Hematologic Malignancies

**Hodgkin Lymphoma**

A Comprehensive Update on Diagnostics and Clinics

von
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1st Edition.

Springer 2010

Verlag C.H. Beck im Internet:
www.beck.de
ISBN 978 3 642 12779 3

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schnell und portofrei erhältlich bei beck-shop.de DIE FACHBUCHHANDLUNG
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<tr>
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**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 this group of 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 chapter, I will review studies on viral involvement in HL with a focus on classical HL (cHL), since nodular lymphocyte predominance HL is considered a separate disease entity. The association with EBV will be discussed with an emphasis on recent data and findings that support a causal role for EBV in this malignancy. Studies investigating involvement of other candidate viruses in this disease will be summarized.

### 2.2 Hodgkin Lymphoma and Epstein–Barr Virus

EBV is a gamma-herpesvirus with a worldwide distribution [10, 11]. 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 [12]. 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 infection are usually asymptomatic [11].

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 [11]. In B-cells transformed by EBV in vitro, six EBV nuclear antigens (EBNA-1, -2, -3a, -3b, -3c, and LP; also called EBNA-1–6) are expressed alongside three latent membrane proteins (LMP1, LMP2A, and LMP2B) [10]. In addition, noncoding viral RNAs are expressed in all latently infected cells [10]. These include two small nonpolyadenylated transcripts, the EBERs, and a large number of viral microRNAs derived from the BARTs (BamHI A rightward transcripts) and the primary EBNA transcript [10, 13–16]. Expression of the full set of latent genes is known as latency type III and is associated with transformation of B-cells [10]. EBV gene expression in EBV-positive lymphomas occurring in the context of immunosuppression frequently follows this pattern; however, more restricted patterns of EBV gene expression are also observed [11]. The EBNA-3 family proteins are immunodominant and the other latent antigens elicit only subdominant or weak cell-mediated immune responses [17, 18]. 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 HL, the Hodgkin and Reed–Sternberg (HRS) cells are infected by EBV and the infection is clonal, i.e., all the tumor cells are derived from a single infected cell [19–22]. The virus is present in all the HRS cells and EBNA-1, LMP1, LMP2A, and 2B as well as the EBER RNAs and BARTs are expressed; the remaining EBNAs are downregulated [20, 22–25]. This pattern of gene expression is referred to as latency type II [11]. 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 (Fig. 2.1). Reagents for both assays are commercially available.

#### 2.2.1 EBV and the Pathogenesis of Hodgkin Lymphoma

The molecular pathogenesis of HL and the origin of HRS cells are described in detail in the following chapter 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 [26, 27]. A pathognomonic feature of these cells is the global suppression of
B-cell signature genes and inappropriate expression of genes usually associated with other hemopoietic lineages [28, 29]. Importantly, HRS cells do not express B-cell receptors. Survival of germinal center B-cells normally requires signaling through both B-cell receptors and CD40; HRS cells must therefore have acquired a non-physiological survival mechanism. Functional studies of EBV, and LMP1 and LMP2A, in particular, support a role for the virus in HRS cell survival.

In 2005, three independent groups published data showing that germinal center B-cells lacking B-cell receptors could survive and be immortalized by EBV [30–32]. In elegant experiments, Mancao and Hammerschmidt [33] later showed that this survival function was dependent on LMP2A expression. A series of in vivo and in vitro studies from the Longnecker laboratory have further defined LMP2A function [34–36], and shown that this viral protein can mimic an activated B-cell receptor and provide a survival signal to B-cell-receptor-negative B-cells [35]. 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 HL-derived cell lines [37]. Constitutive activation of Notch1 by LMP2A, and subsequent inhibition of E2A and downregulation of EBF, two transcription factors that regulate B-cell development, appear to be involved in both survival signaling and transcriptional regulation [34]. Thus, LMP2A is likely to play a key role in both the survival and reprogramming of EBV-positive HRS cells.

