Epstein–Barr virus-associated Hodgkin’s lymphoma

Survivors of Hodgkin’s lymphoma (HL) frequently have many years to experience the long-term toxicities of combined modality therapies. Also, a significant proportion of HL patients will relapse or have refractory disease, and less than half of these patients will respond to current salvage strategies. 30–50% of HL cases are Epstein–Barr virus associated (EBV-positive HL). The virus is localized to the malignant cells and is clonal. EBV-positive HL is more frequent in childhood, in older adults (>45 years) and in mixed cellularity cases. The survival of EBV-positive HL in the elderly and the immunosuppressed is particularly poor. Despite improvements in our understanding of EBV-positive HL, the true contribution of EBV to the pathogenesis of HL remains unknown. Increased knowledge of the virus’ role in the basic biology of HL may generate novel therapeutic strategies for EBV-positive HL and the presence of EBV-latent antigens in the malignant HL cells may represent a target for cellular immunotherapy.

Following Thomas Hodgkin’s initial description of Hodgkin’s lymphoma (HL) in 1832, there was much controversy as to whether HL was a malignant, inflammatory or infectious process. The current consensus is that the Hodgkin Reed-Sternberg (HRS) cells in HL are neoplastic, and it is somewhat ironic that there is now increasing interest in the notion that HL may have an infectious aetiology. The association of Epstein–Barr virus (EBV) with HL has been intensely investigated over the last few years. Despite improvements in our understanding of EBV-associated HL (EBV-positive HL), the true contribution of EBV to the pathogenesis of HL remains unknown. Increased knowledge of the role of EBV in the basic biology of HL may generate novel therapeutic strategies for EBV-positive HL. Further, irrespective of the role of EBV in the aetiology of HL, the presence of EBV-latent antigens in the malignant HL cells may represent a target for cellular immunotherapy (Khanna et al, 2001). This review summarizes the data on the possible role of EBV in the pathogenesis of EBV-positive HL, examines the epidemiology of EBV-positive HL, and discusses therapeutic options.

Histopathology of HL

HL is a unique clinico-pathological disorder in which the malignant cells constitute only a minority (1–2%) of the total tumour mass. The lesion is characterized by the disruption of normal lymph node architecture and the presence of a small number of large mono-nucleated (Hodgkin) and multinucleated (Reed Sternberg) cells amidst a non-neoplastic inflammatory infiltrate. HL is classified into two distinct clinicopathological entities: nodular lymphocyte predominant (NLPHL) – which represents 5% of all HL cases, has a germinal centre genotype and is not typically EBV associated (Weiss et al, 1991), and classical HL (cHL) which has a postgerminial centre genotype. cHL can be divided into four morphological subtypes (Jaffe et al, 2001): nodular sclerosis (NSHL) which accounts for the majority of cases, mixed cellularity (MCHL), lymphocyte-rich (LRHL), and lymphocyte-depleted (LDHL). LRHL and LDHL each comprise less than 5% of all cHL cases, and as a result much of the data on EBV-positive HL is derived from the study of NSHL and MCHL. The exact origin of the HRS cells has long been a topic of intense debate. The phenotype of HRS cells has no obvious normal cellular counterpart. However, elegant studies from Küppers and colleagues have produced compelling evidence to suggest that these malignant cells are predominantly lymphocytes of B cell lineage (Küppers et al, 2003).

Background to EBV

EBV is a ubiquitous γ human herpes virus with a seroprevalence of 95% world wide (reviewed in (Rickinson, 2002). EBV infection is usually asymptomatic in childhood, but infection in adolescence frequently results in infectious mononucleosis (IM), which manifests to haematologists as the production of activated T cells (atypical lymphocytes) on the peripheral blood smear. Oropharyngeal infection results in a localized lytic (replicative) infection followed by infection of circulating B cells and amplification of infection as the virus drives the proliferation of latently infected B cells into the systemic circulation. Latent infection of circulating B cells in IM is characterized by the type III latency pattern, involving expression of six EBV nuclear antigens (EBNA 1, 2, LP, 3A, 3B, 3C), three latent membrane proteins (LMP1, LMP2A and LMP2B), EBV encoded small RNA (EBER1) and EBER2 and the transcripts from the BamH1A region. EBNA1 is the virus genome maintenance protein, the remaining EBNAs are transcriptional regulators and LMP1 is the major effector of...
virus-induced cellular change. Type III latency is associated with B cell transformation (immortalization), via exploitation of the B cell specific transcription activator Pax 5 (also expressed in HRS cells (Foss et al., 1999). The type III latency pattern is highly immunogenic, resulting in an expansion of EBV-specific lymphocytes, which results in the IM syndrome and regression of type III latently infected cells. A small proportion of infected B cells evade lysis by adopting an altered form of latency, reflecting the ability of EBV to adopt different forms of latency at different times. Although B cells expressing type III latency are never subsequently detected in the peripheral blood of healthy carriers (Miyashita et al., 1997), EBV infection persists for life in an incompletely defined cell–virus relationship. Evidence for latent infection in all healthy EBV seropositive donors includes the observation that 'spontaneous' lymphoblastoid cells lines (LCL) can be regularly established from healthy seropositive donors after explantation of peripheral blood lymphocytes or lymph node tissue (Rickinson et al., 1974).

There is now compelling evidence that this latent infection is controlled by a population of EBV-specific (largely CD8) cytotoxic T lymphocytes (CTL). These recognize epitopes frequently derived from EBNA proteins 2, 3A, 3B and 3C (Rickinson & Moss, 1997). Although the mechanisms involved in controlling intermittent virus reactivation from latency have not yet been completely defined, it is clear that the interaction between specific CTL and their targets form a key element in preventing unchecked viral proliferation.

