The Role of Viruses in the Genesis of Hodgkin Lymphoma

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Abbreviations

BARTs	BamHI fragment A rightward transcripts
BHRF1	BamHI-H rightward open reading frame 1
cHL	Classic Hodgkin lymphoma
DDR1	Discoidin domain receptor 1
EBER	EBV-encoded small RNAs
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
HHV	Human herpesvirus
HL	Hodgkin lymphoma
HLA	Human leukocyte antigen
HPyV	Human polyomavirus
HRS	Hodgkin and Reed-Sternberg

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IHC	Immunohistochemistry
LMP	Latent membrane protein
MCHL	Mixed cellularity Hodgkin lymphoma
MCPyV	Merkel cell polyomavirus
miRNAs	MicroRNAs
MV	Measles virus
NSHL	Nodular sclerosis Hodgkin lymphoma
ORF	Open reading frame
PyV	Polyomavirus
SNP	Single nucleotide polymorphism
TSPyV	Trichodysplasia spinulosa polyomavirus
TTMDV	Torque teno midi virus
TTMV	Torque teno mini virus
TTV	Torque teno virus

2.1 Introduction

Hodgkin lymphoma (HL) is a heterogeneous condition. Seminal papers published in 1957 and 1966 suggested that HL in younger and older adults had different etiologies and further suggested an infectious etiology for young adult HL [1, 2]. Subsequent epidemiological studies provide broad support for these hypotheses [3, 4]. Data linking young adult HL with a high standard of living in early childhood and lack of child-child contact suggest that delayed exposure to common childhood infections may be involved in the etiology of these cases [5, 6]. There is now compelling evidence that a proportion of cases of HL are associated with the Epstein-Barr virus (EBV). Paradoxically, older adult and childhood cases of HL are more likely to be EBV-associated than young adult cases [7–9]. In this article, we review studies on viral involvement in HL with a focus on classic HL (cHL), since nodular lymphocyte-predominant HL is considered a separate disease entity. The association with EBV will be discussed with an emphasis on findings that support a causal role for EBV in this malignancy. Studies investigating the direct involvement of other exogenous viruses will be summarized.

2.2 Hodgkin Lymphoma and Epstein-Barr Virus

EBV is a herpesvirus with a worldwide distribution [10–13]. Over 90% of healthy adults are infected by EBV, and, following primary infection, the virus establishes a persistent infection with a reservoir in memory B-cells [14]. Although EBV is an extremely efficient transforming agent, the virus is kept under tight control by cell-mediated immune responses, and both primary and persistent infections are usually asymptomatic [10, 15].

EBV infection can be lytic or latent. Lytic infection is associated with expression of a large number of viral genes, production of progeny virus, and death of the infected cell; in contrast, latent infection is associated with expression of a small number of EBV genes, persistent infection, and growth transformation [10]. In B-cells transformed by EBV in vitro, six EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C, and LP, also called EBNA1–6) and three latent membrane proteins (LMP1, LMP2A, and LMP2B) are expressed [10]. In addition, noncoding viral RNAs are transcribed in latently infected cells [16]. These include two small non-polyadenylated transcripts, the EBERs, and over 44 viral microRNAs (miRNAs) located within introns of the BARTs (BamHI fragment A rightward transcripts) or around the coding region of the BHRF1 (BamHI-H rightward open reading frame 1) gene [16–22]. Expression of the full set of latent genes is known as latency III [10, 13]. EBV gene expression in EBV-positive lymphomas occurring in the context of immunosuppression frequently follows this pattern, but more restricted patterns of EBV gene expression are observed in other malignancies, including cHL [10, 12, 13]. The EBNA3 family proteins are immunodominant, and the other latent antigens elicit only subdominant or weak cell-mediated immune responses [23, 24]. The pattern of gene expression in EBV-associated malignancies most probably depends on both the lineage and stage of differentiation of the infected tumor cells and the host EBV-specific immune response.

In EBV-associated cHL (also referred to as EBV-positive cHL), all of the tumor cells, the Hodgkin and Reed-Sternberg (HRS) cells, are infected by EBV [25–27]. The EBV infection within tumors is also clonal suggesting that all of the tumor cells are derived from a single infected cell [28, 29]. The HRS cells express EBNA1, LMP1, LMP2A, and 2B, but the remaining EBNAs are downregulated (Fig. 2.1); the non-coding EBER RNAs and BART miRNAs are also expressed [25, 26, 30–33]. This pattern of gene expression is referred to as latency type II [10]. EBV infection of HRS cells can be readily demonstrated in sections of routinely fixed, paraffin-embedded material using either EBER in situ hybridization or LMP1 immunohistochemistry (IHC) (Fig. 2.1) [25, 26]. Reagents for both assays are commercially available.

2.2.1 Epstein-Barr Virus and the Pathogenesis of Hodgkin Lymphoma

The molecular pathogenesis of cHL and the origin of the HRS cell are described in detail in Chap. 3. Briefly, HRS cells have clonally rearranged immunoglobulin genes with evidence of somatic hypermutation, indicating a derivation from B-cells that have participated in a germinal center reaction [34, 35]. A pathognomonic feature of these cells is the global suppression of B-cell signature genes and inappropriate expression of genes associated with other hematopoietic lineages [36, 37]. Importantly, HRS cells do not express B-cell receptors (BCRs). Survival of germinal center B-cells normally requires signaling through both BCRs and CD40; HRS cells must, therefore, have acquired a non-physiological survival mechanism(s). Functional studies of EBV, and LMP1 and LMP2A, support a role for the virus in HRS cell survival, transcriptional reprogramming, and immune evasion, as summarized below (Fig. 2.2).

