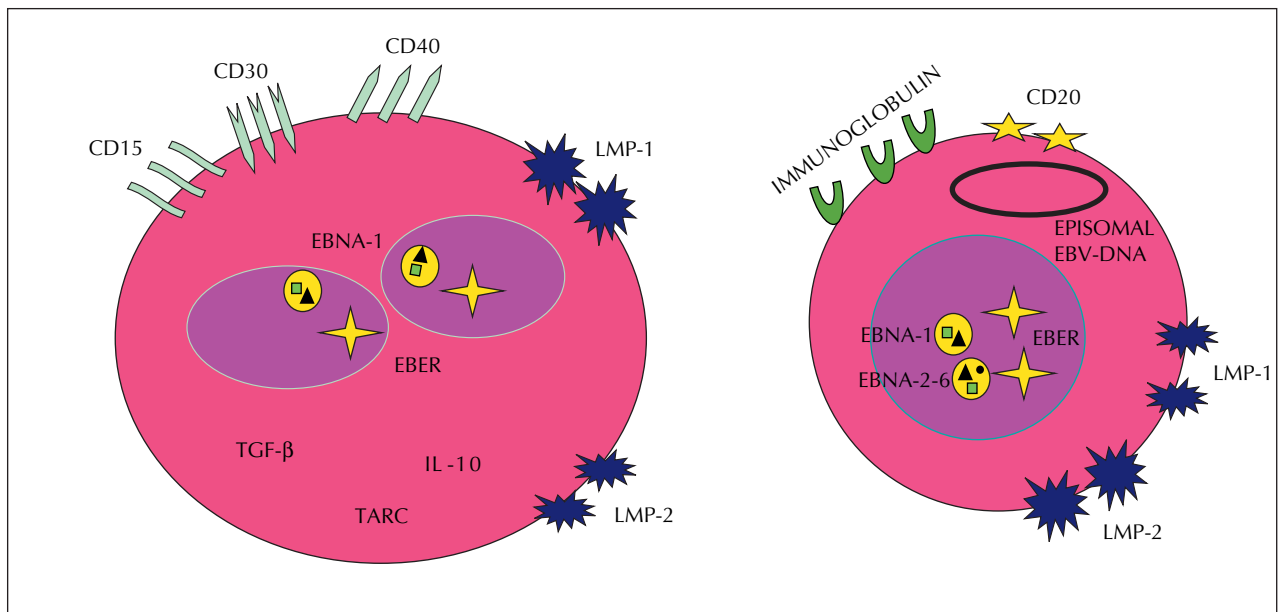


Figure 1:

The left cell illustrates the characteristic phenotype of the bizarre-looking large mono- or multinucleated Hodgkin/Reed-Sternberg cell. These cells often only comprise 1–2% of the total tumour burden in Hodgkin's lymphoma cases and are now classified as B-lymphocytes. CD15, CD30, and CD40 are regularly expressed on their cell surfaces but no immunoglobulin (Ig) and few Epstein-Barr virus (EBV) specific proteins involved in full virus replication (such as EBV nuclear antigen [EBNA]-1, latent membrane protein [LMP]-1 and LMP-2). Furthermore, they are also hybridization-positive in the nucleus for EBV-encoded RNAs (EBER). Intracytoplasmic and transforming growth factor (TGF-β), interleukin (IL)-10 and thymus activation-regulated chemokine (TARC) protein expression are often noticed. The right cell illustrates the normal phenotype of an EBV-infected B-lymphocyte that expresses many more EBV-specific proteins and harbours EBV-DNA in an episomal form in the cytoplasm. These cells are CD20-positive and express Ig on their cell membranes.

antibody titres to different EBV antigens (discussed below), in patterns which suggest that viral reactivation and enhanced virus replication precede HL development. These findings, and the fact that EBV has well-characterized B-lymphocyte oncogenic transforming properties *in vivo* and *in vitro*, suggest that EBV may be a major suspect in HL aetiology.²⁰

Accumulating data indicate that EBV is involved in the pathogenesis of a proportion of HL cases.²¹ The frequency of circulating EBV-DNA-infected B-cells was significantly higher in pre-treatment samples of peripheral blood mononuclear cells (PBMC) taken from EBV-associated HL cases compared with non-EBV-associated cases or healthy seropositive individuals.²¹ This finding was based on studies in which PBMC was purified into the CD20-positive B-cell fraction and a real-time polymerase chain reaction (PCR) assessment of quantitative EBV DNA was then performed. Thus, in patients with EBV-associated disease, the virus persisted in peripheral blood, predominantly in memory B-cells. Furthermore, in patients over 50 years old, EBV positivity was associated with a significantly poorer outcome and increased tumour-associated death ($P=0.003$).²² This population-based study assessed the impact of EBV status on survival in a stratified cohort of adults with classical HL.²² In patients with EBV-associated disease, the virus persisted in peripheral blood, predominantly in memory B-cells. This phenotype – consistent with that seen in healthy EBV-seropositive controls, transplant recipients and patients with IM – may suggest that an individual with less qualitative control of EBV latency, carrying higher EBV-DNA positivity in peripheral blood, has a greater risk of developing HL.