EBV is associated with a variety of human malignancies including Burkitt’s lymphoma (BL), nasopharyngeal carcinoma (NPC), post-transplant lymphoproliferative disease (PTLD) and HL (reviewed in Khanna & Burrows, 2000). Like many other EBV-associated malignancies, the malignant cell in HL is characterized by its unique viral and cellular phenotype. Along with NPC, HRS cells display a type II form of latency with viral antigen expression limited to EBNA1, LMP1, LMP2, along with NPC, HRS cells display a type II form of latency. Type III latency is associated with B cell transformation (immortalization), via exploitation of the B cell specific transcription activator Pax 5 (also expressed in HRS cells (Foss et al., 1999). The type III latency pattern is highly immunogenic, resulting in an expansion of EBV-specific lymphocytes, which results in the IM syndrome and regression of type III latently infected cells. A small proportion of infected B cells evade lysis by adopting an altered form of latency, reflecting the ability of EBV to adopt different forms of latency at different times. Although B cells expressing type III latency are never subsequently detected in the peripheral blood of healthy carriers (Miyashita et al., 1997), EBV infection persists for life in an incompletely defined cell–virus relationship. Evidence for latent infection in all healthy EBV seropositive donors includes the observation that 'spontaneous' lymphoblastoid cells lines (LCL) can be regularly established from healthy seropositive donors after explantation of peripheral blood lymphocytes or lymph node tissue (Rickinson et al., 1974).

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**Diagnosis of EBV-positive HL**

EBV DNA and RNA can be detected in HL tissues using a variety of sensitive techniques including the polymerase chain reaction [PCR for DNA and reverse transcription (RT)-PCR for RNA] and nucleic acid sequence-based amplification (NASBA) (Herbst et al., 1991; Brink et al., 1997). These techniques have research applications but lack wide applicability in the diagnosis of EBV-positive HL because they are unable to localize DNA within diseased tissues to the HRS cell. Southern blot analysis has been used to determine the clonality of EBV-infected tissues based on the presence of variable numbers of terminal repeat fragments at the ends of each DNA molecule. In a large series, Gulley et al. (1994) demonstrated that clonal EBV genomes are an abundant feature of all EBV-positive HL cases [HRS cells positive by in situ hybridization (ISH) for EBER1 transcripts], but in no cases of EBV-negative HL (including cases where the occasional background lymphocyte was EBER positive), implying that infection with EBV occurred either prior to or at any early stage of transformation of the HRS cell.

Both immunohistochemical and ISH techniques permit localization of the virus within biopsy tissue, and can be applied to routinely fixed paraffin-embedded lymph nodes. LMP1 antibodies are readily available, and give a strong signal localized to the cytoplasmic and surface membrane. On some occasions, weak focal positivity can occur. LMP1 immunohistochemistry is relatively rapid, but may be falsely negative in poorly fixed tissues, and care must be taken to distinguish false positive staining in eosinophils and plasma cells. EBER transcripts are expressed in latently infected cells at high copy number at levels approaching one million copies per cell (Clemens, 1993), and therefore represent an excellent target for localizing and detecting EBV latently infected HRS cells by RNA-ISH. A variety of commercially available probes that label a conserved region present in both EBER1 and EBER2 are available. In positive cells, the probe localizes to the nucleus. To prevent false-negatives, it is essential to demonstrate that RNA is preserved and available for hybridization. With both LMP1 and EBER tests, the morphology and distribution of stained cells should be matched with the haematoxylin and eosin slide to enable correct interpretation, and distinguish reactive cells from HRS cells. A recent guideline (Gulley et al., 2002) recommends LMP1 and EBER assays in combination as more effective than either assay alone for the diagnosis of EBV-positive HL.

A study on pretreatment HL serum samples, used both conventional and quantitative PCR to amplify EBV-DNA (Gallagher et al., 1999). Conventional PCR detected EBV-DNA in approximately 90% of known EBV-positive HL, and quantitative PCR detected 75% of cases, but the latter technique had the advantage of detecting few false positives from EBV-negative HL samples. Adult and paediatric patients who enter remission demonstrate a significant reduction in plasma viral load to low or undetectable levels, whereas in poorly responding patients disease progression is associated with rapidly increasing DNA levels (Lei et al., 2000; Wagner et al., 2001). The significant correlation of plasma/serum viral load with therapeutic response does not appear to hold if peripheral blood mononuclear cells (PBMC) are used as the source to measure EBV DNA (Gallagher et al., 1999; Wagner et al., 2001), which is consistent with the source of viral DNA from HRS cells that have been shed into the periphery, rather than viral replication at other sites. The identification of identical HRS immunoglobulin gene rearrangements in biopsy and serum samples demonstrates that HRS cells do shed DNA into peripheral blood (Kornacker et al., 1999). This notion is further supported by the use of DNase digestion to plasma/serum samples, which demonstrate that the detected viral DNA in HL patients is ‘naked’ rather than from packaged.
viral particles; the latter would be expected if active viral replication were taking place (Chan et al, 2003).

Epidemiology

The epidemiology of HL is complex. This most likely represents a multifactorial aetiology involving an interaction between genetic and environmental factors. That there is an inherited susceptibility to HL is indicated by distinct incidence patterns between ethnic groups (Parkin & Muir, 1992), by human leucocyte antigen (HLA) associations (Klitz et al, 1994), by its male predominance and by familial aggregation of the disease (Mack et al, 1995). One of the principal reasons underlying the belief that there is an infectious origin for HL is its distinct pattern of incidence within age-groups. The conventional notion is of a bimodal age distribution, with the highest rates occurring in young adults in developed countries (MacMahon, 1966) and in childhood in developing countries. This is over-simplistic. Age-specific incidence rates vary with both ethnicity and histological sub-type (Perkins et al, 1995), with most marked bimodality for NSHL occurring in Caucasians, while the age-specific incidence rate for MCHL is more comparable across races (Cozen et al, 1992).

A population-based cohort study demonstrated a positive association between IM and EBV-positive HL but not EBV-negative HL (Hjalgrim et al, 2003). The median time from IM to EBV-positive HL onset was 4 years. Mueller et al (1989) showed that IgG and IgA antibody titres against EBNA, viral capsid antigen and early antigen D, taken from the serum of HL patients in the years antedating their diagnosis, were significantly higher than in the serum taken at the same time from healthy controls. Results were not stratified according to the EBV-status of the HRS cells. Ambinder’s group recently observed that serum EBV DNA is elevated in the years preceding a diagnosis of EBV-positive HL, with an upward trend near the time of diagnosis, whereas no difference in detection of EBV DNA was observed between EBV-negative HL cases and healthy controls (unpublished observations). However, none of these observations necessarily implicate EBV as having a causal role in pathogenesis but may reflect a predating reduced ability to control EBV infection.

