The Biology and Clinical Utility of EBV Monitoring in Blood

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Abstract Epstein-Barr virus (EBV) DNA in blood can be quantified in peripheral blood mononuclear cells, in circulating cell-free (CCF) DNA specimens, or in whole blood. CCF viral DNA may be actively released or extruded from viable cells, packaged in virions or passively shed from cells during apoptosis or necrosis. In infectious mononucleosis, viral DNA is detected in each of these kinds of specimens, although it is only transiently detected in CCF specimens. In nasopharyngeal carcinoma, CCF EBV DNA is an established tumor marker. In EBV-associated Hodgkin lymphoma and in EBV-associated extranodal NK-/T-cell lymphoma, there is growing evidence for the utility of CCF DNA as a tumor marker.

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Abbreviations

EBV	Epstein-Barr virus
PBMC	Peripheral blood mononuclear cells
CCF	Circulating cell-free
NK	Natural killer
HIV	Human immunodeficiency virus
IM	Infectious mononucleosis
CAEBV	Chronic active Epstein-Barr virus
IL-6	Interleukin-6
TNF-α	Tumor necrosis factor alpha
HAART	Highly active antiretroviral therapy
MGUS	Monoclonal gammopathy of uncertain significance
PTLD	Post-transplantation lymphoproliferative disorder
NPV	Negative predictive value
NPC	Nasopharyngeal carcinoma
PPV	Positive predictive value
PFS	Progression-free survival
DFS	Disease-free survival
OS	Overall survival
CR	Complete response
PET/CT	Positron emission tomography/computed tomography
HSCT	Hematopoietic stem cell transplantation
HLH	Hemophagocytic lymphohistiocytosis
DLBCL	Diffuse large B-cell lymphoma
ENKTL	Extranodal NK-/T-cell lymphoma
PTCL	Peripheral T-cell lymphoma
PCNSL	Primary CNS lymphoma
LDH	Lactate dehydrogenase
HL	Hodgkin lymphoma
CSF	Cerebrospinal fluid
BAL	Bronchoalveolar lavage

1 Introduction

Measurement of viral nucleic acids in blood now plays an important role in the diagnosis and management of a variety of viral diseases including HIV and hepatitis B and C among others. For EBV DNA, there are three approaches to quantitation that have been investigated: measurements in whole blood, measurements in peripheral blood mononuclear cells (PBMC), and measurements in plasma or serum. DNA in plasma or serum falls into the broader category of circulating cell-free (CCF) DNA. CCF DNA has been increasingly appreciated as providing

a window on cellular compartments that are distinct from cells circulating in the blood. It is now clear that there are situations in which measurements of viral sequences in CCF DNA yield very different information than measurements of viral DNA in PBMC.

This chapter reviews the literature with regard to EBV measurements in various blood specimens in health and disease. It also touches on viral DNA in other body fluid specimens. The chapter concludes with some thoughts about the interpretation of quantitative EBV DNA measurements in various settings.

1.1 Replication of EBV DNA

EBV DNA replicates by two distinct mechanisms (Hammerschmidt and Sugden 2013). In latently infected cells, EBV DNA is generally present as a closed circular nuclear plasmid. Replication proceeds in synchrony with cell cycle and requires only a single viral protein, EBNA1. Replicated plasmids segregate to daughter cells with mitosis. Latency viral replication is not inhibited by antiviral agents such as acyclovir, ganciclovir, or their congeners. Lytic viral replication proceeds through double-stranded multigenome length linear concatemers that are cleaved to give rise to genomes that can be packaged in virions. The process requires many viral proteins including the viral DNA polymerase. Lytic replication is inhibited by antiviral agents that inhibit the viral DNA polymerase including acyclovir and ganciclovir, although lytic EBV replication is less sensitive to inhibition than herpes simplex or varicella zoster virus (Coen et al. 2014).

1.2 CCF DNA

CCF DNA is present in healthy individuals but increases with tissue injury such as stroke, myocardial infarction, surgery, or inflammation, and also with tissue growth and development as accompanies normal pregnancy or neoplasia (Diaz and Bardelli 2014). Important differences in the character of DNA associated with circulating cells versus CCF DNA are illustrated by studies of noninvasive prenatal testing. In plasma, the ratio of fetal to maternal DNA is nearly 1000-fold greater than the ratio of fetal to maternal cells in the blood (Bischoff et al. 2005). The CCF fetal DNA predominantly derives from DNA released from the fetus rather than from fetal cells in the maternal circulation. Furthermore, cells detected in the maternal circulation may reflect past pregnancies, whereas CCF DNA is short-lived and reflects the active gestation. Monitoring CCF DNA after 9- to 10-weeks of gestation has proven more sensitive and specific than invasive screening tests (Lo et al. 2014).