The proportion of EBV-associated HL cases, however, varies with geographical site and age; cases in younger children and older adults are more frequently associated with EBV than cases occurring in early adulthood.^{21,23} The relationship between IM and HL in early childhood

suggests that recent primary infection is a risk factor for developing EBV-associated HL.²⁴ For older adults, reduced immunosurveillance – a consequence of advancing years – may cause increased viral replication,²⁵ which may explain the higher proportion of EBV-associated HL cases in these people.

Aspects of the relationship between cases associated with IM and tumours harbouring EBV have been problematic. The geographical regions in which HL is consistently associated with EBV are those in which the IM syndrome is least common.²⁶ Conversely, in regions where IM is most common, EBV is infrequently found in tumours.

Associations between HL and human leucocyte antigen (HLA) subtype have long been reported:²⁷ the HLA region and its relationship with EBV-positive or -negative HL cases has been studied because the presentation of EBV-antigenic peptides can elicit vigorous immune responses.²⁷ Areas within the HLA class I region were much more prevalent in those with EBV-positive disease, strongly suggesting that antigenic presentation of EBV-derived peptides is involved in the pathogenesis of EBV-associated HL. Polymorphisms in the HLA region could explain ethnic variations in HL incidence by affecting proper presentation of EBV antigens to cytotoxic T-lymphocytes.²⁷ Furthermore, elevated levels of antibodies to various EBV-specific antigens correlate with higher numbers of EBV-infected B-lymphocytes and are therefore indirect markers for increased viral replication and reactivation.²⁸

One may also question whether EBV is only associated with HLs that harbour the EBV genome in HRS cells. Alternatively, the virus could be associated with lymphoma in which the EBV genome cannot be detected.²⁹ An association with HL that does not harbour the genome is plausible. EBV infection of a B-lymphocyte may initiate tumourigenesis, perhaps leading to genetic instability or other proliferative changes that ultimately render the viral genome

superfluous to the maintenance of the malignant state. Evidence indicates that EBV upregulates expression of activation-induced deaminases (endogenous enzymes that can regulate gene expression via cleavage of mRNAs), resulting in somatic hypermutation in germinal-centre B-cells.³⁰ EBV-induced genetic instability may result in abortive infection and – in contrast with the tumour virus, whose DNA integrates into host cell DNA – loss of EBV episomes from malignant cells in tissue culture has been documented in EBV tumour cell lines.³¹

EBV – Epidemiological Studies

Some questions have been partially answered by a population-based study of acute IM and malignancy, undertaken in Denmark and Sweden.¹⁸ The relative risk of EBV-associated HL was increased fourfold in patients with serologically-confirmed IM, although no such increased risk was found for EBV-negative HL cases. The median time-interval from IM diagnosis to EBV-associated HL was approximately 4 years. Thus, IM is clearly linked to EBV-positive HL and no evidence suggests that it is linked to EBV-negative HL, although most HL patients have no documented history of IM. Why EBV-associated HL accounts for the highest percentage of HL in regions where IM is least common remains an unresolved question. At the extremes – in children under 10 years of age and adults over the age of 70 – EBV genes can be detected in HRS cells in virtually all cases.^{18,19} However, IM is most commonly associated with primary HL in teenage or early adult years, although interesting evidence regarding measles has also been presented: immunohistochemical, PCR and *in situ* hybridization studies have demonstrated evidence of measles virus in HL tumour tissues in slightly over 50% of patients,³² including patients with or without EBV in their tumours. There is, however, no proof of pathogenesis or mechanisms involved in B-cell transformation at this time-point. Despite extensive investigation, there is no evidence to support a role for human T-cell lymphotropic virus type 1 (HTLV-1) or HTLV-2, cytomegalovirus, varicella zoster virus, mumps, pertussis or the human herpesviruses-6, -7 or -8 in the HL pathogenesis. At least one research group has failed to associate the JC polyomavirus or adenovirus to HL tumour types.³²