Survival of germinal center B-cells requires signaling through surface CD40 as well as B-cell receptors. LMP1 is an integral membrane protein that interacts with several signal transduction pathways to activate NF-κB, Jun N-terminal kinase (JNK), and p38 mitogen-activated protein [38–42]. In this way, LMP1 mimics a constitutively active CD40 molecule, although providing a more potent and sustained signal [10, 11]. Activation of the NF-κB pathway, which is a feature of HRS cells, leads to upregulation of anti-apoptotic genes, including c-FLIP and XIAP, which are likely to contribute to HRS cell survival [43–45].

The EBV genome is normally maintained as an episome in infected cells, i.e., it does not integrate. The EBNA-1 protein is responsible for maintenance of the genome in an episomal form, and also for genome replication and partitioning during mitosis [10, 46]. EBNA-1 can also influence both viral and cellular gene expression and appears to confer a B-cell survival advantage, although the impact of EBNA-1 on oncogenesis in vivo is controversial [10, 47–50]. Interestingly, in the context of HL, overexpression of EBNA-1 in vitro leads to the appearance of multinucleated cells [49]. The precise function of the EBER transcripts is also unclear but expression of these small RNAs appears important for efficient EBV-induced B-cell growth and transformation [10, 51].

The function of the BARTs, which are expressed by HRS cells, remained elusive for many years but recent data show that these complex transcripts contain two clusters of microRNAs [13–16, 23]. Expression of the BART microRNAs has been most studied in relation to nasopharyngeal carcinoma, an EBV-associated malignancy that shares a similar pattern of EBV gene expression to cHL [13–16], and profiling of these transcripts in cHL has not been reported to date. Little information about the targets of these potent gene regulators is currently available but they are likely to have an important role in oncogenesis. In addition to encoding microRNAs, EBV regulates the expression of cellular microRNAs; EBV infection of primary B-cells leads to a conspicuous downregulation of many microRNAs with the notable exception of mIR-155, which is highly expressed by both EBV-positive and -negative HRS cells [52, 53].

### 2.2.2 Risk Factors for EBV-Associated Hodgkin Lymphoma

It is clear that EBV is associated with only a proportion of cHL cases, around one third in industrialized countries [7, 8, 54]. EBV-associated cHL cases are not randomly distributed among all cHL cases, and the demographic features and risk factors for development of EBV-positive and -negative HL show distinctive features [7, 8]. Children (<10 years) and older adults (50+ years) are more likely to be EBV-associated than young adult cases (15–34 years) [8, 9, 54]. Among EBV-associated cases, males predominate with a ratio of approximately 2:1 whereas males and females are more evenly represented among EBV-negative cases [7, 54]. In developing countries, where childhood HL is more common, a higher proportion of cases are EBV-associated [7, 8]. 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 true for older adult cases [54, 55].

EBV infection usually occurs in childhood, and in many parts of the world there is almost universal infection by the age of 5 years. If infection is delayed until adolescence, as is increasingly occurring in industrialized countries, primary EBV infection manifests as infectious mononucleosis in around 25% of individuals [56]. Infectious mononucleosis is associated with an increased risk of cHL, and this increase is focused in EBV-associated cases [57–60]. The increased risk appears short-lived with a median time interval between infectious mononucleosis and HL of approximately 3–4 years [59, 60]. 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. On the basis of the above data, we have proposed an extension of MacMahon’s model of HL that divides cHL into four subgroups on the basis of EBV-association, age at diagnosis, and age at infection by EBV (Fig. 2.2) [2, 61].

Fig. 2.2 The four disease model of classical Hodgkin lymphoma (cHL). This model divides cHL into four subgroups on the basis of EBV-association, 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 more common in developing countries; (2) a disease, most commonly seen in young adults, which occurs following infectious mononucleosis; (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 cHL cases, which accounts for the young adult age-specific incidence peak seen in industrialized countries. The incidence of each of these four disease subgroups will determine the overall shape of the age-specific incidence curve in any particular geographical locale.