Processes of apoptosis and necrosis in tumors also result in cellular debris and release of CCF tumor DNA (Sausen et al. 2014; Diaz and Bardelli 2014). The

amount of circulating DNA derived from tumor is a function of tumor burden, vascularity, location, cell turnover, and the efficiency with which cellular debris is cleared by infiltrating phagocytes. In some instances, more than 90 % of CCF DNA is tumor derived and tumor DNA is present in excess of 100,000 DNA copies per mL of plasma. Although CCF tumor DNA can be detected in early-stage cancers, it is more abundant in late-stage cancers. CCF tumor DNA can be detected in >75 % of patients with a variety of advanced cancers (Bettegowda et al. 2014). In situations where cancer cells or cancer-related cells sharing a particular genetic or epigenetic pattern also circulate in the blood, their detection may indicate tumor, but in some instances reflects the presence of long-lived biologically inactive cells. Like fetal cells from a previous pregnancy, these long-lived but possibly inert cells do not indicate the presence of a growing malignancy. In contrast, the short half-life of CCF DNA including tumor DNA (approximately 2 h) means that the tumor cells from which the CCF tumor DNA derives are turning over. Long-lived cells that are not cycling cannot account for CCF DNA.

2 Healthy EBV Seropositives and Infectious Mononucleosis

2.1 Healthy EBV Seropositives

In healthy EBV-seropositive individuals, viral DNA is present as nuclear double-stranded DNA plasmids in resting memory B lymphocytes (Decker et al. 1996; Thorley-Lawson et al. 2013). There are typically approximately 1–50 EBV-infected cells per 1000,000 B lymphocytes (Khan et al. 1996). While there is variation in the number of infected B lymphocytes among individuals, these numbers appear to be stable over time (Khan et al. 1996; Stevens et al. 2007). EBV DNA levels in mouthwash samples from EBV-seropositive children and adults correlate with levels in PBMCs in some but not all series (Yao et al. 1991; Hug et al. 2010). CCF EBV DNA is detected in only a minority (0–4 %) and, if present, is typically at low levels (Wagner et al. 2001, 2002; Pajand et al. 2011).

2.2 Infectious Mononucleosis

During acute infectious mononucleosis (IM), EBV DNA is readily detected in PBMC and is almost exclusively found within B lymphocyte fractions (Calattini et al. 2010; Fafi-Kremer et al. 2004; Cheng et al. 2007; Balfour and Verghese 2013; Fafi-Kremer et al. 2005; Hadinoto et al. 2008). The EBV DNA in PBMCs has been shown to decrease between day 0 and day 30, but rises again in the majority of patients at day 60 and/or day 90, when most are asymptomatic. EBV

DNA is also often detected in plasma of IM patients, but is very transient and becomes undetectable in most people within a couple of weeks of symptom onset (Wagner et al. 2001; Cheng et al. 2007; Pitetti et al. 2003; Yamamoto et al. 1995; Teramura et al. 2002). In one series, EBV DNA was detected in the plasma of 95 % of IM patients at diagnosis, decreased by day 3 in most patients, and became undetectable in all patients by day 15 (Fafi-Kremer et al. 2005). In another series, IM patients had no EBV DNA detected in plasma by day 7 (Fafi-Kremer et al. 2004). A third study demonstrated that 100 % of IM patients had EBV DNA in plasma during the acute phase of illness (first 14 days), but only 44 % had EBV DNA detected in plasma during the convalescent phase (days 15–40) (Yamamoto et al. 1995). By contrast, all patients had EBV DNA detected in PBMCs during both phases. The highest copy number was seen in plasma specimens collected within 7 days of symptom onset.

The effects of acyclovir or its prodrug valacyclovir have been studied in IM patients in a series of trials. A meta-analysis of 5 randomized controlled trials found a significant reduction in the rate of oropharyngeal EBV shedding at the end of the therapy but no difference in EBV shedding 3 weeks after discontinuation of therapy (Torre and Tambini 1999). In a more recent study, in people with IM treated with valacyclovir, EBV DNA copy number in saliva decreased during therapy and rebounded after treatment, while EBV DNA copy number in whole blood was stable (Vezina et al. 2010). Taken together, these results suggest that in IM patients, EBV DNA in the saliva is largely virion DNA, while EBV DNA in the blood is predominantly latent viral DNA.

In most patients, IM is a self-limited illness. In rare patients, IM is fatal. In one report, patients with fatal IM had 100-fold higher copy number in plasma as compared to those who survived (Yamamoto et al. 1995).

3 Immunocompromised Patients

In immunocompromised patients, increased numbers of latently infected lymphocytes are detected in the circulation (Babcock et al. 1999; Wagner et al. 2002; Fafi-Kremer et al. 2004; Yang et al. 2000; Calattini et al. 2010; Gotoh et al. 2010). The number of EBV genome copies in each infected B lymphocyte varies from individual to individual and in relation to underlying immune function. As in healthy seropositives, in transplant recipients and in HIV patients EBV DNA in the cellular fraction is predominantly harbored by CD19(+) resting B lymphocytes (Calattini et al. 2010; Gotoh et al. 2010; Babcock et al. 1999). In chronic active EBV (CAEBV), viral DNA can also be detected in plasma cells/plasmablasts, monocytes, or in the T cells (Calattini et al. 2010).