Viral Proteins in Hodgkin's Pathogenesis

The EBV latency membrane protein 1 (LMP-1) has been identified as a likely contributor to tumourigenesis in EBV and HL cases.³³ Overexpression of LMP-1 results in B-cell immortalization in a transgenic model. This protein belongs to the tumour necrosis factor receptor superfamily, activates NF- κ B and is very similar to CD40, which is expressed on activated B-cells and HRS cells.³³ In contrast to CD40, however, signalling is constitutively active with LMP-1 and does not require ligand binding for activation.³⁴ Latency membrane protein 2 (LMP-2) is also important,^{35–37} and is regularly expressed in all EBV-associated HRS cells (Figure 1). These cells, which lack functional Ig, would normally die by apoptotic mechanisms. However, LMP-2 provides an overriding signal that is associated with Ig expression and prevents apoptosis of cells lacking Ig expression.^{33–38} EBV nuclear antigen-1 (EBNA-1), which is also found in tissue samples taken from people with Burkitt's lymphoma,³¹ is constitutively expressed in EBV-associated cases.

Diagnosis of EBV-associated HL

The diagnosis of EBV-associated HL is based on the methods normally used to diagnose EBV. Phenotypic evaluation of tumour tissue biopsies include identification of cell-surface markers such as CD15, CD30 and CD40, which are normally expressed on the surfaces of HRS-like cells. Furthermore, a combination of *in situ* hybridization for detecting EBV-encoded EBER (a virus genus that is associated with EBV replication) and immunohistochemical staining of LMP cell surface expression is regularly used to identify EBV in HRS-like cells (Table 1). Elevated EBV antibody titres that are correlated with a higher level of EBV DNA load in B-cells are risk factors for the later development of EBV-positive, compared with EBV-negative, HL.²⁰ In addition, serological results show that the host's antibody response to EBV varies significantly between EBV-positive and EBV-negative HL cases. EBV-positive HL cases are more likely to demonstrate elevated antibody titres against viral capsid antigen (VCA), IgG plus IgA and early antigen-D, as well as abnormal anti-EBNA-1:anti-EBNA-2 ratios.²⁸ An EBNA-1 versus EBNA-2 ratio of <1.0 is a significant positive predictor of high antibody titres in patients with EBV-associated HL.^{28,39} High levels of EBV-specific IgA antibodies are also classically described in EBV-associated nasopharyngeal carcinoma and in individuals characterized with extensive EBV replication in the oropharynx. Early antigen-D refers to EBV-specific antigens that are diffusely spread in the cytoplasm of EBV-infected cells and thus reflects a staining pattern by immunochemical techniques.

Normal EBV Regulation

During acute EBV infection, peripheral blood demonstrates extensive infection in the pool of resting memory B-cells (up to 50% of which carry EBV DNA). However, even at this stage of acute infection, EBV persistence is tightly regulated. Subsequently there is a rapid decline in infected cells in the early weeks post-

Table 1: Molecular approaches for diagnosis of Epstein–Barr virus-positive Hodgkin's lymphoma

Molecular approach	Result
Hodgkin/Reed–Sternberg cells in tumour biopsy	Ig negative, CD15+, CD30+ CD40+ bizarre-looking mono- or multinucleated B-cells
EBER hybridization in tumour biopsy	Nuclear staining
LMP-1 immunohistochemical staining in tumour biopsy	Membrane staining
EBV serology in serum	Anti-VCA increase Ratio anti-EBNA-1: EBNA-2 <1.0
EBV viral load in blood ^a (real time PCR)	EBV-DNA >50/10 ⁶ B-cells

^a Healthy EBV-seropositive individuals have 1–50 EBV-DNA-positive cells per 10⁶ B-lymphocytes in blood.

EBV, Epstein–Barr virus; EBER, EBV-encoded RNAs (EBV-specific genes expressed in the nucleus of EBV-replicating cells); LMP1, latent membrane protein 1 is a membrane associated protein in EBV-infected cells; PCR, polymerase chain reaction; Ig, immunoglobulin; VCA, viral capsid antigen; EBNA, Epstein–Barr nuclear antigen.

infection, with a parallel occurrence of EBV-specific cytotoxic T-cells: up to 50% of all CD8 T-cells may demonstrate EBV specificity in the peak of cell-mediated immune response. This phase is followed by a much slower decline in levels of infected memory cells, which persists over 1 year. Following primary infection, EBV establishes lifelong latency in B-lymphocytes that is not associated with any symptoms or lymphoproliferative conditions. The CD8 T-cell-mediated cytolytic exocytosis pathway, which involves perforin and granzyme expression, is of central importance for the control of acute infection. The presence of neutralizing antibodies is thought to restrict lytic viral replication, preventing the infection of new B-lymphocytes and, typically, anti-VCA antibodies are already present by the time EBV infection is diagnosed. In contrast, anti-EBNA antibodies – initially directed against EBNA-2 and subsequently more prominently against EBNA-1 – are not found until at least 1 and often several months after acute infection. The development of anti-EBNA antibodies occurs parallel to the detection of EBV-specific mature CD8 cytotoxic T-cells.^{28, 38–40}