Racial and ethnic differences in proportions of EBV-associated cHL suggest that genetic factors also contribute to risk of developing EBV-associated cHL [7, 62]. It is now apparent that there are strong associations between human leukocyte antigen (HLA) class I genes and EBV-associated cHL. HL was, in fact, the first malignant disease to be associated with HLA class I, and early studies showed that HLA-A1 was associated with increased susceptibility [63]. At this time the association between EBV and HL was not known and the increased risk associated with HLA-A1 was modest [63]. Recent genotyping studies investigating markers across the entire HLA region initially revealed that microsatellite markers and single nucleotide polymorphisms (SNPs) in the HLA class I region were strongly associated with EBV-positive cHL [64, 65]. The informative markers are in linkage disequilibrium with HLA-A*01 and HLA-A*02, and it was subsequently demonstrated that HLA-A*01 is associated with an increased and HLA-A*02 with a decreased risk of EBV-associated HL [66]. Risk is independently associated with HLA-A*01 and HLA-A*02, i.e., the increased risk associated with HLA-A*01 is not simply due to lack of HLA-A*02, and is dependent on the copy number of each of these alleles [67]. 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 [67]. Cytotoxic T-cell responses, restricted through HLA class I, are critical for the control of EBV infection, and HLA-A*02 is known to present a wide range of peptides derived from EBV lytic and latent antigens, including those expressed by HRS cells [17, 18]. In contrast, there are no well-characterized HLA-A*01-restricted EBV epitopes [68]. The described 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; therefore, the biological basis of the increased risk associated with HLA-A*01 is not straightforward and requires further investigation. Further work is also necessary to determine whether the critical HLA-A-restricted cell-mediated immune responses are directed towards EBV-infected HRS cells, or whether it is the control of persistent EBV infection, and the host–virus equilibrium, which is all important. Given the failure to expand and accumulate EBV-specific
cytotoxic T-cells in cHL tumors, and the counterintuitive association between increased cytotoxic T-cells and inferior outcome, the latter possibility appears more likely [69–72].

As mentioned above, prior infectious mononucleosis is associated with an increased risk of EBV-positive HL [57–60]. Propensity to develop infectious mononucleosis has been associated with the same genotypic markers (microsatellites and SNPs) that were originally associated with EBV-positive HL, albeit with lesser statistical significance [73]. It therefore appeared possible that the association between infectious mononucleosis and EBV-associated HL could result from shared genetic susceptibility rather than a temporal association. HLA class I 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 HL after adjusting for the effects of HLA-A alleles [67]. 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 HL following infectious mononucleosis in HLA-A*02-positive individuals [67]. These results suggest that infectious mononucleosis is associated with an increased risk of EBV-associated cHL and that this risk is modified by the EBV-specific cytotoxic T-cell response restricted through HLA-A*02.

These data are consistent with the idea that there is a window of time following primary EBV infection when there is an increased risk of EBV-associated HL. Genetic factors, specifically HLA-A genotype, can modify risk and this most probably reflects the strength and breadth of EBV-specific cytotoxic T-cell responses. EBV-associated cHL patients have higher numbers of EBV-infected cells than patients with EBV-negative disease [74], and infectious mononucleosis patients have very high numbers of circulating EBV-infected B-cells, which decrease over time [75]. These findings suggest that the total number of EBV-infected cells may be a critical determinant of risk of EBV-associated cHL. If this is indeed the case, then it would theoretically be possible to decrease the risk of EBV-positive cHL by EBV vaccination or by treatment of infectious mononucleosis. EBV-associated cHL occurring in older adults most probably results from reactivation of viral infection; in this situation it is plausible that an age-related decline in immune function is associated with an increased number of EBV-infected B-cells.