In HIV patients, EBV DNA is present in whole blood at levels higher than in healthy, HIV-seronegative patients (Stevens et al. 2002, 2007; Petrara et al. 2012). EBV DNA levels do not correlate with CD4 count or, in some series, HIV viral load. In one study, EBV DNA copy number in PBMCs was higher in patients

with detectable HIV viremia and corresponded to higher levels of pro-inflammatory cytokines such as IL-6 and TNF-α and higher numbers of activated B cells (Petrara et al. 2012). HIV patients with no EBV DNA detected at one time point were unlikely to have EBV DNA detected at time points years later while those who had elevated EBV DNA in PBMCs had detectable levels on follow-up specimens, indicating some stability in levels over time (Stevens et al. 2007). When compared to banked specimens collected during HIV monotherapy, highly active retroviral therapy (HAART) does not seem to decrease the copy number of EBV DNA in whole blood (Stevens et al. 2002). In a study of HIV patients treated with HAART, EBV DNA copy number in PBMCs was noted to stay stable or increase as CD4 counts improved (Righetti et al. 2002). For those who had an increase in EBV DNA copy number in PBMCs with CD4 count recovery, a rise in IgG levels was also observed (Righetti et al. 2002). In a separate study, EBV DNA copy number in B cells was found to be several fold higher in HIV patients with persistent monoclonal gammopathies (MGUS) as compared to HIV patients with transient MGUS and HIV patients without MGUS (Ouedraogo et al. 2013). In hospitalized AIDS patients without lymphoma or with EBV(−) lymphomas, EBV DNA copy number in plasma is typically low if detected, with no clear relationship between EBV DNA copy number and HIV viral load or CD4 count (Fan et al. 2005).

In cancer patients without EBV-associated tumors, CCF EBV DNA is detected more frequently in patients receiving chemotherapy, particularly T-cell-depleting agents or in those with opportunistic infections (Martelius et al. 2010; Ogata et al. 2011). In healthy individuals, CCF EBV DNA is more frequently detected in the elderly perhaps reflecting age-related immune senescence (Stowe et al. 2007).

4 EBV-Associated Tumors

4.1 Nasopharyngeal Carcinoma (NPC)

EBV DNA quantification in PBMCs has no clinical utility as a tumor marker in NPC (Shao et al. 2004). By contrast, CCF EBV DNA is an established tumor marker in undifferentiated NPC (Leung et al. 2014; Lin et al. 2004, 2007; Kalpoe et al. 2006; Shao et al. 2004). More than 90 % of untreated NPC patients have EBV DNA detectable in plasma compared to only a small percentage of healthy controls. As a screening tool for NPC, plasma EBV DNA quantification is highly sensitive and specific, with both high PPV and NPV (O et al. 2007). Plasma EBV DNA copy number has been shown to be positively correlated with tumor stage (Ma et al. 2006; Sun et al. 2014; Wang et al. 2013; Lin et al. 2007; Hou et al. 2011; Ferrari et al. 2012). In studies that have evaluated plasma and serum EBV DNA levels, both appear to be sensitive and specific for NPC and correlate well with each other, although there are discrepancies between plasma and serum levels (Jones et al. 2012). In meta-analyses of plasma and serum EBV DNA assessment

for the diagnosis of NPC and its utility in distinguishing those with disease from healthy individuals, plasma has been shown to perform better than serum (Liu et al. 2011; Han et al. 2012).

4.1.1 Screening

In Southern China, EBV DNA in CCF DNA has been investigated for screening for NPC (Ji et al. 2014). With a cutoff of 0 copies/mL plasma, EBV DNA was 87 % sensitive for detecting NPC within a one year, with a PPV of 30 % and NPV of 99.3 %, although sensitivity was lower for early-stage disease. In Hong Kong in an evaluation of prospective screening, individuals with EBV DNA detected in plasma at enrollment and at two-week follow-up were referred for further evaluation with nasal endoscopy (Chan et al. 2013). NPC was diagnosed in 15 % of these patients.

4.1.2 Prognosis

CCF EBV DNA is detectable in the plasma in NPC patients at the time of diagnosis or relapse, but is rarely detectable in NPC patients during periods of remission (Fan et al. 2004). In several series, median plasma EBV DNA copy number for untreated NPC patients with stage II–IV disease ranges from a few hundred to a few thousand copies/mL (Lin et al. 2007; Leung et al. 2014; Tan et al. 2006). Pre-treatment plasma EBV DNA levels of ≥1500 copies/mL have been associated with inferior progression-free survival (PFS), disease-free survival (DFS), and overall survival (OS) in many studies including prospective cohorts (Wei et al. 2014; Wang et al. 2013; Lin et al. 2004, 2007). The association between high pre-treatment plasma EBV DNA levels and inferior OS has also been demonstrated in NPC cohorts using other cutoffs (Chai et al. 2012). Pre-treatment plasma EBV DNA levels have been shown to be a prognostic marker for treatment response and distant metastasis-free survival (Li et al. 2013; An et al. 2011; Hsu et al. 2012; Wang et al. 2010; Leung et al. 2003). In one study, the probability of distant failure in NPC patients was significantly higher in those with pre-treatment EBV DNA copy number of >4000 copies/mL plasma (Leung et al. 2003). In patients with relapsed NPC, preoperative plasma EBV DNA copy number corresponded to tumor burden, positive surgical margins, and subsequent systemic metastasis (Chan and Wong 2014).