Using a highly sensitive PCR-based technique capable of detecting a single EBV-infected cell among $>2 \times 10^5$ uninfected cells, the frequency of EBV-infected cells was found to be considerably higher in the peripheral blood of EBV-associated HL cases than in non-EBV-associated cases.²¹ This assessment was undertaken before anti-tumour treatment was initiated. The differences persisted even after cases with negative EBV-serological results were excluded and adjustments for the effects of age were made, indicating that every HL patient has a generalized defect in immunological surveillance of EBV. One may speculate that the increased frequency of EBV-infected B-cells observed in EBV-associated cases is biologically relevant. Since the cytolytic exocytosis-dependent pathway of cytotoxic T-cells shows a gradual decline in activity with senescence, one may speculate that individuals with selective loss of EBV-specific CD8 T-cells may be at increased risk of late development of EBV-associated HL. However, to prove or disprove this theory requires longitudinal studies of EBV-specific immunity and occurrence of HL, which have yet to be performed.

Treatment of EBV-associated HL with Immunotherapy

Together, HL and non-Hodgkin's lymphomas comprise 8% of all malignancies.⁴¹ These lymphomas are highly sensitive to radiotherapy and chemotherapy, and long-term cure rates above 80% have been established for HL. Although such therapeutic approaches have been used for over 30 years, the magnitude of late treatment-related morbidity and mortality – especially in younger people with HL – has generated much interest in adoptive immunotherapy. Since EBV-positive HL cases clearly express identified EBV antigens in tumour-specific HRS cells, they could (in principle) be targets for adaptive immunotherapy with viral antigen-specific T-cells (Figure 1). However, like most tumour-associated antigens in the immunosuppressed host, these potential targets are only weakly immunogenic and consist primarily of LMP-1 and -2 antigens. Furthermore, HL tumours possess a range of tumour evasion strategies. For example, HRS cells secrete immunosuppressive cytokines including interleukin (IL)-10 and transforming growth factor- β , as well as the thymus activation-regulated chemokine, which selectively recruits IL-4-secreting T-lymphocyte helper (Th)-2 cells.⁴²

This results in inhibition of Th-1 cell-mediated cytotoxic T-cell responses. HRS cells also recruit CD4+, CD25+, FoxP3+ regulatory T-cells (T_{reg} -cells) into the tumour environment. T_{reg} -cells can block antigen-presenting cells, tumour-specific CD4 helper T-cells and cytotoxic T-cells, and therefore may exert local immunosuppression, which may be a central mechanism for persistence of tumour mass.

Consequently, the value of immunotherapy with EBV-specific cytotoxic effector cells has been questioned. Both allogeneic and autologous EBV-cytotoxic T-cells (CTL) have, however, been administered in EBV-associated HL cases.^{43–46} The combination of gene marking, tetramer staining and functional analysis of infused cells has been used to track the fate and activity of such inserted CTL cells.⁴⁴ It has been demonstrated that infused effector cells expand *in vivo* and contribute to the pool of memory CD8 T-cells that traffic to tumour sites.⁴⁵ Tetramer staining and functional analysis show that these T-cells respond predominantly to LMP-2-expressing HRS cells, which decrease the viral load in treated patients and thereby demonstrate the biological activity of adoptive immunotherapy. Clinically, EBV CTLs are well tolerated and result in control of the classical 'type B' symptoms that are found in HL cases (namely fever, night sweats and weight loss); they also have anti-tumour activity in 30 – 40% of cases.^{43–46} Even if it has been difficult to demonstrate LMP-2-specific CD8 and CD4 T-cells in infused stimulated T-cells *in vitro*, the expansion *in vivo* has led to increased numbers of cells with specificity against LMP-2. Such cells are also known to be capable of infiltrating the tumour sites. Furthermore, LMP-1 tumour-expressing cells disappear after CTL infusion.⁴⁵ The *in vitro* expansion of these cells without the presence of negative regulatory T-cells may facilitate the development of EBV-specific cytotoxic T-cells *in vitro*.

Conclusions

Abortive infection of EBV in germinal B-cells may result in survival of mutated Ig-negative B-cells that are normally proven to undergo apoptosis. Induction of anti-apoptotic transcription factors and subsequent NF- κ B activation, which have both been found in HRS cells, indicate that EBV may play an important role in tumour genesis. Adoptive treatment by induction of LMP-1 and LMP-2-specific cytotoxic T-cells may be a therapeutic choice in addition to established cytotoxic drugs and radiation therapy. There is, however, no evidence that EBV is involved in HL cases that do not express EBV genes.

Conflicts of Interest

No conflicts of interest were declared in relation to this article.

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