CLINICAL MICROBIOLOGY REVIEWS, Apr. 2010, p. 350–366 Vol. 23, No. 2
0893-8512/10/$12.00 doi:10.1128/CMR.00006-09

Using Epstein-Barr Viral Load Assays To Diagnose, Monitor, and Prevent Posttransplant Lymphoproliferative Disorder

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INTRODUCTION

Epstein-Barr virus (EBV) is a double stranded DNA virus belonging to the family of herpesviruses. It can infect B lymphocytes as well as malignant cells of several lineages, including T lymphocytes, epithelial cells, and smooth muscle cells. EBV is associated with a wide range of malignancies, including posttransplant lymphoproliferative disorder (PTLD), Hodgkin and non-Hodgkin lymphomas, nasopharyngeal carcinoma, gastric carcinoma, and leiomyosarcoma.

Nearly every human is infected before adulthood. Infection early in childhood is usually asymptomatic, while delayed primary infection is typically manifest by the signs and symptoms of infectious mononucleosis. Once infection occurs, the viral genome is maintained for life in a small fraction of B lymphocytes. Periodic reactivation of the virus occurs in the oral mucosa, where shedding of virions in saliva propagates the infection among human hosts.

Systemic reactivation of an infection is normally kept in check by the healthy immune system that fights lytic replication using cytotoxic T lymphocytes, natural killer cells, and antibody-dependent cell cytotoxicity. The virus persists long-term as a latent infection. EBV is capable of driving B cell proliferation *in vitro* to form immortalized cell lines and also *in vivo* when immune surveillance is inadequate (119, 179). In the setting of allogeneic transplantation when iatrogenic immunosuppression is used to prevent graft rejection, an unintended consequence is failure to suppress active EBV infection, which is accompanied by a heightened risk of developing PTLD (7, 61, 154, 167, 185, 198). PTLD is a potentially life-threatening neoplasm exhibiting a spectrum of histopathologies ranging from reactive-appearing, polyclonal lymphoid infiltrates to sheets of undifferentiated cells that are morphologically indistinguishable from malignant lymphoma or plasma cell myeloma. PTLD is nearly always EBV related, meaning that EBV DNA lies within the nuclei of the proliferating lymphocytes. Elevated levels of EBV DNA are present in blood specimens of affected patients, including intracellular EBV within circu-

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lating B lymphocytes and extracellular EBV DNA measurable in plasma (77). EBV load, as measured by quantitative molecular analysis of the viral genome, serves as a biomarker for predicting and monitoring the course of PTLD (77).

While tumorigenesis is multifactorial, the nearly constant presence of the EBV within the lymphoproliferative lesion implies that EBV drives the process. Since similar EBV-driven lymphoproliferations are seen in other immunosuppressive settings, such as chemotherapy-related immunosuppression for autoimmune disease or age-related decline in immunity, it appears that impaired immunity is the other major tumorigenic cofactor beyond EBV infection (119, 126, 187). In particular, diminished T cell immunity allows uncontrolled infection, which can progress rapidly and systemically if not promptly recognized and treated. Lesional tissue contains EBV-infected cells that are almost always monoclonal, as shown by clonal immunoglobulin (*IGH*) gene rearrangement, by clonal EBV genomes, or by monotypic expression of kappa or lambda light chains (76, 96). It is thought that the tumor arises as an outgrowth of a single infected cell, either a donor B cell in a patient who underwent allogeneic stem cell/marrow transplant or a recipient B cell in a patient who underwent solid organ transplant (163). Undoubtedly, secondary genetic events mark the neoplastic transformation (194).

Clinical Presentation of PTLD

PTLD typically occurs in the first year after transplantation, sometimes within weeks of the onset of immunosuppression. The median onset of PTLD is 2 months after marrow transplant or 6 months after solid organ transplant. Onset is delayed occasionally beyond a year and rarely beyond a decade after transplantation.