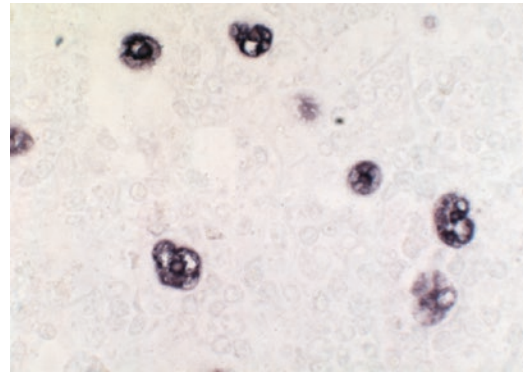

Fig. 2.2 EBV EBER in situ hybridization staining of EBV-positive Hodgkin and Reed-Sternberg cells. The characteristic staining pattern is observed in the nuclei of Hodgkin and Reed-Sternberg cells

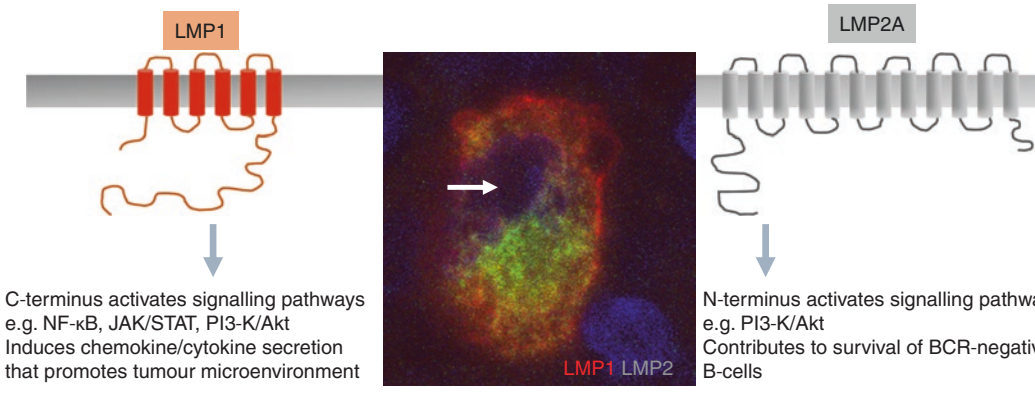

Fig. 2.1 The latent membrane proteins of EBV contribute to the pathogenesis of classic Hodgkin lymphoma. Schematic diagram of LMP1 (left) and LMP2A (right) proteins in the cell membrane (gray bar). Both are transmembrane proteins that signal constitutively through the C-terminus in the case of LMP1 and the N-terminus in the case of LMP2A. The photomicrograph in the center shows the co-expression of LMP1 (red) and LMP2A (green) in the same Hodgkin and Reed-Sternberg cell in a tissue section of classic Hodgkin lymphoma. The nucleus of the Hodgkin and Reed-Sternberg cell stained blue with DAPI is arrowed

In 2005, three independent groups published data showing that germinal center B-cells lacking BCRs could survive and be immortalized by EBV [38–40]. In elegant experiments, Mancao and Hammerschmidt later showed that this survival function was dependent on LMP2A expression [41]. A series of in vivo and in vitro studies from the Longnecker laboratory further defined LMP2A function and showed that this viral protein could mimic an activated BCR and provide a survival signal to BCR-negative B-cells [42–44]. LMP2A expression in B-cells also results in downregulation of B-cell-specific genes and induction of genes associated with proliferation and inhibition of apoptosis, a gene expression profile similar to that seen in cHL-derived cell lines [45]. Constitutive activation of Notch1 by LMP2A, and subsequent inhibition of E2A and downregulation of EBF, two transcription factors that regulate B-cell development, appears to be involved in both survival signaling and transcriptional regulation [44]. Although these data suggest a role for LMP2A in the survival and reprogramming of HRS cells, many of the intracellular molecules involved in BCR signaling are downregulated in HRS cells, and therefore the precise contribution of LMP2A in cHL is not clear.

CD40 signaling plays a critical role in the positive selection of germinal center B-cells expressing high-affinity immunoglobulin and their subsequent exit from the germinal center [46]. EBV LMP1 is an integral membrane protein which interacts with several signal transduction pathways to activate NF-κB, Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase [47–51]. In this way, LMP1 mimics a constitutively active CD40 molecule, although it provides a more potent and sustained signal. Many of the genes that are transcriptionally regulated by LMP1 in germinal center B-cells are also CD40 and NF-κB targets [52]. Activation of the NF-κB pathway, which is a feature of both EBV-positive and EBV-negative HRS cells, leads to upregulation of anti-apoptotic genes and is thought to play a key role in HRS cell survival [53–55]. LMP1 expression in germinal center B-cells also leads to increased expression of Id2, an inhibitor of the E2A transcription factor mentioned above, and repression of B-cell signature genes [52]; therefore, LMP1 may also contribute to transcriptional reprogramming.

The EBV genome is maintained as an episome in infected cells; i.e., it does not normally integrate. The EBNA1 protein is responsible for maintenance of the genome in episomal form, genome replication, and genome partitioning during mitosis [10, 56]. EBNA1 can also influence both viral and cellular gene expression and appears to confer a B-cell survival advantage, although the impact of EBNA1 on oncogenesis in vivo is controversial [10, 57–60]. Interestingly, in the context of cHL, overexpression of EBNA1 in vitro leads to the appearance of multinucleated cells [57].

The EBV EBER RNAs are two small, non-polyadenylated RNA pol III transcripts that are stably expressed in the nuclei of all latently infected cells, including HRS cells. The precise function of the EBERs remains unclear, and, although not essential for transformation, expression of these small RNAs is required for efficient EBV-induced B-cell growth and transformation [16, 61–63].