As discussed earlier, there is now compelling evidence that a proportion of HL cases are associated with EBV and that in these cases the virus is localized to the HRS cells and is clonal. In developed countries the EBV strain is generally type A, but paediatric cases from developing countries frequently exhibit either type A, type B or dual infections, an observation which may reflect the underlying socioeconomic conditions (Tomita et al, 1996; Weinreb et al, 1996). In developed countries such as North America and the European states, a number of predominantly adult-based studies have found that the proportion of EBV-positive HL cases varies from approximately 30–50% (Herbst et al, 1991; Pallesen et al, 1991) (see Table I). By contrast, the prevalence of EBV in HRS cells is extremely high in developing countries (Ambinder et al, 1993; Leoncini et al, 1996), which suggests that EBV may have a complex aetiological role, perhaps in relation to other environmental factors and ethnicity.

The proportion of EBV-positive HL cases is generally higher in childhood (especially under 10 years of age) and in older adults (>45 years) than in younger adults (15–35 years) (Glaser et al, 1997). Other consistent findings are that among EBV-positive HL there is a male preponderance and that a significantly higher proportion of EBV-positive HL cases are MCHL than NSHL (Pallesen et al, 1991; Herbst et al, 1992; Flavell et al, 2000). Although MC disease is relatively more common in the older adult age groups, this effect is seen even after adjustment for age and other variables (Jarrett et al, 1996).

Paradoxically, the best evidence for an infectious aetiology in immunocompetent HL cases is in young adults, who tend to have EBV-negative HL. In these cases, it has been suggested that there has been delayed exposure to an infectious agent (Glaser et al, 2002), but despite a plethora of studies there remains no convincing evidence for another virus consistently detectable in HRS cells (reviewed in Jarrett (2002). Alternatively, it has been proposed that the EBV genome persists as integrated fragments (EBV is normally episomal) or as a defective genome with absent viral gene expression in HRS cells that have been labelled ‘EBV-negative’ by conventional techniques: the ‘hit + run’ hypothesis (Ambinder, 2000). Descriptions of loss of EBV-positivity in HRS cells in relapsed HL support the loss of viral DNA during tumour progression (Deleclosse et al, 1997; Nerurkar et al, 2000). These studies did not determine the clonal origin of either the presentation or relapsed disease, and therefore it remains conceivable that the EBV-negative HRS cells represented a de novo presentation. A recent study using DNA-ISH in LMP1-negative HRS cells (Staratschek-Jox et al, 2000) found no evidence to support the ‘hit and run’ idea. Intriguingly, using a combination of a highly sensitive conventional PCR technique with PCR-ISH to localize a positive signal, Gan et al (2002) recently detected a

Table I. Epidemiology of EBV-associated Hodgkin’s lymphoma.

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<th>EBV-positive HL</th>
<th>EBV-negative HL</th>
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<tr>
<td>Frequency</td>
<td>30–50% cases (&gt;90% in developing countries, &gt;95% in HIV associated cases)</td>
<td>50–70% cases</td>
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<tr>
<td>Histology</td>
<td>Mixed cellularity &gt; nodular sclerosis</td>
<td>Nodular sclerosis &gt; mixed cellularity</td>
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<tr>
<td>Age</td>
<td>Patients typically &lt;10 years or &gt;45 years old</td>
<td>Typically 15–45 years old</td>
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<tr>
<td>Prognosis</td>
<td>Relatively poor in &gt;45 year old patients, conflicting data in younger patients</td>
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defective rearranged EBV genome present in a proportion of EBER-positive and EBER-negative paediatric HL tumours. The authors speculated that defective DNA might have a role with respect to viral and/or cellular gene expression that might promote transformation. However, no evidence of the defective genome was detected in adult biopsies (Gallagher et al., 2003).

EBV-positive HL in the immunodeficient patient

Although not a frequent manifestation of cellular immunodeficiency, there is an increased frequency of HL following solid-organ transplantation (Nalesnik et al., 1993; Bierman et al., 1996) and alloSCT (Rowlings et al., 1999). These cases are virtually always EBV-associated, making it likely that, in the context of immunodeficiency, EBV is involved in the pathogenesis of HL. Further study of these cases may well prove to be beneficial in understanding the immunopathogenesis of EBV-positive HL.

EBV-positive HL is the most frequent non-acquired immunodeficiency syndrome (AIDS) defining cancer diagnosed in human immunodeficiency virus (HIV) patients (Goedert et al., 1998; Dolcetti et al., 2001) (see Table I). Treatment of HL in the HIV patient provides unique challenges. Although chemotherapy (frequently with concomitant retroviral therapy) is feasible and results in high complete remission rates, the risk of opportunistic infections is increased and the rate of freedom from progression is lower than that seen in the non-immunocompromised patient (Spina et al., 2002; Hartmann et al., 2003).

Influence of EBV on prognosis

Studies of the influence of EBV on relapse rates and survival after HL are conflicting. This most probably reflects the methodology employed. However, population-based studies have no selection bias and are therefore particularly informative, and results from such studies appear to be broadly consistent. Two recent population-based series (one in Californian women, the other in Northern England) have found a marked survival disadvantage in older EBV-positive HL patients as compared with EBV-negative HL cases. Clarke et al. (2001) found EBV-positive HL was an adverse prognosticator in women aged 45–79 years (but not in females aged between 19 and 44 years) which was not explained by age, stage or histological sub-type. An analysis of HL in patients ≥60 years by the Northern Lymphoma group of Proctor and associates found the median survival of patients with EBV-positive HL was 20 months but remained undeterminable in the EBV-negative HL group (P = 0.007) (Stark et al., 2002). A third unselected series, this time in a Swedish population, found that patients with EBV-positive HL were more likely to be older, have more B symptoms and advanced disease and showed a trend towards reduced survival as compared with EBV-negative HL patients (Enblad et al., 1999).