2.2.3 **EBV and Hodgkin Lymphoma: A Causative Association?**

In the absence of prevention of 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 provides support for this idea. (1) In healthy individuals, EBV infects 1–50 per million B-cells [76]. EBV is consistently associated with a significant proportion of cHL cases; therefore, it is unlikely that EBV is simply a passenger virus in an HRS cell that has arisen from an EBV-infected B-cell transformed by other mechanisms. (2) In EBV-associated cases, the viral infection is clonal and all HRS cells are infected. Although EBNA-1 facilitates both synchronous replication of the viral episome with cellular DNA and genome partitioning, this process is not 100% efficient [46]. If the virus is not required for maintenance of the transformed phenotype, one would expect to see a gradual loss of viral genomes from the tumor cells. (3) LMP1 and LMP2A have plausible biological function in the pathogenesis of cHL, as described above. (4) Crippling mutations of immunoglobulin genes have been described in a quarter of cHL cases but almost all of these cases have been EBV-positive [77]. This suggests that EBV is required to rescue HRS cells (or precursors) that have destructive mutations of their immunoglobulin genes. (5) Deleterious mutations of the TNFAIP3 gene, a negative regulator of NF-κB, are much more frequent in HRS cells from EBV-negative compared to EBV-positive cases (see Chap. 3) [78]. Likewise, mutations of the gene encoding the NF-κB inhibitor, Iκ-Bα, have been described only in EBV-negative cases [79–82]. This suggests that HRS cells in EBV-negative cHL have developed alternative strategies to constitutively activate NF-κB. (6) EBV-associated cHL cases share risk factors for disease development, which are distinct from those associated with EBV-negative cHL. (7) Development of EBV-associated cHL is temporally related to primary EBV infection in some cases [59, 60].
2.2.4 EBV and the Clinicopathological Features of Hodgkin Lymphoma

Although differences in the molecular pathogenesis of EBV-associated and nonassociated cHL are emerging, the phenotypic expression of both processes appears remarkably similar. Mixed cellularity HL cases are significantly more likely to be EBV-associated than nodular sclerosis HL cases [7, 8]. In most series, around 60–70% of mixed cellularity HL cases are associated with EBV, compared to ~25% of nodular sclerosis HL cases [7, 8]. Despite these differences, it is clear that “barn door” nodular sclerosis HL 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 histological subtyping of cHL. In industrialized countries, nodular sclerosis is more common than mixed cellularity HL, and in our experience the majority (just) of EBV-positive cases are in fact nodular sclerosis HL and not mixed cellularity HL. Gene expression profiling has been successfully applied to the study of HRS cells [28, 29]. Although no systematic comparison of EBV-positive and -negative cases has been reported thus far, there is no evidence that expression profiles of the two groups of cases cluster differently (Ralf Kuppers, personal communication).

Early studies investigating clinical outcome in relation to EBV status in cHL appeared conflicting but a more consistent picture is now emerging [83–86]. Among young adult cases, aged 15–34 years, there appears to be no significant difference in overall survival by EBV status. In contrast, EBV-positivity is associated with inferior outcome among older adult cases, aged 50 years or over. It is not clear whether this difference is related to the disease process itself or whether it is a reflection of the underlying co-morbidity or immune dysregulation that potentially predisposes to EBV-associated cHL. Further studies investigating this issue and alternative treatment options in EBV-positive older patients are required.

2.3 Non-EBV-Associated Hodgkin Lymphoma Cases

As mentioned above, young adult cHL cases are the group of cases least likely to be associated with EBV and yet it is for these cases that there is most epidemiological evidence pointing to viral involvement. Early studies reported consistent associations between young adult HL and correlates of a high standard of living in early childhood [87]. Recent studies have generally not detected associations with the same social class variables and this probably relates to secular changes in living standards; however, one study observed an increased risk of young adult HL in individuals with ≤1 year of preschool attendance [5, 60]. Together, the data suggest that diminished social contact in early childhood is associated with an increased risk of this disease. From this it is inferred that young adult HL may be associated with delayed exposure to a common childhood infection. Interview and questionnaire data generally support the idea that young adult HL patients have experienced fewer common infections in childhood [57, 88].