EBV-encoded miRNAs were identified first in 2004, and their important role in EBV biology and oncogenesis is an area of intense study [16, 17, 22, 64, 65]. Functional analysis of the BHRF1 and BART miRNAs suggests roles in evading the immune response, promoting cell survival and proliferation, inhibiting viral reactivation, and fine-tuning gene expression [16, 22, 64, 65]. EBV-associated malignancies, including cHL, express BART miRNAs, but the BHRF1 miRNAs, which are associated with latency type III, are not expressed [33]. In vitro studies of knockout viruses lacking some or all of the miRNAs suggest that they have an important role in the initial stages of B-cell transformation by EBV; BHRF1 miRNAs play the predominant role with some contribution from BART miRNAs at low multiplicity of infection [22]. In contrast, in vivo studies in a murine huNSG model suggest that the main function of these miRNAs is to attenuate the antiviral T-cell-mediated immune response, leading to increased numbers of EBV-

infected B-cells at later time points [66]. Again, these effects appear to be mediated by the BHRF1 miRNAs, as viruses deficient in only the BART miRNAs produced similar results to wild-type virus in this model system [66]. Ross et al. reported that miRNA BART11-5p downregulates the B-cell transcription factor EBF1, suggesting a plausible role for this miRNA in cHL [67]. EBV also regulates the expression of host miRNAs; infection of primary B-cells leads to a conspicuous downregulation of many miRNAs with the notable exception of mIR-155, which is highly expressed by both EBV-positive and EBV-negative HRS cells [68, 69]. Analysis of host miRNAs in cHL is described in more detail in Chap. 4, but it has been reported that EBV status of tumors is associated with differences in expression pattern [70].

While most studies have investigated the effects of the latent genes in isolation, there is evidence that co-expression of the EBV latent genes is important. For example, it has been shown that LMP1 is transforming when expressed alone in transgenic mouse B-cells [71]. However, when LMP2A is expressed together with LMP1, the resulting mouse B-cells are normal [71]. Comparison of LMP1 and LMP2A in B-cells confirms they have both synergistic and counteracting transcriptional effects [72]. Furthermore, in another study it was shown that LMP1 and LMP2A co-expression in mouse B-cells resulted in tumors, but only if the animals were immunosuppressed suggesting that the combined expression of these latent genes is immunogenic in vivo [73].

There is evidence that the tumor microenvironment in EBV-positive and EBV-negative cHL is different. Thus, the T-helper cells present in EBV-positive cHL are enriched for functional Th1 cells [74]. EBV-positive cHL is also preferentially infiltrated by regulatory Type 1 cells (Tr1), which express ITGA2, ITGB2, and LAG3 and secrete IL-10 [75]. This Th1-biased infiltrate is consistent with previous reports of higher numbers of activated CD8+ T-cells in EBV-positive cHL [76] and is also associated with the presence of predominantly M1-polarized macrophages [77]. There is evidence that the EBV latent genes are responsible, at least in part, for the recruitment and modification of this tumor microenvironment. LMP1, in particular, has been shown to induce expression of many of the chemokines and cytokines secreted by EBV-infected HRS cells [78, 79]. The cHL tumor microenvironment also contributes to the suppression of host anti-EBV-specific immunity. Thus, while LMP1 and LMP2A proteins are targets of CD8+ cytotoxic T lymphocytes, it is clear that immune effectors present in the tumor tissues of EBV-positive cHL cannot kill the virus-infected cells [80, 81]. LMP1 probably also contributes to the suppression of EBV-specific immunity through its ability to induce expression of the immunosuppressive cytokines, including IL-10, and upregulate the immune checkpoint ligand, PD-L1 [82, 83]. LMP1 also upregulates the collagen receptor, discoidin domain receptor 1 (DDR1), a receptor tyrosine kinase expressed by HRS cells [84]. Engagement of DDR1 by collagen leads to the increased survival of lymphoma cells, thus providing a link between the expression of LMP1 and pro-survival signaling from the tumor microenvironment.

2.2.2 Risk Factors for Epstein-Barr Virus-Associated Hodgkin Lymphoma

It is clear that EBV is associated with only a proportion of cHL cases. In industrialized countries around one third of cases are EBV-associated, whereas in Africa and Central and South America, this proportion is significantly higher [8, 9, 85, 86]. EBV-associated cHL cases are not randomly distributed among all cHL cases, and the demographic features and risk factors for the development of EBV-positive and EBV-negative cHL show distinctive features [8, 9, 86]. Childhood (<10 years) and older adult (50+ years) cases are more likely to be EBV-associated than young adult cases (15–34 years) [7, 8, 86]. Among EBV-associated cases, males outnumber females by approximately 2:1, whereas males and females are more evenly represented among EBV-negative cases [9, 86]. In developing countries, childhood cHL is more com-

mon than in industrialized countries, resulting in a higher proportion of EBV-associated cases [9, 86, 87]. Material deprivation is associated with an increased proportion of EBV-positive childhood cHL cases in industrialized countries, and there is some evidence that this also holds for older adult cases [85, 88].

EBV infection usually occurs in childhood, and in many parts of the world, there is almost universal infection by the age of 5 years [11, 89]. If infection is delayed until adolescence, as is increasingly observed in industrialized countries, primary EBV infection manifests as infectious mononucleosis in around 25% of individuals [90]. Infectious mononucleosis has been associated with an increased risk of EBV-associated cHL in some, although not all, studies [6, 91–93]. The increased risk appears relatively short-lived with a median time interval between infectious mononucleosis and cHL of approximately 3–4 years (see Chap. 1: Epidemiology of Hodgkin Lymphoma) [92, 93]. Thus, in both developing and developed countries, there appears to be a period following primary EBV infection, probably lasting several years, in which risk of EBV-associated cHL is increased. cHL occurring in the context of immunosuppression is almost always EBV-associated (see Chap. 1: Epidemiology of Hodgkin Lymphoma) [94, 95], and it is likely that the increased incidence of EBV-associated cHL that occurs in older adults is related to immune senescence. Based on these data, we have proposed an extension of MacMahon's model of HL that divides cHL into four subgroups on the basis of tumor EBV status, age at diagnosis, and age at infection by EBV (Fig. 2.3) [2, 96].