Other studies have generally selected patients accrued into clinical trials (who tend to be younger) and/or have been limited by availability of histology slides for analysis. Several studies have found no effect of EBV HRS status on clinical outcome (Enblad et al., 1997; Axdorph et al., 1999), whilst others have observed that EBV-positive HL is associated with improved disease-free survival (but has no significant effect on overall survival) in young adults (Glavina-Durdov et al., 2001; Flavell et al., 2003). It may be that the differential impact that EBV HRS status has on outcome between young adult and older adult age groups is either a consequence of biologically distinct diseases, or alternatively a decline in EBV-specific cellular immunity with age.

Pathogenesis

Single cell analysis of HRS cells has shown that immunoglobulin gene rearrangements have taken place but that transcription is virtually always absent. In the majority of cases this is due to a functional defect in the immunoglobulin gene regulatory machinery (Marafioti et al., 2000). However, in 25% of cHL cases, lack of gene expression appears due to clonal immunoglobulin gene heavy-chain rearrangement involving ‘crippling’ somatic mutations within the HRS cells (Kanzler et al., 1996). This data supports the notion that HRS cells are derived from preapoptotic postgerminatal centre B cells. ‘Crippling’ mutations are normally incompatible with B cell survival, suggesting that a transforming event, such as EBV infection, has taken place. Interestingly, similar ‘crippling’ immunoglobulin mutations have been recently detected in seven of 11 post-transplant lymphoproliferative disorder (PTLD) cases (Timms et al., 2003). PTLD is typically driven by the uncontrolled proliferation of EBV-transformed B cells due to the diminished EBV-specific CTL response, which leads to subsequent selection of the malignant clone(s). Further, remission induction and also long-term disease-free survival in PTLD are characterized by the recovery of latent EBV-specific CTL frequency (Sherritt et al., 2003). EBV-positive HL has also been reported in solid organ transplant patients following PTLD (Nalesnik et al., 1993; Bierman et al., 1996). Evidence suggests that Burkitt lymphoma (BL) cells are also atypical survivors of postgerminatal centre B cells (Chapman et al., 1996). It is tempting to speculate that EBV infection is a common initiating event in the pathogenesis of atypical postgerminatal centre B cell malignancies, including a subset of HL, BL and PTLD cases. Recent gene expression profiling of HRS cells has identified a range of aberrantly expressed genes (Küppers et al., 2003; Küppers, 2003), with the profile showing many similarities to that of EBV-transformed LCLs.

LMP1 expression was associated with multinularity in an EBV-negative LMP1-transfected HRS cell line (Knecht et al., 1996) and a functional analysis of LMP1 sequences associated with HL has found that these sequences appear to be more oncogenic than LMP1 sequences from normal EBV-infected B cells (Mehl et al., 1998). These studies, together with reportedly
high levels of LMP1 expression in HRS cells, support the contention that EBV plays an important role in the direct pathogenesis of HL. LMP1 functions as a constitutively activated tumour necrosis factor (TNF) receptor and many of the phenotypic and growth transforming effects of LMP1 are the result of its ability to activate a variety of signalling pathways, including nuclear factor κB (NFκB), through two C-terminus activating regions, CTAR1 and CTAR2 (Huen et al, 1995). Constitutively activated NFκB is a unique and common characteristic of HRS cells with consistent overexpression observed in cells from HL-infiltrated lymph nodes (Bargou et al, 1997), while Krappmann et al (1999) demonstrated direct involvement of NFκB in both induction of cell proliferation and inhibition of apoptosis of HRS cells (Krappmann et al, 1999). One possible explanation for this constitutive activation of NFκB in HRS cells may involve deregulation of IκBα-mediated control. Using single cell PCR of HRS cells, Jungnickel et al (2000) detected clonal deleterious mutations in the IκBα gene in two of three cases of EBV-negative HL. However, no such alterations were observed in two EBV-positive HL cases. Since the IκBα gene is an inhibitor of NFκB activation, the presence of mutations in only EBV-negative HL cases implies that IκBα may serve as a tumour suppressor gene, and also suggests an alternate (i.e. non-IκBα mutation dependent) mechanism of NFκB activation in EBV-positive HRS cells.

Normal germinal centre B cells that lack B cell receptor expression are eliminated by apoptosis. The majority of HRS cells are of B cell lineage yet lack B cell receptor expression, indicating that they must be rescued by a transforming event. LMP1 plays a critical role in the protection of B cells from apoptotic death by the up-regulation of several antiapoptosis genes. This survival signal has been proposed to play a crucial role in protecting a variety of cells, including EBV-positive HRS cells, from TNF-mediated apoptosis (Devergne et al, 1996; Asso-Bonnet et al, 1998). It has been proposed that the observed protection may be coincident with the increased expression of the anti-apoptotic protein Bcl-2 (Henderson et al, 1991), although data on the association of bcl-2 and LMP1 are conflicting (Jiwa et al, 1995).

Kim et al (2000) suggested a novel mechanism for the development of EBV-positive HL by LMP1 via CD99, a glycosylated transmembrane protein encoded by mic2. This follows earlier studies where in vitro down-regulation of CD99 in B lymphocytes was reported to generate cells with HRS phenotypes as seen in the lymph nodes of patients with Hodgkin’s disease, implicating the association of loss of CD99 with HL pathogenesis (Kim et al, 1998). In contrast to the well-documented functions of LMP1 as an activator of many cellular signal transduction pathways, Kim et al (2000) reported that LMP1 also acts as a transcriptional repressor on CD99 and demonstrated that the NFκB activation domains in the cytoplasmic terminus of LMP1 were associated with CD99 repression.

The EBV latent protein, LMP2A has also been implicated in the pathogenesis of HL although its role in the malignant phenotype is less clear. Recent research carried out by Portis et al (2003) utilizing DNA microarray technology to study changes in gene transcription following LMP2A expression in B cells of transgenic mice and human LCL, demonstrated that LMP2A interferes with global transcription factor gene expression and activity during normal B-cell development, with many alterations in gene expression induced by LMP2A similar to those recently described in HRS cells. Of particular significance in the LMP2A mice during B lymphopoiesis, was the two- to threefold down-regulation of the transcription factors E2A, EBF and Pax-5 in bone marrow and splenic B cells. Additionally, the DNA binding activity of E2A in these cells was significantly inhibited as shown by the diminished binding to the promoters of its target genes, including EBF and Pax-5, while the expression of two E2A inhibitors, Id2 and SCL were up-regulated in the splenic B cells (Portis et al, 2003).