It has frequently been suggested that EBV is involved in all cases of HL but uses a hit-and-run mechanism in “EBV-negative” cases. This possibility is very difficult to exclude but the available data indicate that this mechanism cannot account for all cases in which EBV is not detected. Importantly, not all cases are EBV infected; in fact, we found that EBV-negative cHL cases in the 15–24 year age group were more likely to be EBV-seronegative than age-matched controls [89]. In addition, there is no evidence for retention of fragments of integrated EBV genomes in “EBV-negative” HL biopsies [89, 90].

We therefore believe that another viral agent is involved in EBV-negative HL. This agent is likely to be a virus that infects many people early in life; therefore, candidate agents include herpesviruses and polyomaviruses. These are discussed in further detail below. The Anellovirus genus, which includes Torque teno virus (TTV) and related viruses, also fit these criteria. zur Hausen and de Villiers [91] have suggested that TTVs and TTV-like viruses could play a role in the development of leukemias and lymphomas that are associated with a “protected childhood environment.” In their model, it is 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. TTVs have been detected in HL [92–94]; however, further knowledge of these extremely common and genomically diverse viruses is required before their potential involvement in HL can be evaluated.
2.3.1 Hodgkin Lymphoma and Herpesviruses Other than EBV

At present, there are eight known human herpesviruses (HHVs), including EBV (officially HHV-4). With the exception of herpes simplex virus 2 and HHV-8, all are widespread in distribution and most adults are infected. Like EBV, HHV-8 is a gamma-herpesvirus that is associated with human lymphomas, but there is no evidence that this virus is associated with cHL [95–97]. The α-herpesviruses, herpes simplex virus 1 and varicella zoster virus, have also not been detected in HL biopsies [96]. In contrast, genomes of the β-herpesviruses, human cytomegalovirus, HHV-6, and HHV-7 have been detected in HL tumors using sensitive molecular assays. Schmidt et al. [97] detected human cytomegalovirus genomes by PCR in 8/86 HL biopsies, although smaller case series failed to identify this virus in tumor samples [96, 98–100]. HHV-7 has been detected in 20–53% of HL biopsies by PCR [96–98]; however, using Southern blot analysis, which is much less sensitive than PCR but would still be expected to detect a virus present in all HRS cells, negative results have been obtained [101]. There is, therefore, no evidence that HHV-7 is directly involved in HL pathogenesis.

HHV-6 deserves special mention because serological studies have shown that HHV-6 antibody titers and, in some studies, seroprevalence, are higher in HL cases than controls [102–104]. Furthermore, we 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 has been consistently detected in HL biopsies using PCR, with detection rates varying from 12.5 to 79% [96–98, 105–107]; however, studies of reactive lymph nodes have reported similar detection frequencies [98, 107]. There is no evidence from in situ hybridization and immunohistochemical studies that the virus is localized to HRS cells [107–109], and Southern blot studies have largely been negative following exclusion of cases with integrated HHV-6 [95, 104, 107, 110]. Current data do not, therefore, favor a direct role for HHV-6 in disease pathogenesis. It remains possible that HHV-6 is a marker for another virus that is associated with HL. The ability of HHV-6 to integrate into chromosomal DNA also suggests novel mechanisms in which this virus could interact with the host genome and contribute to oncogenesis [110, 111].

In order to search for novel members of the herpesvirus family, we and others have designed degenerate PCR assays that amplify herpesvirus polymerase and glycoprotein B gene sequences [96, 112]. 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 [113]. Using herpesvirus polymerase assays we did not detect novel herpesviruses in HL tumors although the assays had sufficient sensitivity to detect EBV in EBV-associated cases, and to pick up HHV-6 and HHV-7 sequences in a significant minority of cases [96].