Symptoms are often quite nonspecific, e.g., fever, malaise, and anorexia (190), and some patients are asymptomatic. PTLD frequently presents as a rapidly enlarging mass in the grafted organ, in lymph nodes, filling the marrow space, or in extranodal sites such as upper airway or intestine.

In young children, primary EBV infection often occurs after iatrogenic immunosuppression commences, either when an infected graft is introduced or later in the posttransplant period. Although the infection is primary, one is cautioned against making a diagnosis of infectious mononucleosis, since that diagnosis implies a self-limited process, whereas an immunosuppressed transplant recipient requires vigilant monitoring of the infection. PTLD can present with symptoms reminiscent of infectious mononucleosis, but PTLD is a much more serious illness.

RISK FACTORS FOR PTLD

Nearly all transplant recipients are infected or eventually become infected by EBV, yet only a fraction will develop PTLD. Risk factors for PTLD are as follows: EBV seronegativity at the time of transplant, active primary EBV infection at the time of transplant, underlying disease leading to transplantation, prior splenectomy, second transplant, patient age (children and older adults), coinfection by cytomegalovirus and other viruses, acute or chronic graft-versus-host disease, immunosuppressive drug regimen and intensity, cytokine polymorphisms, HLA type and extent of HLA mismatch, and the presence of multiple risk factors on this list. In a study by Landgren et al. of 21,686 stem cell transplant patients (104), a low incidence of PTLD (0.2%) was found in patients with no risk factors, while the incidence was 8.1% when there were three or more risk factors for PTLD. Incidence also varies by the organ that was transplanted (Table 1.)

TABLE 1. Incidence of posttransplant lymphoproliferative disorder by organ type

Organ transplanted	Incidence (%)		References
	Overall	In children	
Kidney	0.5–1	1–10	18, 100, 127, 190
Marrow and stem cell	0.5–1	13	104, 116, 183
Liver	1.6–5	4–15	59, 93, 100, 210
Heart or lung	1.9–10	6–20	100, 171
Intestinal		12	100, 152

A major predisposing factor is EBV seronegativity at the time of solid organ transplant, with young children being most likely to have avoided exposure to EBV prior to transplantation (139). Interestingly, advanced age was also a risk factor for PTLD among renal and stem cell transplant recipients (23). Active primary EBV infection is a contraindication to transplantation (11).

A critical risk factor is the drug regimen used to prepare the patient for transplant as well as the ongoing immunosuppressive drugs used to prevent graft rejection (122, 142). For example, anti-thymocyte globulin depletes T cells and thus protects from graft rejection, but its use clearly contributes to subsequent PTLD (104, 105, 172). Fludarabine, azathioprine, and other agents causing profound T cell suppression or mutagenicity are also implicated in PTLD pathogenesis (99, 119, 172). Ironically, agents diminishing both B and T cell immunity are not as problematic (104). Patients having multiple bouts of rejection with corresponding interventions to heighten immunosuppression may be at higher risk of PTLD.

Particular HLA types influence recognition of cells expressing foreign viral proteins, which in turn influences the pathogenesis of EBV-driven lymphoproliferation (66, 179, 208). For example, the HLA-A3 allele is associated with a sevenfold risk of PTLD among patients who were seronegative at the time of lung transplantation (208). It is suggested that HLA type influences the immune system's ability to present certain foreign epitopes for immune destruction. Recently, there is increased acknowledgment of the role of host cytokine polymorphisms (e.g., mutated gamma interferon [IFN-γ], transforming growth factor β [TGF-β], and interleukin-10 [IL-10]) in defense against EBV and other viral pathogens (13, 80, 108, 179). Likewise, EBV genomic polymorphism is emerging as a potential contributor to tumorigenesis (51, 74, 118, 165, 199). Variants in viral LMP1 gene sequence have been linked to pathogenesis and severity of lymphoid neoplasia (74, 165).