EBNA1 is the only viral protein that is consistently expressed in all EBV-associated malignancies, as well as in chronic active EBV infection (Yoshioka et al, 2003), and is essential for viral DNA replication and maintenance of the viral episome in infected cells. EBNA1 binds to the symmetrical sequences of the origin of replication of the viral DNA and together with cellular proteins co-ordinates replication of viral episomes with cellular DNA (Yates et al, 1985). Although no direct oncogenic activity of EBNA1 has been observed in tissue culture assays, expression of EBNA1 in transgenic mice induces B cell follicular lymphoma (Wilson et al, 1996). This demonstration that EBNA1 is oncogenic in vivo suggests that it may also play a direct role in the pathogenesis of EBV-associated malignancies. Both LMP1 and EBNA1 are regulated by the JAK-STAT (Janus kinase-signal transducers and activators of transcription) signalling transduction pathways, with STAT3 constitutively activated in HRS cells (Chen et al, 2001), suggesting that dysregulation of the JAK-STAT pathway may precede the development of EBV-associated tumorigenesis.

**Immune evasion**

The HL has evolved multiple strategies to both evade and subvert the immune response. Ineffective immunity against HRS cells is most probably linked to a well established cell-mediated immune deficiency that is present early in the disease (reviewed in (Poppema et al, 1999). It remains uncertain whether this immune deficiency predates oncogenesis and results in an increased susceptibility to HL, or conversely is a sequela generated by the malignancy itself. Clinically, this immune deficiency is reflected in an increased susceptibility to fungal, viral and bacterial infections. Prior to the advent of modern chemo-radiotherapeutic strategies, infection was a major cause of death due to HL (Colby et al, 1981). Diminished cellular immunity, as evidenced by a reduced capacity to reject skin allografts and impaired delayed type hypersensitivity reactions, has long been known (Eltringham &
Kaplan, 1973). These observations may in part be explained by a selective loss of circulating CD4 T cells (perhaps as a result of an influx of CD4 T cells within the inflammatory infiltrate that surrounds HRS cells), and also due to impaired lymphocyte functions (Gaines et al., 1973).

Lymph nodes involved by cHL are characterized by a minority of neoplastic cells, and an overwhelming admixture of reactive cells including T-helper type 2 cells (Th2), fibroblasts, plasma cells, eosinophils and histiocytes. The composition of the inflammatory infiltrate varies according to histological sub-type of the cHL, but CD8 T cells and natural killer (NK) cells are generally sparse. The nature of the aberrant immune response in the vicinity of the HRS cells is a matter of great interest (reviewed in Maggio et al., 2002; Skinnider & Mak, 2002). TH2 (including Thymus and Activation Regulated Chemokine (TARC), macrophage-derived chemokine (MDC), interleukin (IL)-5, IL-6, IL-13 and eotaxin) and immunosuppressive (IL-10 and transforming growth factor-beta) chemokines and cytokines produced either by HRS or infiltrating cells, might be one explanation for the maintenance of a favourable micro-environment for HRS cells to proliferate, escape from apoptosis and survive host anti-tumour defences. The TH1 cytokine IL-12 is detected within reactive lymphoid cells present in close proximity to (but not within) HRS cells (Schwaller et al., 1995). EB13 is an EBV-induced cytokine homologous to the p40 subunit of IL-12, and is strongly expressed in HRS cells independently of EBV status, and it has been postulated that this may serve as a functional antagonist of IL-12 to inhibit a localised TH1 immune response (Niedobitek et al., 2002). Flow cytometry of infiltrating lymphocytes from HL lymph nodes has shown significant populations of CD4⁺ IL-10⁺ and CD4⁺ IL-12⁺ regulatory T cells, and it is likely that this represents another mechanism by which HRS cells evade an effective cellular response (Marshall et al., 2003a). In healthy subjects, LMP1 has been shown to inhibit in vitro T cell proliferation and this response appears to be mediated by IL-10 secretion from CD4⁺ regulatory T cells (Marshall et al., 2003b). Intriguingly, IL-10 may also be induced by EBERs present in EBV-positive HRS cells as has been found for EBV-associated BL (Kitagawa et al., 2000).

In EBV-positive HL, patients are paradoxically able to mount an effective anti-EBV response after the episode of initial EBV infection, but unable to successfully reject EBV-positive HRS cells. Based on studies in healthy individuals and HL patients, the EBV-specific CTL response is strongly focussed through epitopes within the EBNA2 and 3A, 3B and 3C proteins with minimal class I reactivity against LMP epitopes and none within EBNA1 (Chapman et al., 2001, Unpublished observations). Since HRS cells express a type II latency pattern, i.e. only EBNA1 and LMP1 & 2 antigens, the existing EBV-specific CTL repertoire in HL patients may have a limited capacity to control this potentially immunogenic tumour in vivo. Frisan et al (1995) demonstrated that EBV-specific CTL could be detected in the peripheral circulation but not in the immediate vicinity of EBV-positive HL HRS cells. In contrast they found that EBV-specific tumour infiltrating CTL were present in EBV-negative HL, and in one EBV-negative HL case demonstrated the presence of a dominant LMP2A specific CTL response (Dolcetti et al., 1995).