4.1.3 Detecting Residual or Relapsed Disease After Therapy

EBV DNA in plasma after local radiotherapy is associated with the presence of distant metastatic disease (Lin et al. 2004, 2007; Twu et al. 2007; Hou et al. 2011; An et al. 2011; Chan et al. 2002). Persistent plasma EBV DNA one

week after therapy was associated with inferior OS and DFS compared to those with undetectable EBV DNA in their plasma after therapy (Lin et al. 2004). In another study, post-treatment levels of >500 copies/mL plasma were associated with inferior PFS and OS (Chan et al. 2002). Return of plasma EBV DNA positivity after therapy has been associated with subsequent relapse, and patients who develop distant metastatic disease have been shown to have corresponding increases in EBV DNA copy number in plasma on serial evaluation (Ferrari et al. 2012; Kalpoe et al. 2006; Chan et al. 2004b). At the end of therapy, those achieving a complete response (CR) had EBV DNA copies remain below 500 copies/mL, while those in apparent CR who later relapsed had EBV DNA copy number increase from <500 copies/mL at the end of therapy to >500 copies/mL 2–16 months prior to clinically detectable relapse (Chan et al. 2004a). PET/CT has been shown to be valuable in detecting distant metastatic disease in patients with low degree of spread to regional lymph nodes but high EBV DNA copy number in plasma (Tang et al. 2013). In treated NPC patients in remission who were prospectively monitored for relapse with serial plasma EBV DNA measurements, all patients with EBV DNA in plasma on follow-up were found to have disease recurrence on PET, whereas no recurrences were detected in patients with undetectable EBV DNA in the plasma, even if there were symptoms or radiographic findings suggestive of potential relapse (Wang et al. 2011).

4.1.4 EBV DNA Copy Number Kinetics

Intraoperative plasma EBV DNA levels were checked one hour after NPC resection and were found to be undetectable in most patients and significantly lower than preoperative levels in remaining patients (Chan and Wong 2014). One week post-op, all patients had undetectable EBV DNA in plasma. This finding reinforces the conclusion that in NPC patients, viral DNA detect in CCF DNA is not derived from circulating tumor cells nor from virions released from lymphocytes. Since the viral DNA disappears with excision of the tumor, the tumor itself must be the source of the viral DNA.

Molecular investigations have investigated whether the CCF EBV DNA was encapsidated (virion) DNA or was DNA released from cells. Three techniques have been used to make this differentiation. Ultracentrifugation will pellet virions but not free DNA. Virions but not free DNA will be relatively protected from DNase digestion. Finally, DNA released from apoptotic cells is exposed to nucleases that clip DNA not protected by nucleosomes. The result is DNA fragment lengths that are 180–200 bps or multiples thereof forming a characteristic “ladder” on sizing gels. All three approaches to distinguishing viral sequences released from latently infected cells and virion DNA have shown that very little if any of the DNA detected is virion DNA.

The clearance rate of plasma EBV DNA with treatment has been found to be prognostic for response and overall survival (Hsu et al. 2012; Wang et al. 2010). Plasma EBV DNA half-life at a cutoff of >7 or 8 days also predicted treatment

response and OS in patients with metastatic or relapsed disease (Hsu et al. 2012; Wang et al. 2010). On multivariate analysis, pre-treatment plasma EBV DNA levels in combination with assessment of half-life added additional prognostic information for OS (Hsu et al. 2012). Serial assessment of EBV DNA copy number in plasma showed a decline to undetectable levels within three weeks of starting therapy in responding patients (Kalpoe et al. 2006). In another study, most patients had undetectable EBV DNA in plasma after four weeks of therapy and the presence of EBV DNA in plasma at this mid-treatment time point was associated with inferior PFS, OS, and distant failure (Leung et al. 2014). In patients with metastatic NPC, those whose EBV DNA in plasma became undetectable after one cycle of chemotherapy had better survival than the others (An et al. 2011).

4.2 EBV Post-Transplant Lymphoproliferative Disease (EBV-PTLD)

In lung transplant patients, EBV DNA copy number in whole blood was associated with lower rates of graft rejection (Ahya et al. 2007). Similarly, in heart transplant patients, detectable EBV DNA levels in whole blood were associated with higher drug levels of calcineurin inhibitors (Doesch et al. 2008). Thus, some have used EBV DNA copy number in blood as a marker of adequate immunosuppression in transplant recipients.

A rise in EBV DNA in PBMCs can be observed weeks prior to the onset of clinical symptoms or signs suggestive of EBV-PTLD providing the rationale for the monitoring strategies leading to preemptive interventions employed by some transplant centers (Meerbach et al. 2008). However, detection of EBV DNA in PBMCs of transplant patients is not associated with EBV-PTLD in most cases. EBV DNA copy number thresholds that should lead to the initiation of preemptive interventions are not well established. The organ transplanted, the particular immunosuppressive regimen, and host factors may all be important (Tsai et al. 2008; Meerbach et al. 2008; Ono et al. 2008; Wagner et al. 2002). While EBV DNA in the PBMCs of transplant patients is not diagnostic or highly predictive of EBV-PTLD, the absence of EBV DNA in PBMCs has a high NPV (Tsai et al. 2008).