HISTOLOGIC FEATURES, IMMUNOPHENOTYPE, AND GENETICS

PTLD is divided into four major histopathologic subtypes with corresponding clinical and biologic features (28), as described in the World Health Organization (WHO) subclassification scheme (187). These include early lesions, polymorphic

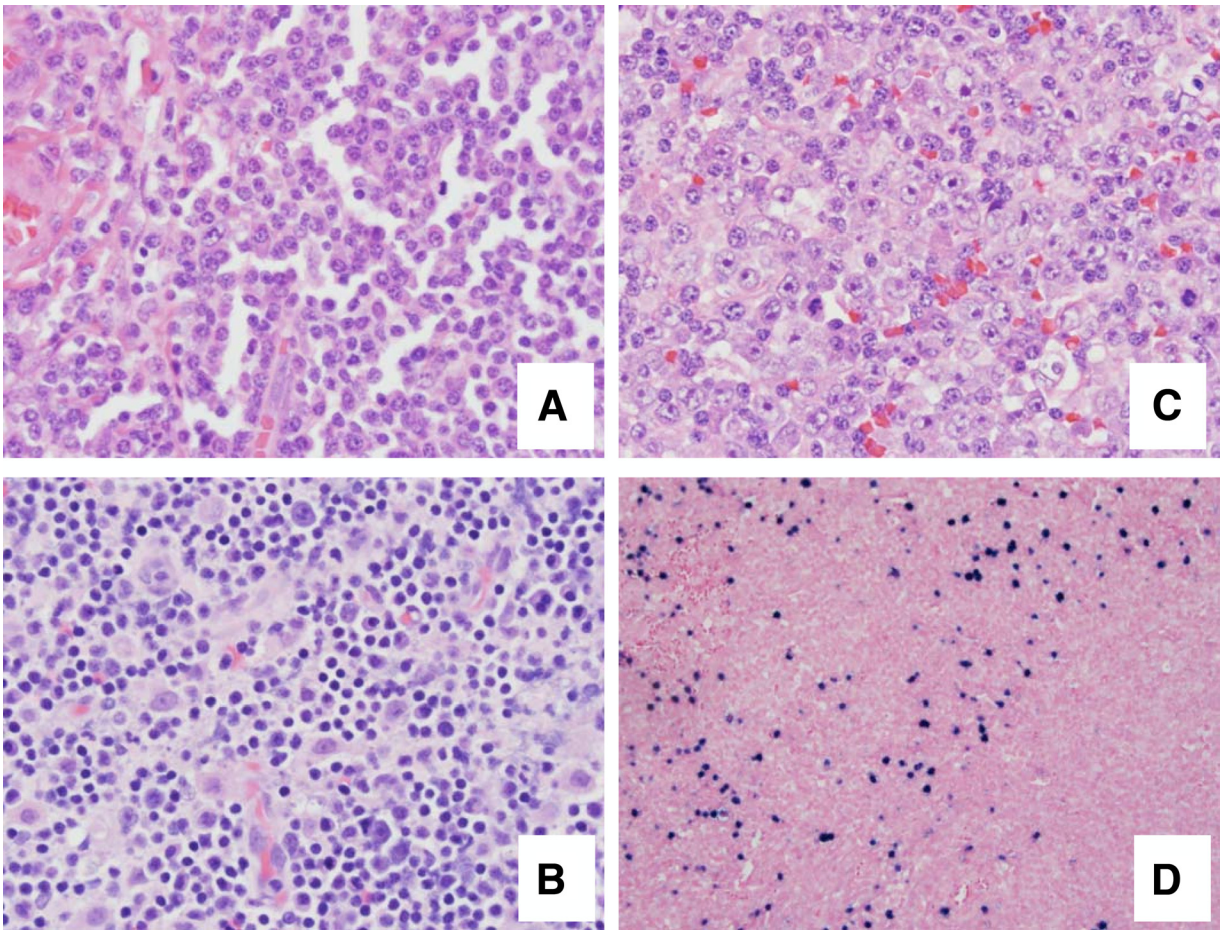

FIG. 1. Histopathologic features reflect clinical subtypes of PTLD. (A) An early lesion (plasmacytic hyperplasia) contains mature polyclonal plasma cells that expand but do not replace tissue architecture. (B) In polymorphic PTLD there is a mixture of small and large lymphocytes. (C) In monomorphic PTLD there are sheets of large lymphoid cells reminiscent of diffuse large B cell lymphoma. (D) EBER *in situ* hybridization reveals a purple EBER signal localized to the nuclei of tumor cells. (Photomicrographs courtesy of Yuri Fedoriw, University of North Carolina at Chapel Hill; reproduced with permission.)

PTLD, monomorphic PTLD, and classical Hodgkin lymphoma-type PTLD (Fig. 1). Although these lesions may bear microscopic resemblance to diseases arising sporadically in otherwise healthy individuals (e.g., infectious mononucleosis, diffuse large B cell lymphoma, myeloma, Hodgkin lymphoma, and age-related B cell lymphoproliferative disorder), their occurrence in the setting of transplantation warrants a diagnosis of PTLD given that the natural history and recommended therapy for PTLD differ from those for lesions having similar histologic features in nonimmunocompromised hosts. In terms of natural history, PTLD almost always progresses quite rapidly to a fatal conclusion unless promptly recognized and treated (141). The ability to reduce or eliminate immunosuppressive drugs is a helpful strategy for restoring natural antiviral and antineoplastic immunity.

Early Lesions

Early lesions usually arise within 6 months after graft placement in a seronegative recipient. Unlike other subtypes of PTLD, early lesions are often polyclonal and exhibit architectural preservation of the affected tissue (188) (Fig. 1A). Reducing immunosuppression allows immune recognition and control of the EBV-driven B cell proliferation. If untreated, a single neoplastic clone may emerge to generate a monoclonal process with eventual histologic progression and effacement of tissue architecture, signifying a transition to one of the more advanced subtypes of PTLD described below (188).