2.3.2 Polyomaviruses and Hodgkin Lymphoma

There are currently five known human polyomaviruses, namely JCV, BKV, KIV, WUV, and Merkel cell polyomavirus (MCV or MCPyV) [114–116]. JCV and BKV were discovered almost 40 years ago but the latter viruses have all been discovered since 2007 with the advent of modern molecular techniques for virus discovery. Recent seroprevalence studies suggest that the majority of adults are infected by BKV, KIV, WUV, and MCV and a significant minority (35–39%) are infected by JCV [117–119]. Infection generally occurs in early childhood, with infection by JCV occurring slightly later than infection with the other viruses [117]. MCV is detectable in around 80% of Merkel cell carcinomas and is the only human polyomavirus to be unambiguously associated with a specific malignancy [115, 120]; however, other polyomaviruses clearly have oncogenic potential.

Using sensitive quantitative PCR assays, we found no evidence of JCV or BKV genomes in 35 cHL biopsies [121]. Hernandez-Losa et al. [98] detected JCV in 1/20 and BKV in 2/20 cHL samples using a multiplex, nested PCR. Similarly, Shuda et al. [122] detected MCV in only 1/30 HL samples examined by quantitative PCR. To date, there have been no reports on KIV or WUV prevalence in adult cHL samples. Degenerate PCR assays based on conserved sequences in the T antigen and structural proteins of polyomaviruses
have been applied to the study of HL [121, 123]. Volter et al. [123] 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. We examined 35 cases of cHL, including 23 EBV-negative cases, using three degenerate polyomavirus assays based on the large T antigen and, similarly, obtained negative results [121]. The latter assays were designed before the discovery of KIV, WUV, and MCV; sequence alignment suggests that the assays would be able to detect KIV and WUV but not MCV. Overall, these results provide no evidence for polyomavirus involvement in the pathogenesis of cHL but it remains possible that an unknown polyomavirus has escaped detection using the available assays.

### 2.3.3 Measles Virus and Hodgkin Lymphoma

In 2003, Benharroch et al. reported an association between measles virus (MV) and cHL [124]. They subsequently reported that MV proteins were detectable by immunohistochemistry, using at least two antibodies, in HRS cells from the majority of HL cases [125]. MV RNA was also detected by RT-PCR and in situ hybridization in a significant minority of the cases examined [125]. Subsequent studies have failed to confirm these associations [126, 127]. Our group found no evidence of MV in 97 cHL cases examined by immunohistochemistry and 20 cHL cases investigated using RT-PCR [127]. 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 [126]; the latter cases had previously scored positive for MV antigens [125]. Epidemiological studies have also failed to show that MV infection is a risk factor for development of cHL; on the contrary, the data suggest a mild protective effect of prior MV infection [57, 88].

### 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 HL. This does not exclude viral involvement. HL is a notoriously difficult disease to investigate, and virus discovery studies present particular challenges. 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 HL [113]. Next-generation sequencing methods have opened new avenues for virus discovery and have led to the identification of several novel viruses in the last few years [115, 116, 128]. Digital transcriptome subtraction [115], the technique used in the discovery of MCV, is now being applied to the study of HL. It is likely that, in the not too distant future, complete sequencing of HRS cell DNA will also be performed. 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. Deleterious mutations of inhibitors of the NF-kB pathway, including genes encoding A20 and IκBα, appear to be present in the HRS cells of many cases of EBV-negative HL (see Chap. 5) [78–82], 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 still appears attractive. Identification of a virus in EBV-negative cHL would open up possibilities for disease prevention as well as novel therapeutic targets, and so it is important to resolve whether, or not, such an agent exists. Exciting times are ahead.

**Acknowledgments** To Scamp, my faithful old feline friend who died during the preparation of this manuscript. Thanks to Tina Rich for reading the manuscript. Work in our laboratory is supported by the Leukaemia Lymphoma Research and the Kay Kendall Leukaemia Fund.

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