Recent data also suggest that humoral and cell-mediated responses to EBV modulate risk of EBV-associated cHL. Levin and colleagues examined anti-EBV antibody profiles in serum samples from military personnel (mainly young men) that had been collected several years before the diagnosis of cHL [97]. Individuals who subsequently developed EBV-positive, but not EBV-negative, cHL were more likely to have elevated

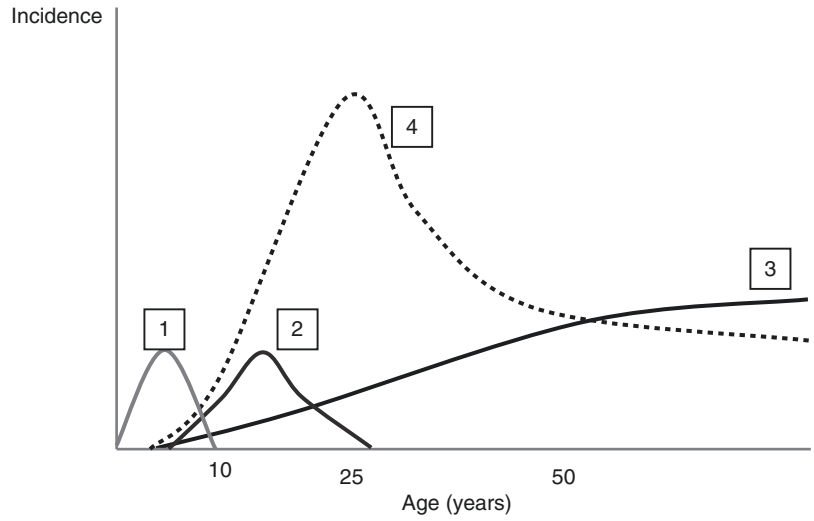

Fig. 2.3 The four-disease model of classic Hodgkin lymphoma. This model divides classic Hodgkin lymphoma into four subgroups based on EBV tumor status, age at diagnosis, and age at EBV infection. Three groups of EBV-associated disease are recognized: (1) a childhood disease, usually occurring below the age of 10 years, which is commoner in developing countries; (2) a disease, most commonly seen in young adults, which occurs following infectious mononucleosis; and (3) a disease associated with poor control of EBV infection, which is typified by the older adult cases but can occur at other ages, particularly in the context of immunosuppression. (4) Superimposed on these is a single group of EBV-negative classic Hodgkin lymphoma cases, which account for the young adult age-specific incidence peak seen in industrialized countries. The relative incidence of each of these four disease subgroups will determine the overall shape of the age-specific incidence curve in any geographical locale

antibody titers to EBV viral capsid and early antigens and an anti-EBNA-1/anti-EBNA2 antibody ratio ≤1.0 when compared to controls. Decreased anti-EBNA-1/anti-EBNA2 antibody ratios have been previously associated with EBV-associated cHL [98], and it has been suggested that a ratio ≤ 1.0, which persists for more than 2 years after infectious mononucleosis, indicates defective control of persistent EBV infection [99]. Variations in EBNA-1 titer are significantly associated with polymorphisms in the human leukocyte antigen (HLA) region [100], suggesting that titers may, in part, be genetically determined and relate to the findings described below.

Data from HLA association studies and genome-wide association studies (GWAS) show clear associations between cHL risk and both HLA alleles and single nucleotide polymorphisms (SNPs) in this region. Although some SNPs appear to be associated with all cHL, independent of EBV status, most HLA associations differ between EBV-positive and EBV-negative subgroups [101–108]. Both HLA class I and II alleles are associated with EBV-positive cHL, whereas EBV-negative cHL is largely associated with class II alleles [102, 103, 105, 107]. Since class I and II alleles present peptides from pathogens to CD8- and CD4-positive T-cells, respectively, this suggests that genetically determined differences in the cell-mediated response to EBV influence disease risk. HLA-A∗01 is associated with an increased risk of EBV-associated cHL, whereas HLA-A∗02, specifically A∗02:01, is associated with decreased risk [102, 103]. Associations with these alleles are independent, i.e., the increased risk associated with A∗01 is not simply due to lack of A∗02, and effects are dependent on the copy number of each of the alleles [103] (Fig. 2.4). As a result, there is an almost tenfold variation in odds of EBV-associated cHL between HLA-A∗01 homozygotes and HLA-A∗02 homozygotes [103]. More recent data suggest that B∗37:01 is also associated with an increased risk of EBV-positive cHL [105, 107]. Class II alleles have been less extensively studied, but Huang et al. reported an increased frequency of DR10 alleles in patients with EBV-positive cHL compared to controls, and we have detected protective effects of DRB1∗15:01 and DPB1∗01:01 [105, 107]. In addition, the SNP rs6457715, which is located close to the HLA-DPB1 gene, is strongly associated with EBV-positive but not EBV-negative cHL [108].

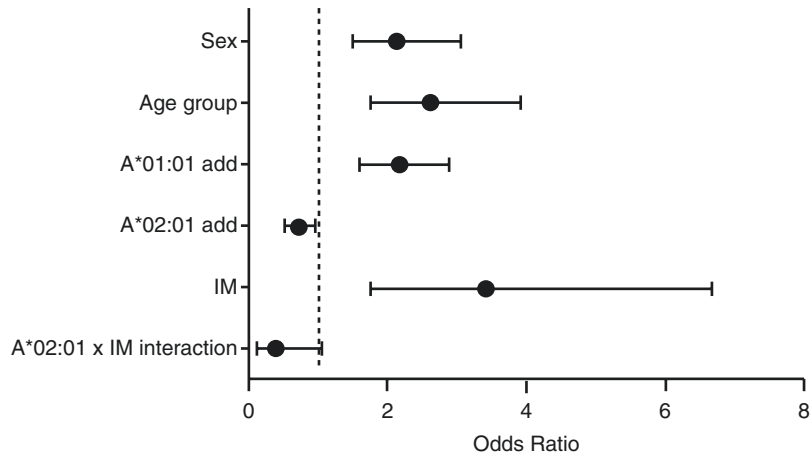

Fig. 2.4 Risk factors for EBV-associated classic Hodgkin lymphoma in adults. Forest plot showing odds ratios and 95% confidence intervals for development of EBV-associated Hodgkin lymphoma from a case series analysis of HLA and non-HLA risk factors [103]. Increased risk is associated with male sex, older age (age ≥50 years versus 15–34 years), possession of HLA-A∗01:01 alleles (add, additive effect), and prior history of infectious mononucleosis (IM). Possession of HLA-A∗02:01 alleles is associated with decreased risk, and abrogation of the increased risk associated with IM