EBNA1 is protected from processing and presentation via the conventional major histocompatibility complex (MHC) class I pathway by virtue of its internal Glycine-Alanine repeat (GAr) domain (Levitskaya et al., 1995), which acts as a cis-inhibitory signal to prevent ubiquitin/proteasome dependent protein degradation (Levitskaya et al., 1997). EBNA1 also appears to reduce its own synthesis via Gar-mediated inhibition of EBNA1 messenger RNA translation (Yin et al., 2003). This may be why early investigators were unable to detect EBNA1-specific CTL using autologous virus-infected stimulation to detect EBNA1-expressing target cells from blood taken during primary infection (Steven et al., 1996) or from healthy virus carriers (Murray et al., 1992). Subsequent work using EBNA1 peptides, EBNA1-protein loaded dendritic cells and MHC class I EBNA1-specific tetramers have identified EBNA1-specific CD8⁺ T cells that are much more abundant than hitherto imagined, being seen both in the primary and memory phases of infection at magnitudes at least the equal of responses to immunodominant epitopes from the ‘conventionally processed’ EBNA3 proteins (Blake et al., 2000). It is likely that dendritic cells exogenously acquire EBNA1 (via viral proteins released from infected cells or by take up of apoptotic-infected cells themselves) and present the antigen via the MHC class I pathway, (a process termed ‘cross-priming’). CD4⁺ CTL may also be important in targeting EBNA1, and there is evidence to suggest this may occur by an HLA-DM independent, class II pathway (Khan et al., 1997). To date, only a few HLA class II epitopes and their class II restrictions have been determined (Voo et al., 2002; Kruger et al., 2003).

The majority of LMP1 and LMP2 T cell epitopes are processed by a trypsin activation peptide (TAP)-independent pathway (Khan et al., 1996). TAP-deficient LCL that express LMP proteins are more efficiently recognized than TAP-positive LCLs. It is therefore tempting to speculate that EBV-positive HRS cells have evolved to maintain TAP expression and so limit the presentation of LMP epitopes, thus facilitating their escape from immune surveillance. It has also been proposed that LMP1 and LMP2A enriched exosomes that are secreted in association with MHC class II, may be taken up by infiltrating T-lymphocytes, where LMP1 could exert an anti-proliferative effect to enable HRS cells to evade the cellular immune response (Flanagan et al., 2003).

Limitations of current therapy

Although the majority of patients with HL can be cured with conventional modality treatments, up to 30% of patients with advanced HL will progress or relapse (Connors et al., 2001). Although new, more intensive dose regimens have significantly improved outcome (Diehl et al., 2003), less than half of relapsed and refractory patients will respond to conventional...
salvage strategies. Recent data from the German Hodgkin’s Lymphoma Study Group confirms that some of these patients will be successfully treated by high dose therapy and autologous haematopoietic stem cell transplantation (autoSCT) (Schmitz et al, 2002). Whilst this study demonstrates that many patients with relapsed but chemosensitive disease can be salvaged, the outcome for patients with primary refractory disease remains poor (Horning, 1998). A registry study of autoSCT cases reported to the European Blood and Marrow Transplantation group, found that only one-third of patients who fail to enter remission following initial induction therapy will be alive at 5 years (Sweetenham et al, 1999). Despite the recent advent of retroviral therapy, survival rates in those with advanced HL and concomitant HIV infection (Spina et al, 2002) are substantially inferior to non-HIV associated HL patients. Older patients with HL have a worse prognosis than young adults, and the survival of EBV-negative HL elderly HL patients is particularly poor (Stark et al, 2002).

Many younger survivors have a long life expectancy subsequent to treatment, providing a long time to experience the non-fatal and fatal long-term toxicities of combined modality therapies (Aisenberg, 1999). In one study of over 1000 young patients (aged 15–29 years) in continuing remission after second line treatment, overall survival at 20 years (the average career span of a Consultant Haematologist) was only 84% compared with 98.5% in age-matched healthy controls (Vaughan Hudson et al, 1994). Even good-risk patients have been found to have a sustained excess in treatment-related mortality (Ng et al, 2002). Those patients with relapsed and refractory disease who go on to receive stem cell transplantation, will be at risk of an additional multitude of long-term side-effects (Curtis et al, 1997; Gandhi et al, 2003a). Therefore, it remains essential that haemat-oncologists resist the temptation to become complacent about HL, and that research continues to be directed towards protocols that maximize efficacy but are associated with fewer and less severe side-effects.

### Graft versus HL

Indirect evidence that cellular immunotherapy may be of benefit for EBV-negative HL comes from allogeneic haematopoietic stem cell transplantation (alloSCT). Results from alloSCT for poor-risk HL patients, provides several lines of evidence to suggest a graft versus HL effect exists. Firstly, although conventional alloSCT for HL is associated with high rates of procedural-related mortality (Gajewski et al, 1996), survivors appear to have a reduced relapse rate (Akpek et al, 2001), and this may be associated with graft versus host disease (GvHD). Secondly, investigators using reduced intensity allogeneic stem cell transplantation (RIST) to harness the immunotherapeutic benefits of alloSCT but with reduced toxicity, reported that the progression-free survival at 1 year was significantly better for those with HL than with high-grade non-HL (Robinson et al, 2002). The MD Anderson group found that, in six heavily pre-treated HL patients who had failed autoSCT, a fludarabine based RIST regimen resulted in three complete remissions (Anderlini et al, 2000). Relapsed HL patients have subsequently responded to donor lymphocyte infusions (DLI) (Marks et al, 2002). Perhaps the most direct evidence that a graft versus HL effect can occur is data from DLI in patients given no preparative regimen. Porter et al (1999) induced a sustained remission (over 2 years) in one of three HL patients following administration of DLI as ‘primary therapy’ for HL relapsing following autoSCT.

One mechanism, by which graft versus HL may be mediated in EBV-positive HL patients, is that EBV-latent antigens act as tumour-associated antigens for EBV-specific CTL. It has been shown that herpes-virus-specific CTL, including CTL specific for latent EBV-antigens can be transferred in EBV-seropositive donors via the donor allograft, and that primary EBV-specific CTL can be generated in the recipient within the first 6 months following transplant from donor progenitor cells (Gandhi et al, 2003b). The relative response to alloSCT in a large series of EBV-negative HL and EBV-positive HL patients has yet to be compared.