Polymorphic PTLD

Polymorphic PTLD is termed polymorphic because of the mixture of small to large lymphocytes and immunoblasts that efface lesional tissue by microscopic examination (Fig. 1B). These cells include EBV-infected neoplastic B cells as well as reactive CD4 and CD8 T cells. The mitotic rate may be brisk, and clonality assays reveal monoclonal B cells (77). Reducing the level of immunosuppression is often but not always effective in reversing cell growth (14, 171).

Monomorphic PTLD

In monomorphic PTLD there are sheets of atypical lymphocytes mimicking one of the conventional histopathologic types

of B cell malignancy, i.e., diffuse large B cell lymphoma, immunoblastic lymphoma, Burkitt lymphoma, anaplastic large cell lymphoma, or myeloma (Fig. 1C). The immunophenotype usually demonstrates B cell lineage, although rare cases of T cell or NK lineage are reported (186). The vast majority of cases are EBV infected, but occasional cases lack EBV, and these are more likely to occur late (beyond 1 year) after transplantation (186), implying that they are akin to lymphoma of the conventional type. Conventional lymphoma therapy is not necessarily needed, however, since monomorphic PTLD can be managed in some instances by reducing immunosuppression (67, 100).

Classical Hodgkin Lymphoma-Type PTLD and Other Rare Variants

Rare cases of classical Hodgkin lymphoma-type PTLD occur late after transplant (beyond the first year) and invariably contain EBV within the malignant Reed-Sternberg/Hodgkin cells (148). The immunophenotype is that of nodular sclerosis or mixed cellularity Hodgkin lymphoma, and response to therapy is generally favorable (174). Other rare histologies include primary effusion lymphoma with coinfection by EBV and human herpesvirus 8 (HHV8), well-differentiated lymphoma of the marginal zone or mucosa-associated lymphoid tissue (MALT) subtype, and florid follicular hyperplasia, which could be an early histologic manifestation of PTLD (195).