Cytotoxic T-cell responses, restricted through HLA class I, are critical for the control of EBV infection, and A∗02 is known to present a wide range of peptides derived from EBV lytic and latent antigens, including those expressed by HRS cells [23, 24]. In contrast, there are no well-characterized A∗01-restricted EBV epitopes [24], and EBV-specific T-cell responses restricted through A∗01:01 have not been described [109]. The observed associations with HLA-A, therefore, seem biologically plausible. However, HLA-A∗01 is in strong linkage disequilibrium with HLA-B∗08, which is associated with immunodominant EBV-specific cytotoxic T-cell responses, and yet there is no protective effect associated with this allele [107]. The biological basis underlying associations between HLA alleles and EBV-associated cHL is therefore not clear. Further work is also necessary to determine whether the critical HLA-A-restricted cell-mediated immune responses are directed toward EBV-infected HRS cells or whether it is the control of persistent EBV infection, i.e., the host-virus equilibrium, which is all-important. The increased risk associated with individual class I alleles favors the idea that failure to respond to a particular protein, or very restricted group of proteins, determines risk; this focusses attention on EBV proteins expressed by HRS cells. Consistent with this, no EBNA1, LMP1, or LMP2 epitopes restricted by B∗37:01 have been identified although a B∗37:01-restricted EBNA3C epitope has been described [24].

As mentioned above, prior infectious mononucleosis has been associated with an increased risk of EBV-positive cHL [91–93, 110]. Infectious mononucleosis has also been associated with the same genotypic markers (microsatellites and SNPs) in the HLA class I region as EBV-positive cHL, albeit with lesser statistical significance [111]. These data raised the possibility that the association between infectious mononucleosis and EBV-associated cHL resulted from shared genetic susceptibility. However, HLA-A typing of over 700 cHL cases with available self-reported history of infectious mononucleosis revealed that prior infectious mononucleosis was independently associated with EBV-associated cHL after adjusting for the effects of HLA-A alleles [103]. In addition, a statistically significant interaction between prior infectious mononucleosis and HLA-A∗02 was detected; the effect of this was to abrogate the increased risk of EBV-associated cHL following infectious mononucleosis in HLA-A∗02-positive individuals [103]. These results suggest that the increased risk of EBV-associated cHL following infectious mononucleosis is modified by the EBV-specific cytotoxic T-cell response restricted through HLA-A∗02. Thus, it is possible that different HLA alleles exert their effects at different stages in the natural history of EBV-associated cHL.

Associations with childhood cHL and infectious mononucleosis suggest that there is a window of time following primary EBV infection when there is an increased risk of EBV-associated cHL and that genetic factors, specifically HLA-A genotype, modify this risk. EBV-associated cHL patients have higher numbers of EBV-infected cells than patients with EBV-negative disease [112], and infectious mononucleosis patients have very high numbers of circulating EBV-infected B-cells, which decrease over time [113]. The number of EBV-infected cells carried by an individual is therefore likely to influence the risk of EBV-associated cHL. It may, therefore, be possible to decrease the risk of EBV-positive cHL by EBV vaccination, even in the absence of sterilizing immunity [114], or by treatment of infectious mononucleosis with antiviral agents.

2.2.3 Epstein-Barr Virus and Hodgkin Lymphoma: A Causative Association?

In the absence of good animal models and the ability to prevent EBV infection, it is difficult to prove that the association between EBV and cHL is causal; however, consideration of the viral, molecular, and epidemiological data pro-

vides support for this idea. (1) The EBV infection in EBV-positive cHL tumors is clonal indicating that all the tumor cells are derived from a single EBV-infected cell. (2) In EBV-associated cases, all HRS cells are infected by the virus. Although EBNA1 facilitates both synchronous replication of the viral episome with cellular DNA and genome partitioning, this process is not 100% efficient [56]. If the virus were not required for maintenance of the transformed phenotype, a gradual loss of viral genomes from the tumor cells would be anticipated. (3) EBV is present in the tumor cells of a significant proportion of cHL cases. Although most adults are infected by EBV, only 1–50 per million B-cells are EBV-infected in healthy individuals [115]. If EBV were simply a passenger virus, i.e., present in a B-cell that was subsequently transformed by other mechanisms, EBV-associated cHL would be a rare occurrence. (4) LMP1 and LMP2A have plausible biological functions in the pathogenesis of cHL, as described above. (5) Crippling mutations of immunoglobulin genes have been described in a quarter of cHL cases, and almost all of these cases are EBV-associated [116]. This is consistent with the idea that EBV rescues HRS cells (or precursors) that have destructive mutations of their immunoglobulin genes from apoptosis. (6) Recent studies show that EBV-positive cHL has significantly fewer cellular mutations, including chromosomal breakpoints and aneuploid autosomes than EBV-negative cHL [117, 118]. Deleterious mutations of the *TNFAIP3* and *NFKBIA* genes, which are both negative regulators of NF-κB signaling, are also much more frequent in HRS cells from EBV-negative cases (see Chap. 3) [119–123]. (7) EBV-associated cHL cases share genetic risk factors for disease development, which are generally distinct from those associated with EBV-negative cHL [101–105, 107, 108, 124, 125]. (8) In some cases, the development of EBV-associated cHL is temporally related to primary EBV infection [92, 93, 95]. (9) Individuals who subsequently develop EBV-associated cHL have abnormal EBV antibody profiles before diagnosis [97].