CCF EBV DNA is not routinely detected in transplant recipients. However, it may be detected after intensified pharmacologic immunosuppression for graft rejection, antithymocyte globulin, or T-cell-depleted stem cell transplant (Barkholt et al. 2005; van Esser et al. 2001; Haque et al. 2011). A progressive rise to high levels is usually indicative of EBV-PTLD (Loginov et al. 2006). In comparison with PBMCs, EBV detection in plasma has superior specificity, PPV, and NPV and comparable sensitivity in detecting EBV-PTLD (Tsai et al. 2008; Ruf et al. 2012; van Esser et al. 2001).

When monitored after hematopoietic stem cell transplant (HSCT), cell-free EBV DNA is typically not detected until ~60 days after transplant, occurring after engraftment and with the recovery of lymphocyte counts (Clave et al. 2004; van Esser et al. 2001). In pediatric transplant patients with chronically elevated EBV DNA copies in whole blood (>5000 copies/mL for >60 months), none developed PTLD and the majority did not have EBV DNA detected in plasma (Gotoh et al. 2010). In adult transplant patients, EBV DNA was detected in 24 % of whole blood specimens but only 6 % of plasma specimens (Wada et al. 2007). In another study of transplant patients monitored for PTLD, EBV DNA was commonly detected in the whole blood and was often persistent, but was not associated with EBV-PTLD unless EBV DNA was also detected in the plasma (Tsai et al. 2008). Several studies report that among transplant patients who develop systemic EBV-PTLD, all have EBV DNA detected in plasma at diagnosis (Wada et al. 2007; Ishihara et al. 2011; Tsai et al. 2008; Meerbach et al. 2008). Conversely, EBV DNA is rarely detected in the plasma of transplant patients without EBV-PTLD, with EBV(−) PTLD, or with central nervous system (CNS)-only EBV-PTLD (Tsai et al. 2008). In screening for EBV-PTLD, the NPV associated with a plasma EBV DNA copy number <1000 copies/mL can be as high as 100 % (van Esser et al. 2001; Ruf et al. 2012). At diagnosis, patients often have cell-free EBV DNA copy numbers upward of 10,000 copies/mL (Wagner et al. 2001; Haque et al. 2011; van Esser et al. 2001). CCF EBV DNA can be detected weeks in advance of the development of clinical signs and symptoms of EBV-PTLD (van Esser et al. 2001). Furthermore, patients with a log increase in plasma EBV DNA copy number are at increased risk for developing EBV-PTLD, speaking to the potential value of serial measurements. However, while the copy number is predictive, CCF EBV DNA quantification alone is inadequate for diagnosis.

Whereas EBV DNA levels can often be elevated in whole blood, PBMCs, and B lymphocyte fractions even during PTLD-free episodes, clinically meaningful fluctuations in EBV DNA levels that correspond to PTLD disease activity and treatment response are more reliably observed in plasma (Ruf et al. 2012; Wagner et al. 2001). With treatment of EBV-PTLD, plasma EBV DNA has been shown to decline or become undetectable in responding patients (Tsai et al. 2008; Savoldo et al. 2006). In solid organ transplant patients at high risk for EBV-PTLD or with PTLD who were treated with autologous EBV-specific cytotoxic T cells, EBV DNA copy number in plasma was detectable prior to therapy, transiently increased early in therapy, and then declined in line with clinical response (Savoldo et al. 2006). In HSCT patients treated preemptively for EBV-PTLD with a single dose of rituximab if they had EBV DNA >1000 copies/mL plasma, EBV DNA became undetectable in plasma at a median of eight days in responding patients (van Esser et al. 2002).

It is worth considering the detection of cytomegalovirus DNA versus EBV DNA in CCF in the post-transplant setting. Detection of cytomegalovirus DNA is always indicative of active lytic replication. In the absence of resistance mutations, cytomegalovirus DNA will always clear with inhibitors of lytic viral replication. In contrast, latently infected EBV lymphocytes may increase in number without

lytic replication. Cell turnover alone such as occurs in neoplasia will result in release of viral DNA fragments into CCF blood. Although these DNA fragments may be indicative of EBV-associated pathology, they are typically not responsive to inhibition with antivirals. Furthermore, the viral DNA need not reflect the presence of infectious virions at all and whereas it is entirely appropriate to refer to cytomegalovirus "viral load," referring to the EBV DNA debris from proliferating cells as "viral load" is misleading and should be avoided. The term "EBV copy number" is less likely to be misinterpreted as an indication of infectious virions in the blood.

4.3 EBV(+) Lymphomas and Lymphoproliferative Disorders

CCF EBV DNA has been investigated as a potential tumor marker in patients with EBV(+) lymphoma and other EBV-associated diseases. Patients with untreated EBV(+) lymphomas or EBV-associated hemophagocytic lymphohistiocytosis (EBV-HLH) consistently have EBV DNA detected in plasma, often at high copy number (Martelius et al. 2010; Lei et al. 2001, 2002; Kanakry et al. 2013; Musacchio et al. 2006; Donati et al. 2006; Gallagher et al. 1999; Elazary et al. 2007; Teramura et al. 2002; Morishima et al. 2014; Suwiwat et al. 2007; Beutel et al. 2009). Across EBV(+) lymphomas of B-, T-, and NK-cell lineage, EBV DNA has been demonstrated to be detectable in the plasma prior to therapy in the majority of patients and to be markedly elevated in many (Machado et al. 2010; Au et al. 2004; Kanakry et al. 2013). By contrast, immunocompetent patients with EBV(−) lymphomas rarely have EBV DNA detected in plasma (Au et al. 2004; Machado et al. 2010; Kanakry et al. 2013).