Histochemical Stains in Biopsy Specimens

Biopsy is necessary to confirm a diagnosis of PTLD and to rule out other neoplastic or infectious lesions (11). Histochemical stains are helpful in narrowing the differential diagnosis. *In situ* hybridization targeting EBV-encoded RNA (EBER) is the single best laboratory procedure for localizing EBV to neoplastic cells, thus defining a PTLD as an EBV-related neoplasm (129) (Fig. 1D). Because RNA is labile, negative EBER stain results should always be interpreted in the context of a control assay to demonstrate that RNA is preserved and available for hybridization. Immunohistochemistry is somewhat less reliable, since viral proteins such as LMP1, LMP2, EBNA1, and EBNA2 may be expressed focally or inconsistently in PTLD cases with EBV infection (40, 175).

B Cell Lineage and Viral Reprogramming

CD20 stains are typically positive in PTLD, consistent with B cell lineage and predicting response to anti-CD20 antibody therapy. Interestingly, some cases of PTLD lack CD20 expression, and it is hypothesized that downregulation of CD20 and other B cell markers occurs in response to EBV infection (8, 170). An analogous process is thought to account for virus-mediated reprogramming of the B cell phenotype in classical Hodgkin lymphomas (176). Immunotherapy specifically targeting B cells, such as anti-CD20 antibody therapy, might be ineffective when the B cell phenotype is reprogrammed (170). Likewise, EBV may interfere with programmed cell death in B cells, potentially contributing to viral persistence, accumulation of somatic mutations, and neoplastic transformation (20, 21, 27, 169, 189).

Viral Gene Expression Patterns

To evade immune destruction and promote lifelong persistence of viral infection, EBV gene expression is normally restricted to nonimmunogenic factors such as EBER transcripts and microRNAs (type 0 latency) that can inhibit apoptosis (39, 46, 88, 132). Some mucosal B lymphocytes additionally express EBNA1 (type 1 latency), which maintains the EBV genome and ensures its propagation to dividing cells (209), and LMP1 and LMP2 (type II latency), which mimic B cell receptor stimulation with JAK/STAT signaling to promote cell proliferation, inhibit apoptosis, and generate long-lived memory B cells (22, 200). In PTLD, neoplastic cells often express an even wider range of viral factors, including those listed above plus EBNA-2, -3A, -3B, -3C, and -LP (type III latency), which are associated with NF-κB activation, MYC upregulation, major histocompatibility complex (MHC) class 1 repression, and rapid cell proliferation (9, 40, 54, 120, 132, 175). Each cell division is accompanied by susceptibility to secondary genetic mutation that could further promote clonal neoplastic cell growth (73).

Acquired Mutations

The genetic alterations driving neoplastic cell growth are not well characterized (195). Karyotype and comparative genomic hybridization studies reveal acquired gross chromosomal defects in about half of PTLD cases (42, 149, 156, 195). Mutation or rearrangement of *BCL6* (3q27), *MYC* (8q24), *PAX5* (9p13), *PIM1* (6p21), *RHOH* (4p13), or *NRAS* (1p13) may relate, at least in part, to somatic hypermutation occurring naturally in B cells (27, 42, 136, 149, 194, 195). Methylation-induced inactivation of tumor suppressor genes (*DAPK1* and *MGMT*) has also been described (24, 35, 159). These genetic defects seem to arise in a background of chronic inflammation that, in turn, is caused by graft versus host disease, various infections, acute or chronic rejection, and other inflammatory processes typical of transplant recipients. Gene expression profiling reveals different patterns of human gene transcription in EBV-positive and EBV-negative patients (35). Notable differences include overexpression of interferon-regulated factors and downregulation of B cell receptor signaling molecules in virus-infected tumors. The differences suggest that EBV is not an innocent bystander but rather is associated with specific biochemical alterations promoting evasion of immune recognition, proliferation, and persistence (35). The PTLD profile is distinct from that of B cell lymphomas and is similar to that of memory or activated B cells (194).