2.2.4 Epstein-Barr Virus and the Clinicopathological Features of Hodgkin Lymphoma

Although the above data indicate that EBV-positive and EBV-negative cHL have distinct natural histories, the phenotypic expression of both processes appears remarkably similar. Gene expression profiling of HRS cells suggests that EBV has only a small influence on the transcription profile of established HRS cells [126]. However, EBV status does show clear associations with histological subtype. In a meta-analysis of published studies of EBV and cHL, Lee et al. reported that 66% of MCHL cases are EBV-associated, compared to 29% of NSHL cases [86]. Despite this difference, it is clear that "barn door" NSHL cases can be EBV-positive, and so the lack of a complete correlation between histological subtype and EBV status is not simply due to the criteria used in, and subjective nature of, histological subtyping. In industrialized countries, NSHL is much more common than MCHL, and in our experience, the majority (just) of EBV-positive cases in the UK are, in fact, NSHL and not MCHL.

Early studies investigating clinical outcome in relation to EBV status in cHL appeared conflicting, and the meta-analysis performed by Lee et al., which was not able to stratify patients by age, did not find any associations with survival. However, a consistent picture has emerged from population-based studies with age stratification of patients [127–130]. In young adult patients, there appears to be no significant difference in overall survival by EBV status. In contrast, EBV positivity is associated with inferior outcome among patients aged 50 years and over. It is not clear whether this difference is related to the disease process itself or whether it reflects an underlying comorbidity or immune dysregulation that potentially predisposes to EBV-associated cHL. EBV status is not routinely used in therapeutic decisions, but it is possible that this group of patients would benefit from alternative treatments, such as third-party cytotoxic lymphocyte infusions or novel therapies tar-

geting EBV. Biomarker levels may also vary by EBV status; for instance, CCL17 (TARC) levels are lower in patients with EBV-associated cHL, but monitoring of plasma EBV levels (a form of circulating tumor DNA) can be used to assess treatment response and detect relapse in these patients [131, 132]. Further studies investigating these issues are required.

2.3 Epstein-Barr Virus-Negative Hodgkin Lymphoma Cases

Adolescent and young adult cHL cases are the group least likely to be associated with EBV, and yet it is for these cases that there is most epidemiological evidence suggesting viral involvement. Early studies reported consistent associations between young adult HL and correlates of a high standard of living in early childhood [133]. Many of these associations with social class variables have not been detected in recent studies, most probably reflecting societal changes; however, an increased risk of young adult HL in individuals with less than 1 year of preschool attendance has been observed [6, 93]. Collectively, the data suggest that diminished social contact in early childhood is associated with an increased risk of this disease. Interview and questionnaire data generally support the idea that young adult HL patients have experienced fewer common infections in childhood [91, 134]. This has led to speculation that young adult HL is associated with delayed exposure to one or more common childhood infections.

A frequent suggestion is that EBV is involved in all cases of cHL but uses a hit-and-run mechanism in "EBV-negative" cases. This possibility is very difficult to exclude, but the available data indicate that it cannot account for all "EBV-negative" cases. Importantly, not all cases are EBV-infected [98, 135]; in fact, we found that EBV-negative cHL cases in the 15- to 24-year age group were more likely to be EBV-seronegative than age-matched controls [135]. Also, there is no evidence for integration of incomplete EBV genomes in "EBV-negative" cHL biopsies [135, 136].

Alternative hypotheses are that lack of exposure to pathogens in early life shapes the microbiome and immune defenses, leading to an increased risk of developing cHL in young adulthood [137], or that EBV-negative cHL is associated with delayed exposure to another common virus that is directly involved in disease pathogenesis. Candidate viruses that are common and have transforming potential include herpesviruses and polyomaviruses. Any virus with a direct transforming role would be expected to be present in all HRS cells within tumors.

2.3.1 Hodgkin Lymphoma and Herpesviruses Other Than Epstein-Barr Virus

At present, there are nine known human herpesviruses (HHVs), including EBV (officially HHV-4). All are widespread in distribution, except herpes simplex virus 2 (HHV-2) and HHV-8. EBV and Kaposi sarcoma herpesvirus (KSHV, officially HHV-8) belong to the gammaherpesvirus subfamily of herpesviruses; both infect lymphoid cells and are tumor viruses. KSHV causes Kaposi sarcoma and rare forms of lymphoma but is not associated with cHL [138–141]. There is also no evidence of involvement of the alphaherpesviruses, herpes simplex virus 1, and varicella zoster virus [140]. In contrast, genomes of the betaherpesviruses, human cytomegalovirus, HHV-6A, HHV-6B, and HHV-7, have been detected in cHL tumors using sensitive molecular assays. Schmidt et al. detected human cytomegalovirus genomes by PCR in 8/86 HL biopsies [139], although smaller case series failed to identify this virus in tumor samples [140, 142–144]. HHV-7 has been detected in 20–68% of HL biopsies by PCR [139, 140, 144, 145]. However, negative results were obtained using Southern blot analysis, which is much less sensitive than PCR but would be expected to detect a virus present in all HRS cells [146], and there is no evidence that the virus is present in HRS cells [145]. There is,

therefore, no evidence for direct involvement of HHV-7 in cHL pathogenesis.

HHV-6 deserves special mention because this lymphotropic virus has been consistently linked with cHL. HHV-6 is now classified as two distinct viruses, HHV-6A and HHV-6B [147], rather than two variants, but until recently many studies have not distinguished between the two viruses. Serological studies have shown that HHV-6 antibody titers and, in some studies, seroprevalence are higher in HL cases than controls [148–150]. We also found that young adults with non-EBV-associated HL had higher titers of HHV-6 antibodies than age-matched cases with EBV-associated disease (unpublished results). HHV-7 antibody titers were similar in the two groups of cases suggesting a specific association between HHV-6 and cHL.