In diffuse large B-cell lymphoma (DLBCL), EBV DNA is detected in the plasma of the majority of patients with EBV(+) tumors but not in patients with EBV(−) tumors or in healthy controls (Morishima et al. 2014). In EBV(+) extranodal NK-/T-cell lymphoma (ENKTL), more patients had EBV DNA detected in plasma than in PBMCs at diagnosis (Suzuki et al. 2011). In patients with EBV(+) peripheral T-cell lymphoma (PTCL) or EBV(+) T-cell proliferative diseases, EBV DNA is commonly detected in plasma, at significantly higher levels than seen in patients with EBV(−) PTCL or lymphoproliferative diseases (Suwiwat et al. 2007). On DNase I digestion, EBV DNA in plasma became undetectable or very low in all patients studied, suggesting that the circulating viral DNA is not encapsidated and is likely tumor derived (Suwiwat et al. 2007). In patients with HIV-associated EBV(+) lymphomas, EBV DNA was detected in plasma or serum in all patients in two series, but was undetectable in the plasma of HIV(+) controls matched for CD4 count and HIV viral load (Fan et al. 2005; Ouedraogo et al. 2013). However, in EBV(+) primary CNS lymphoma (PCNSL), EBV DNA may be detected in the plasma in only a minority of patients and at low copy number (Bossolasco et al. 2002, 2006; Fan et al. 2005), perhaps reflecting a "brain–blood" barrier for tumor DNA.

In many EBV(+) lymphomas, CCF EBV DNA has been shown to fall to undetectable levels for those achieving remission, to remain elevated in those with refractory disease, and to rise prior to clinically detectable relapse (Au et al. 2004; Machado et al. 2010; Lei et al. 2001; Martelius et al. 2010; Jones et al. 2012). By contrast, changes in cellular EBV DNA over time were not associated with response in patients with EBV(+) lymphomas (Jones et al. 2012). In patients with B-cell malignancies and high EBV DNA copy number in plasma, a rapid decrease in copy number has been shown to occur with rituximab treatment (Martelius et al. 2010). As rituximab targets CD20(+) B lymphocytes, it should be noted that a fall in EBV DNA copy number in PBMCs after rituximab may not as accurately reflect tumor response (Yang et al. 2000). In patients with HIV, EBV DNA was detected in plasma in all patients with untreated EBV(+) lymphomas and fell to undetectable in the majority of responding patients, some as quickly as three weeks into therapy (Fan et al. 2005).

In ENKTL, CCF EBV DNA copy number at diagnosis correlated with lactate dehydrogenase (LDH) and disease stage, with high copy number found to be associated with inferior treatment response rates and DFS (Au et al. 2004; Lei et al. 2002; Kwong et al. 2014; Ito et al. 2012). In patients with early-stage ENKTL treated with radiotherapy, high EBV copy number in pre-treatment plasma (> 500/mL) was associated with B symptoms, high LDH values, and inferior OS (Wang et al. 2012a). In another study, plasma EBV DNA copy number of 0, <1000 copies/mL, and ≥1000 copies/mL stratified patients into three prognostic groups for OS, with high copy number patients having the worst outcomes (Suzuki et al. 2011). CCF EBV DNA has also been shown to be a marker of disease status in patients with ENKTL where changes in plasma EBV DNA levels on serial assessment corresponded to degree of treatment response (Suzuki et al. 2011; Lei et al. 2002). Plasma EBV DNA positivity appears to be an early indicator of relapse in ENKTL, as patients in apparent clinical remission but with elevated EBV DNA in plasma have been observed to subsequently relapse (Lei et al. 2002). Furthermore, patients with detectable EBV DNA in plasma after therapy had inferior PFS and OS outcomes, where undetectable EBV DNA in plasma after chemotherapy was the best predictor of good OS (Kwong et al. 2014; Wang et al. 2012b). While plasma specimens have been more frequently studied in ENKTL, whole blood and plasma EBV DNA measurements have been shown to be highly correlated (Ito et al. 2012). However, pre-treatment plasma EBV DNA appears to be a better indicator of clinical stage, B symptoms, performance status, and prognosis than PBMC measurements in these patients (Suzuki et al. 2011).

In patients with classical Hodgkin lymphoma (HL), detection of EBV DNA in plasma is highly specific for EBV(+) disease and appears promising as a prognostic marker and indicator of treatment response. EBV DNA copy number in plasma was higher in HL patients with advanced disease, higher prognostic scores, and B symptoms (Hohaus et al. 2011). In one study, EBV DNA was detectable in the plasma of all untreated patients with EBV(+) HL and was undetectable in all responding EBV(+) HL patients after therapy, as well as patients with EBV(−) HL (Gandhi et al. 2006). By contrast, there was no association between EBV