### Cellular immunotherapy

The viral and cellular phenotype of EBV-positive HL provides an excellent opportunity for targeted cellular immunotherapy (Khanna et al, 2001) (see Table II). Initial studies have been with poor-risk patients, although arguably the optimal role for such therapies may be in the setting of minimal residual

<table>
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<th>Table II. Immunotherapeutic strategies for EBV-positive Hodgkin’s lymphoma patients with chemo-refractory or multi-relapsing disease.</th>
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<td><strong>Cellular immunotherapy</strong></td>
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<tr>
<td>1. AlloSCT ± DLI (not known whether there is differential benefit in EBV-positive versus EBV-negative HL patients)</td>
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<td>2. Adoptive transfer of allogeneic autologous LMP1/2A-specific CTL</td>
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<td><strong>Antibody immunotherapy</strong></td>
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<td>1. Immunotoxin-conjugated antibodies (can be used irrespective of EBV-HRS status)</td>
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<td>1. LMP1: abrogates oncogenic phenotype and enhances immunogenicity</td>
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AlloSCT, allogeneic stem cell transplantation; DLI, donor lymphocyte infusions; CTL, cytotoxic T-lymphocyte; HRS, Hodgkin Reed-Sternberg cell.
disease or perhaps in combination with SCT. Successful T cell-based immunotherapy for EBV-positive HL relies on the premise that the malignant cells are susceptible to recognition by the host immune system. It has been demonstrated that the majority of EBV-positive HRS cells in fresh tumour biopsies and long-term established lines express the essential components for CTL recognition (Lee et al, 1998; Murray et al, 1998). These include normal levels of expression of TAP-1 and TAP-2, the proteasome components LMP-2 and LMP-7, and high levels of HLA class I and class II alleles on the cell surface (EBV-negative HL also express TAP1 and TAP2 proteins but have reduced or low level HLA class I molecule expression). In vitro cytotoxicity assays indicate that EBV-negative HRS cell lines expressing recombinant EBV proteins from recombinant vaccinia vectors are highly susceptible to CTL-mediated lysis (Sing et al, 1997; Lee et al, 1998).

Adoptive transfer of polyclonal EBV-specific autologous CTL into patients with advanced HL has been reported (Roszkrow et al, 1998). In this study, all patients who received this adoptive therapy had measurable biologic responses, including reduction of high viral load, increase in virus-specific CTL precursor frequency, and resolution of some symptoms with transient stabilization of the disease. Although it was encouraging to see a short-term therapeutic effect, no patients with aggressive EBV-positive HL were cured. Perhaps the major limitation of this approach was the expansion of CTL by stimulating peripheral blood lymphocytes with autologous LCL, which preferentially stimulates T cells specific for EBV antigens rather than LMP1 and LMP2. Further refinement of the CTL expansion protocol may allow selective expansion of LMP-specific CTL (Rooney et al, 1998), and gene-modified CTL may be protected from the inhibitory cytokines secreted by HRS cells (Bollard et al, 2002). It is possible to isolate and expand LMP-specific CTL from the peripheral blood of healthy virus carriers and HL patients which can efficiently lyse LMP-expressing target cells (Sing et al, 1997; Chapman et al, 2001).

In vitro stimulation of T cells with autologous dendritic cells (DC) transduced with an adenoviral construct that encodes for 90% of the LMP1 protein, has successfully been used to selectively expand LMP1-specific CTL which have minimal reactivity to other EBV antigens (Gottschalk et al, 2002). However, the use of full-length LMP1 antigen to activate specific CTL for adoptive immunotherapy in a clinical setting may be significantly constrained by the fact that these proteins have both oncogenic potential and probable immunosuppressive properties (Dukers et al, 2000; Marshall et al, 2003b).

However, a large panel of HLA class I-restricted CTL epitopes within LMP1 and LMP2 latent proteins have now been identified (Duraiswamy et al, 2003a). These epitopes are presented by a wide range of HLA class I alleles, covering more than 90% of the Caucasian population, and are highly conserved in EBV isolates from a variety of other ethnic populations. Although LMP-specific CTL frequencies are low in newly diagnosed HL patients (Chapman et al, 2001), preliminary studies by our group using healthy virus carriers and HL patients have indicated that fresh autologous PBMC coated with synthetic immunogenic LMP1 and LMP2 peptide epitopes can be successfully used to expand large numbers of LMP1- and LMP2-specific CTL. These T cells efficiently kill virus-infected target cells. Human cells infected with a recombinant vaccinia-virus polyepitope construct that encoded six HLA A2-restricted LMP1-specific epitopes were efficiently recognized by LMP1-specific CTL lines from HL A2 healthy individuals (Duraiswamy et al, 2003b). Furthermore, immunization of HLA A2/K b mice with this polyepitope vaccine consistently generated strong LMP1-specific CTL responses to five of the six epitopes which were readily detected by both ex vivo and in vitro assays. More importantly, this polyepitope vaccine successfully reversed the outgrowth of LMP1 expressing tumours in HL A2/K b mice. Recently, similar results have been obtained via another polyepitope vaccine, this time utilizing the replication-deficient adenoviral Ad5/F35 vector. This vector permits highly efficient gene transfer into haematopoietic stem cells and dendritic cells (Yotnda et al, 2001). The Ad5/F35-LMP polyepitope encodes for thirteen HLA class I LMP1 and LMP2A epitopes restricted through 14 different HLA class I alleles, which span >90% of the population in different ethnic groups. In humans, in vitro stimulation with Ad5/F35-LMPpolyepitope generates rapid expansion of LMP-specific CTL. These expanded T cells display strong lysis of autologous target cells sensitized with LMP1 and/or LMP2 CTL epitopes. As with the vaccinia LMP1 polyepitope, the adenoviral vaccine was successfully used to reverse the outgrowth of LMP1-expressing tumours in HL A2/K b mice (Duraiswamy et al, 2004).