HHV-6 genomes have been consistently detected in HL biopsies using PCR although detection rates range from 8% to 79% [139, 140, 144, 150–155], and some studies have reported similar detection rates in reactive lymph nodes [144, 152]. Differences in PCR assay sensitivity and the amount of DNA assayed most probably account for the differences in detection rate since viral genome copy numbers within biopsies are often low. Up to 87% of NSHL cases have been reported to be HHV-6-positive [155, 156], but it is clear that these PCR-positive cases include both EBV-associated and EBV-non-associated cases [140, 152, 155, 156]. Both HHV-6A and B have been detected within biopsies with four studies showing a clear bias toward HHV-6B [140, 151, 152, 155], one detecting a higher proportion of HHV-6A-positive tumors [139], and one detecting HHV-6A and B as well as dual infections [156]. The low viral genome copy in many tumors suggests that the virus cannot be present in every HRS cell and raises the suspicion that the virus is in cells in the reactive component of tumors. Very high viral copy numbers must also be interpreted with caution since inherited chromosomally integrated HHV-6 (iciHHV-6) is transmitted in the germline in around 1% of individuals and gives rise to high viral loads since viral genomes are present in every nucleated cell in the body [157–159]. Following exclusion of cases with iciHHV-6, studies using the less sensitive technique of Southern blot analysis have largely been negative suggesting a low viral copy number within tumors [138, 150, 152, 153, 160]. In contrast, in EBV-associated cHL, EBV genomes are almost always detectable using this technique [7, 20, 128]. The critical question is whether HHV-6 infects HRS cells and, if so, is the virus present in every HRS cell.

Early studies using in situ hybridization and IHC reported that the virus was present in cells in the tumor microenvironment, either exclusively [152, 161] or with occasional positive HRS cells [162, 163]. However, two recent studies described HHV-6-positive HRS cells [156, 164], and we detected HHV-6 transcripts in an RNAseq analysis of HRS cells enriched from an EBV-negative cHL biopsy (unpublished data), thus renewing interest in cHL and HHV-6. Lacroix et al. made a polyclonal antiserum to the DR7 open reading frame (ORF) of HHV-6B (designated DR7B) to examine the cellular localization of the virus in PCR-positive cases [164]. They selected this particular ORF because the equivalent HHV-6A ORF has transforming properties and the translated protein binds p53 and inhibits p53-activated transcription [153, 165]. It is likely that the DR7 ORF is expressed as the second exon of DR6, a larger nuclear protein [166, 167]. Using this antiserum, cytoplasmic staining of HRS cells was identified in 28/38 PCR-positive biopsies [164]. In 17 cases, positive staining was exclusive to HRS cells, and in further 17 cases, positive staining of cells in the microenvironment was noted. In 15 of the 38 biopsies, HRS cells were also positively stained using an antibody to the HHV-6 gp116/64/54 glycoprotein. Further analyses suggested that DR7B bound p53, upregulated NF-κB p105 and p65 promoters, significantly increased NF-κB activation, and induced upregulation of Id2. In the second study, Siddon et al. investigated biopsies from 21 NSHL cases, including 18 that were HHV-6-positive by PCR, using multiple approaches [156]. In ten cases, staining of HRS cells was demonstrated using a commercially available monoclonal antibody raised

against virus lysate (Santa Cruz Biotechnology); scattered positive HRS cells were also demonstrated using antibodies to the late viral proteins p41 and p98. Laser capture microdissection coupled with PCR confirmed the presence of HHV-6 DNA in pooled HRS cells from eight of the ten IHC-positive biopsies. This study provides the most convincing evidence to date that HHV-6 can infect HRS cells but does not show that the virus is present in every HRS cell. Furthermore, the IHC staining pattern suggests lytic replication (or abortive replication) rather than latent infection, and so the outcome of viral infection in these cells is not clear.

As mentioned above, some individuals inherit HHV-6 in the germline [157, 159]. The first study to demonstrate chromosomally integrated HHV-6 investigated three patients with high viral loads in peripheral blood, including one with cHL [168, 169]. To determine whether iciHHV-6 is associated with cHL, we examined 936 cHL cases and 563 controls but found no evidence that iciHHV-6 was overrepresented among cases [170].

Overall, the data do not support the idea that HHV-6 has a direct role in disease pathogenesis. However, it is possible that HHV-6 is frequently reactivated in cHL tumors. CD134 is the cellular receptor for HHV-6B [171], and it is possible that CD134-positive T-cells in the cHL microenvironment [74] facilitate replication of HHV-6B. Robust in situ hybridization assays for HHV-6 are required to confidently rule out a direct role in cHL.

To search for novel members of the herpesvirus family, we and others have designed degenerate PCR assays which amplify herpesvirus polymerase and glycoprotein B gene sequences [140, 172]. The primer sequences in degenerate assays are derived from well-conserved peptide motifs in amino acid sequences of proteins; therefore, these assays should have the ability to detect genomes from known and currently unknown viruses [173]. Using herpesvirus polymerase assays, we have not detected novel herpesviruses in cHL biopsies although the assays had sufficient sensitivity to detect EBV in EBV-associated cases, as well as low-level HHV-6 and HHV-7 infection [140] (and unpublished results).

2.3.2 Polyomaviruses and Hodgkin Lymphoma

There are now (at least) 14 human polyomaviruses (HPyVs) [174–178]. JCV and BKV were discovered over 40 years ago, but the others have all been discovered since 2007 with the advent of modern molecular techniques for virus discovery. Seroprevalence studies suggest that the majority of adults are infected by BKPyV, KIPyV, WUPyV, MCPyV, HPyV6 and 7, and TSPyV and a significant minority by JCPyV, HPyV9, and HPyV12 [176, 179–181]. Among this expanding list of HPyVs, only JCPyV, BKPyV, TSPyV (associated with trichodysplasia spinulosa in immunosuppressed persons), and MCPyV show clear disease associations. MCPyV is associated with Merkel cell carcinoma and has been categorized by IARC as a group2A carcinogen (probably carcinogenic to humans) [175, 182, 183]. It is the only HPyV to be unambiguously linked with a specific malignancy; however, other polyomaviruses have oncogenic potential.