DNA copy number in PBMCs and EBV(+) HL disease activity (Gandhi et al. 2006). In a study of children with EBV(+) HL, 85 % had EBV DNA detectable in plasma prior to therapy and those with post-treatment plasma specimens and complete response had no EBV DNA detectable after therapy (Sinha et al. 2013). In adults with HL, plasma EBV DNA status was closely concordant with tumor EBV status by tissue-based techniques and pre-treatment plasma EBV DNA positivity was associated with inferior PFS on multivariate analysis (Kanakry et al. 2013). At month six of therapy, patients who were plasma EBV DNA-positive had significantly inferior PFS compared to those who were plasma EBV DNA-negative (Kanakry et al. 2013). Other studies have also shown that declines in plasma EBV DNA copy number are associated with treatment response, while increases in plasma EBV DNA copy number precede disease relapse (Spacek et al. 2011). Plasma EBV DNA copy number has also been associated with higher numbers of tumor-associated macrophages and soluble CD163 levels, both of which may also have prognostic significance in HL (Jones et al. 2013; Hohaus et al. 2011). There is evidence to suggest that very little of the EBV CCF DNA detected in Hodgkin lymphoma is virion DNA (Ryan et al. 2004) and we (Kanakry, Ambinder) have unpublished data to corroborate these findings.

Aggressive NK leukemia is EBV(+), and in one series, all patients had EBV DNA detected in their serum at diagnosis (Zhang et al. 2013). Those who responded to chemotherapy had a decrease in serum EBV DNA copy number post-therapy, with undetectable post-treatment levels only observed in patients with a clinical complete remission (Zhang et al. 2013). Serum EBV DNA levels were noted to rise one to two weeks prior to clinically detectable relapse (Zhang et al. 2013).

In CAEBV patients undergoing HSCT, plasma EBV DNA copy number at diagnosis of CAEBV was significantly higher in those who died after transplant as compared to survivors (Gotoh et al. 2008). Patients who had a disappearance of clinical symptoms of CAEBV after HSCT had an accompanying decrease in EBV DNA in the plasma, whereas patients who had relapsed/refractory disease did not have a decrease in EBV DNA in the plasma (Gotoh et al. 2008). In EBV-HLH patients, EBV DNA was no longer detected in the plasma at four months in patients responding to therapy, although EBV DNA copy number prior to therapy or at two months did not distinguish responders from non-responders (Teramura et al. 2002). Taken together, CCF EBV DNA is a promising potential biomarker of EBV-associated lymphomas and lymphoproliferative disorders which may have diagnostic and prognostic value akin to its clinical utility in NPC, although further studies are still needed.

5 Other Patient Populations and Other Body Fluids

5.1 Critical Illness

CCF EBV DNA is less often detected in the absence of an EBV-associated cancer or lymphoproliferative disorder (Fafi-Kremer et al. 2004; Hakim et al. 2007). In hospitalized non-transplant patients, EBV DNA is rarely detected in plasma and, when detected, is typically observed in patients who are critically ill or immunocompromised by HIV, immunosuppressive drugs, or malignancy (Martelius et al. 2010). In one prospective series, one-third of patients with sepsis had EBV DNA detectable in plasma, whereas in non-septic critically ill patients and healthy controls, EBV DNA was detected in plasma in 5 % and 0.6 % of cases, respectively (Walton et al. 2014). Septic patients who were plasma EBV DNA-positive were more likely than plasma EBV DNA-negative septic patients to have fungal infections (Walton et al. 2014). Detection of EBV DNA in plasma correlated with longer intensive care unit stay, but did not correlate with mortality risk scores.

5.2 Malaria

The relationship between endemic Burkitt lymphoma and malaria remains poorly understood but has generated interest in possible interplay between the parasitic illness and the viral infection. In children with acute *Plasmodium falciparum* infection, EBV DNA is detected more frequently and at higher copy number than in plasma from children without malaria (Donati et al. 2006). Treatment of malaria has been reported to be associated with decreases in EBV DNA copy number in plasma (Chene et al. 2011; Donati et al. 2006). In whole blood, EBV DNA is more frequently detected among those with a history of severe malaria infection compared to those with a history of mild malaria infection and whole blood EBV DNA levels have been shown to be correlated with the number of malaria attacks (Yone et al. 2006). However, whether malaria is associated with more virions in the blood or the release of viral DNA from latently infected cells has not yet been determined.

5.3 Other Body Fluids

It is quite common to periodically detect EBV DNA in the saliva of EBV-seropositive individuals (Ling et al. 2003). Seasonal variation of EBV DNA shedding has been demonstrated, with higher frequency of shedding in spring and fall (Ling et al. 2003). In college students followed longitudinally and diagnosed with primary EBV infection, EBV DNA was undetectable in the saliva prior to

the onset of symptoms but became detectable in the oral cell pellet at diagnosis, remaining positive for a median of 175 days (Balfour and Verghese 2013). In the oral supernatant of these IM patients, EBV DNA copy number was lower and detected more transient (Balfour and Verghese 2013). In patients with HIV, EBV DNA is very commonly detected in the saliva, even among those on effective antiretroviral therapy (Jacobson et al. 2009; Griffin et al. 2008). However, patients with lower HIV RNA viral loads or those on HAART have been shown to have EBV DNA less frequently detected in saliva and, if detected, present at lower copy number (Ling et al. 2003; Griffin et al. 2008). In some studies, EBV DNA is frequently detected in saliva regardless of CD4 count (Jacobson et al. 2009), while other studies have shown HIV patients with higher CD4 counts to be less likely to shed EBV DNA in saliva (Griffin et al. 2008). Patients with endemic BL have been shown to have high EBV DNA copy number in saliva, with a frequency of detection of 100 % in one series (Donati et al. 2006).