Lessons learnt from immunotherapeutic studies of the β herpes virus, cytomegalovirus (CMV), reviewed in (Gandhi et al, 2003c), may have application for adoptive transfer protocols for LMP-specific CTL. Avoidance of in vitro expansion of CTL by positive selection of HLA class I tetramer-labelled CMV pp65-specific CD8+ T cells has been successfully applied for the adoptive transfer of sibling-donor CMV pp65-specific CD8+ T cells in alloSCT recipients (Cobbold et al, 2003). However, given the relatively low frequency of LMP-specific CTL as compared with CMV pp65-specific CTL, this approach will probably prove challenging for the treatment of EBV-positive HL and would need to be modified to include further expansion of tetramer-positive CTL. HLA class I tetramers have been used for rapid cloning for adoptive immunotherapy in healthy CMV seropositive subjects (Szmania et al, 2001). Szmania and colleagues performed initial expansion of CTL using peptide-pulsed mature DC, then two re-stimulations with peptide-pulsed autologous LCL followed by positive selection of HLA class I tetramer-labelled LMP-specific CD8+ T cells and further expansion of the purified population. Alternatively, one approach which does not require characterization of immunodominant viral peptides would be to use viral antigen (Peggs et al, 2003). Such a strategy has the additional advantage of inducing CD4 and CD8 virus-specific T cell responses.
Although adoptive transfer of autologous LMP-specific CTL appears promising, it will be difficult for such a technically demanding cell-based immunotherapy to be sufficiently ‘scaled up’ to treat large numbers of patients. One way to circumvent this in the future might be to use HLA-partially matched allogenic EBV-specific CTL. A similar strategy has successfully been used for the treatment of PTLD (Haque et al., 2002). Cytotoxicity to donor phytohemagglutinin (PHA) blasts was used to screen for alloreactivity. No patients developed either GvHD or graft rejection. An alternate approach would be immunization using LMP peptide-pulsed DC, as has been successfully achieved in patients with naso-pharyngeal carcinoma (Lin et al., 2002). Even wider applicability would be achieved using an LMP polyepitope construct for active therapeutic immunization. This could be expressed as either a viral vector or as a DNA vaccine, as reviewed in (Khanna et al., 2001). Humoral responses to attenuated viral vaccines are minimal in the first year following autoSCT (Gandhi et al., 2001), making the generation of a neutralizing antibody response against the viral vector less likely, were the vaccine administered within this time-frame to relapsed or refractory Hodgkin’s patients following autograft.

**Proteosomal targeting**

Recently, investigators have explored a novel strategy to circumvent the restricted class I processing of EBNA1 (see Fig 1). It has been demonstrated that the GAr-mediated proteosomal block on EBNA1 can be reversed by specifically targeting this antigen for rapid degradation by a process of co-translational ubiquitination combined with N-end rule targeting (Khanna et al., 1996; Tellam et al., 2001). The subsequent enhanced intracellular degradation of EBNA1 led to the induction of a very strong EBNA1-specific CTL response, and restoration of the endogenous processing of HLA class I-restricted CTL epitopes within EBNA1 for immune recognition by human EBV-specific CTLs.

Co-translational ubiquitination combined with N-end rule targeting of LMP1 results in enhanced degradation of LMP1 (Fig 1). This is associated with decreased activation of NFκB and STAT in human cells and a subsequent increase in sensitivity to apoptosis of ubiquitinated-LMP1-expressing fibroblasts. There was abrogation of the oncogenic phenotype in nude mice, whilst in BALB/c mice an enhanced CD8+ T cell response to a model epitope fused to the C-terminus of LMP1 was observed following immunization with ubiquitinated LMP1 (Tellam et al., 2003). It is important to stress that a model epitope was used in this study to assess the immunogenicity of ubiquitinated LMP1, and formal validation of this approach would require further studies based on an LMP1-derived epitope. These observations raise the possibility of developing therapeutic strategies to modulate the stability of LMP1 in normal and HRS cells. Through manipulation of the ubiquitin-dependent proteolytic machinery, specifically targeted E3 ubiquitin-protein ligases could potentially lead to the enhanced degradation of LMP1, thereby increasing this protein’s sensitivity to apoptosis.

**Antibody-based immunotherapy**

Monoclonal antibodies can be manufactured to clinical scale and HRS cells potentially offer a number of attractive antigens for targeted immunotherapy. Definitive evidence of the extracellular accessibility of LMP1 and LMP2A is lacking (Flanagan et al., 2003). Antibody-based targeting of LMP1 and LMP2 extracellular domains on EBV-positive HRS cells may have therapeutic (and diagnostic) possibilities. Alternatively, CD25 and CD30 represent attractive antigens for targeted immunotherapy. This approach has the added theoretical advantage of therapeutic efficacy in both EBV-positive HL and non-EBV HL cases. Although immunotoxins have showed in vivo antitumour activity in a severe combined immunodeficient mouse model (Barth et al., 2000), phase I/II clinical trials have seen only modest responses in relapsed HL patients (Schnell et al., 2002).

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**Fig 1.** Enhanced presentation of EBNA1/LMP1 via proteosomal targeting. (A) Ubiquitin-dependent protein degradation, resulting in peptide presentation to cytotoxic T lymphocytes (CTL). (B) Co-translational ubiquitination combined with N-end rule targeting of EBNA1 or LMP1, restores/enhances intracellular degradation of EBNA1/LMP1 and restores/enhances the endogenous processing of HLA class I-restricted CTL epitopes within EBNA1/LMP1 for immune recognition by peptide-specific CTLs.
Sixteen heavily treated refractory HL patients have been treated with a bi-specific antibody against CD16 and CD30, resulting in a 25% response rate. This approach enables the antibody to simultaneously bind to CD30 on the HRS cell and CD16 on NK cells to induce tumour directed cytoxicity (Hartmann et al, 2001). Borchmann et al (2002) have demonstrated similar efficacy with a CD30/CD64 bi-specific antibody. One major obstacle for broader applicability of these approaches is the immunity that is induced against murine antibodies and the toxin components, which can result in a vascular leak type syndrome. Further research is on-going to generate humanized antibodies to reduce side effects (Borchmann et al, 2003).

Conclusion and future directions
The past few decades have seen enormous advances in the treatment of HL. Yet therapeutic challenges still remain, particularly with respect to minimizing long-term side effects, designing an effective salvage strategy for relapsed and refractory disease, and the need to identify better prognostic markers to enable patients with poor-risk disease to be stratified towards novel therapies. Although our understanding of the epidemiology and immunopathogenesis of HL has made important strides, several issues still need clarification, including the potential aetiological role of other factors with regard to both EBV-positive HL and EBV-negative HL. Further, irrespective of the persisting controversy over whether EBV has a direct or indirect role in the biology of HL, the presence of EBV-latent antigens in the malignant HL cells represents an attractive opportunity for targeted immunotherapy.

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