Several laboratories have looked for evidence of HPyV genomes in cHL biopsies. Using sensitive quantitative PCR assays, we found no evidence of JCV or BKV genomes in 35 cHL biopsies [184]. Hernandez-Losa et al. detected JCV in 1/20 and BKV in 2/20 cHL samples using a multiplex, nested PCR [144]. Robles et al. reported that MCPyV seroprevalence was slightly higher in HL cases than controls, 84.4% compared to 81.2%, but differences were not statistically significant [185]. Two quantitative PCR studies detected MCPyV genomes in a small proportion (1/30 and 3/41) of cHL tumors [186, 187]; viral copy numbers were low making it extremely unlikely that this virus is playing any role in disease pathogenesis. To date, there have been no reports on the prevalence of the more recently identified viruses in cHL.

Degenerate PCR assays have also been applied to the study of PyVs and HL [184, 187]. Volter et al. examined five cases of HL using a degenerate PCR assay based on the viral VP1 protein but did not detect any evidence of polyomavirus infection [187]. We examined 35 cases of cHL, including 23 EBV-negative cases, using 3 degenerate PyV assays based on the large T antigen, and also obtained negative results [184]. The latter assays were designed before 2006 and therefore before most HPyVs were discovered. Alignment of large T antigen amino acid sequences from the recently identified viruses suggests that our assays would be able to detect KIPyV, WUPyV, TSPyV, and HPyV9 and 10 but not MCPyV, HPyV6, and HPyV7; however, given the tropism of the latter viruses for skin, it is unlikely that they are involved in cHL [176]. Overall, these results provide no evidence for HPyV involvement in the pathogenesis of cHL, but it remains possible that an unknown HPyV has escaped detection.

2.3.3 Measles Virus and Hodgkin Lymphoma

In 2003, Benharroch and colleagues reported an association between measles virus (MV) and cHL [188]. They subsequently reported that MV proteins were detectable by IHC in HRS cells from most HL cases [189]. MV RNA was also detected by RT-PCR and in situ hybridization in a significant minority of the cases examined [189]. Subsequent studies have failed to confirm these associations [190, 191]. Our group found no evidence of MV in 97 cHL cases examined by IHC and 20 cHL cases investigated using RT-PCR [191]. Similarly, Maggio et al. found no evidence of MV genomes or transcripts in HRS cells microdissected from biopsies from 18 German and 17 Israeli HL cases [190]; the latter cases had previously scored positive for MV antigens [190]. Epidemiological studies have also failed to show that MV infection is a risk factor for the development of cHL; on the contrary, the data suggest a mild protective effect of prior MV infection [91, 134, 192].

2.3.4 The Virome, Anelloviruses, and Hodgkin Lymphoma

It is now recognized that the microbiome, which is thought to play an important role in shaping the immune system, includes a large number of viral species (the virome). Anelloviruses account for around 70% of these viruses [193]. The anellovirus family includes a large number of genetically diverse viruses with small, circular, single-stranded DNA genomes, which are classified in the Torque teno virus (TTV), Torque teno midi virus (TTMDV), and Torque teno mini virus (TTMV) genera in humans. They are widely distributed, acquired early in life, and establish persistent infections, but have not yet been associated with any disease; however, it has been suggested that they can modulate both innate and adaptive immune responses [194]. In 2004, Jelcic et al. reported the isolation of 24 novel TTVs from a spleen of an HL patient [195]. This led zur Hausen and de Villiers to suggest that TTVs could play a role in the development of leukemias and lymphomas that are associated with a "protected childhood environment" [196]. In their model, they postulated that TTVs and related anelloviruses increase the risk of chromosomal abnormalities and that anellovirus load is increased in individuals who have experienced fewer infections [196]. Increased TTV loads could also contribute to cHL through modulation of immune defenses. TTVs have also been identified in cHL tumor biopsies by other groups [197, 198], but these studies detected TTVs at a similar frequency in other lymphomas [197] and reactive nodes [198]. In a recent metagenomic analysis, Pan et al. analyzed the virome in blood samples from 19 HL patients, 252 non-Hodgkin lymphoma patients, and 40 healthy controls from China [199]. Eleven novel, but closely related, TTMVs were identified in three of the HL patients but not in the other patients or controls. The significance of these findings is currently unclear. Further investigation of the virome in both cHL patients and individuals with lack of social contact in early childhood is required to understand the potential contribution of anelloviruses, the virome, and the microbiome to the risk of cHL.

2.4 Conclusions

While the evidence suggesting a causal relationship between EBV and a proportion of cHL cases appears strong, current data do not show a consistent and specific association between any virus and EBV-negative cHL. This does not exclude viral involvement since the difficulty of obtaining large numbers of highly enriched HRS cells has precluded the use of certain techniques, such as representational difference analysis, in the analysis of cHL [137]. Next-generation sequencing methods have opened new avenues for virus discovery and have led to the identification of numerous novel viruses in the last few years [139, 140, 156]. These techniques provide our best hope of discovering a new virus in EBV-negative HRS cells. It is possible that cellular mutations substitute for the functions of EBV genes in EBV-negative HRS cells [126]. Deleterious mutations of inhibitors of the NF-κB pathway, including genes encoding A20 and IκBα, appear to be present in the HRS cells of many cases of EBV-negative cHL (see Chap. 3) [90–94], and it is possible that these mutations substitute for LMP1. However, there is no obvious link between these mutations and the epidemiological features of cHL, and involvement of another virus(es) with either a direct or indirect role still appears attractive. Understanding the role of viruses in EBV-negative cHL could potentially open up possibilities for disease prevention as well as novel therapeutic targets and is a goal worth pursuing.

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