In HIV-PCNSL, EBV DNA is detected in cerebral spinal fluid (CSF) in over 75 % of patients, but does not correlate with EBV DNA copy number in plasma (Bossolasco et al. 2002, 2006). In patients with imaging studies showing central nervous system mass lesions, the presence of viral DNA in the CSF is viewed by many as adequate to establish a diagnosis of PCNSL. EBV DNA can also be detected in the CSF of patients with central nervous system involvement by an EBV(+) tumor, such as leptomeningeal involvement by NPC (Ma et al. 2008).

In NPC patients, EBV DNA was detected in the urine of 56 % of untreated patients and patients with EBV DNA in urine had significantly higher plasma EBV DNA copy numbers (Chan et al. 2008). Urine and plasma values were positively correlated and it is presumed that small DNA fragments from plasma are filtered through the glomerulus into the urine.

In lung transplant patients, EBV DNA was detected in 44 % of patients' bronchoalveolar lavage fluid (Bauer et al. 2007). By contrast, EBV DNA was detected in 5 % of BAL fluid specimens from healthy controls. Over 33 % of lung transplant patients had both EBV and CMV detected in BAL fluid, and EBV and CMV DNA copy number in BAL fluid were positively correlated. The clinical implications of EBV DNA in BAL fluid of transplant patients, however, are not clear.

6 Summary

EBV is associated with many diseases but most often not associated with any disease at all. EBV DNA can be measured in PBMC, serum or plasma, or in whole blood. As a tool for basic investigation, measuring EBV DNA in PBMC and in other cellular fractions has provided important insights into the biology of persistence and the role of the resting memory B cell. In the most sensitive assays, viral DNA can be detected in PBMC in almost all subjects who have been infected. Among organ transplant recipients, higher copy number of viral DNA in PBMC may be indicative of the level of immunosuppression achieved and is

perhaps useful in guiding pharmacologic immunosuppression. However, present evidence suggests that most of the viral DNA even in immunocompromised patients is not in lymphoblastoid-like immortalized cells but is in resting memory B cells. In any case, absent or very low viral DNA copy number in PBMC makes a diagnosis of EBV-PTLD unlikely. Although high copy number should raise suspicion for EBV-PTLD and may be useful in guiding adjustment of immunosuppression in the organ transplant setting, high copy number alone is not adequate for diagnosis. Typically, the addition of acyclovir or ganciclovir has little if any impact on viral copy number in PBMC insofar as at least a large fraction of the viral DNA is latent. In patients with EBV-PTLD, the administration of rituximab or other anti-B-cell therapy typically eliminates measurable copy number in PBMC even when the EBV-PTLD continues to progress and thus further measurements in PBMC have little value. In other settings such as patients with HIV infection and lymphoma, in patients with NPC, or in patients with HL, EBV copy number in PBMC does not seem promising with regard to either tumor diagnosis or monitoring. Measurement of viral sequences in CCF DNA is a very different measurement insofar as viral DNA is not detected in most seropositives in the absence of disease. However, the viral sequences detected in CCF DNA may be either virion DNA or may be DNA released from cells. As with detection of viral DNA in PBMC, inhibition of viral lytic replication does not impact on viral DNA sequences released in association with turnover of latently infected cells. It is perhaps worth noting that some virion DNA is present in patients with acute IM and some in patients with CAEBV, although the relative contributions of EBV from latently infected cells versus that from virion DNA have not yet been well defined. In NPC and HL, present evidence suggests that in most instances, the viral CCF DNA derives from tumor cells and is not packaged as virions. Persistence of CCF DNA appears to correlate closely with the presence of residual tumor. In NPC, assays of CCF DNA have become fairly standard in tumor monitoring. This is not yet the case in HL but seems a promising path for further investigation. EBV DNA is assayed in whole blood in many settings. This has the virtue of identifying high copy number in either cells or CCF DNA and of involving minimal processing. And on the other hand, assay of whole blood obscures the differences between compartments. In particular circumstances, assay of body fluids, particularly cerebrospinal fluid in patients with HIV and central nervous system lesions

Table 1 EBV DNA in blood compartments

PBMC
Latent infection in lymphocytes
Lytic infection in lymphocytes including cell-associated virions
Plasma or Serum
Virions
Lytically replicated DNA released from cells
Latently replicated DNA released from lymphocytes by processes of apoptosis, necrosis, and secretion

Table 2 DNA in blood compartments

Cellular
Normal blood cells, endothelial cells, fetal cells (that may circulate and persist long after parturition), circulating tumor cells. The half-life of the cells is a function of the particular cell type but may be measured in days, months, or years.
Circulating cell-free DNA
Derived from cells in association with cell death (apoptosis, necrosis), secretory processes, or lytic viral replication. The source of the DNA includes but is not limited to circulating cells. The half-life of CCF DNA is measured in hours.

on imaging, may be useful to establish a diagnosis of primary central nervous system lymphoma (see Tables 1 and 2).

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