EBV LOAD AS A MARKER OF PTLD

EBV viral load measurement has become a routine test for monitoring transplant recipients at high risk of PTLD or under therapy for PTLD. PTLD patients nearly always have high levels of EBV DNA in whole blood and in plasma (206). Indeed, high circulating EBV levels serve as a measure of tumor burden that can be monitored during treatment (67, 68, 193, 196, 197, 206). Even before the onset of signs and symptoms, high EBV levels serve as a harbinger of impending PTLD, thus permitting preemptive intervention to avert illness

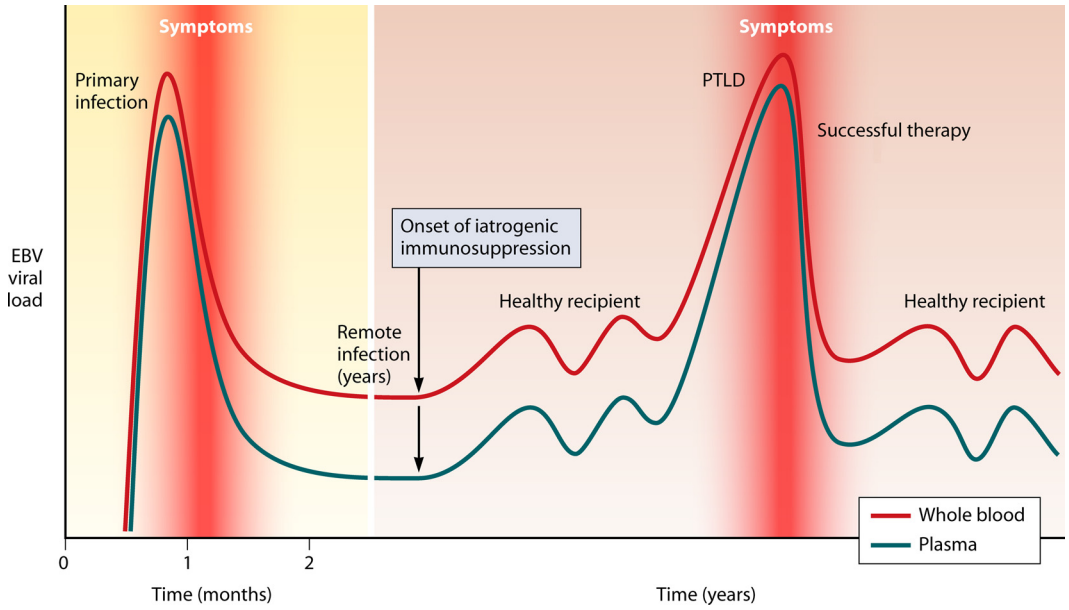

FIG. 2. EBV load, as measured by quantitative PCR in whole blood or plasma, mirrors clinical status in transplant recipients. Nearly every human becomes infected during childhood or adolescence, at which time the viral load climbs until the infection is brought under control by the immune system. Humoral and cell-mediated immunity established during primary infection helps maintain viral quiescence for the remainder of the person's life, with latent EBV DNA retained for life in a small subset of B lymphocytes. Healthy carriers have measurable EBV DNA in whole blood, whereas plasma rarely contains EBV DNA at levels exceeding the lower limit of detection. When a transplant patient is iatrogenically immunosuppressed to prevent graft rejection, active viral infection results in higher baseline viral loads in both whole blood and plasma. Levels often rise before clinical diagnosis of posttransplant lymphoproliferation (PTLD), allowing preemptive intervention in high-risk patients who are routinely monitored for EBV levels. Successful intervention is marked by a return to baseline. A child or a rare adult who was never infected before onset of iatrogenic immunosuppression lacks prior immunity, placing the patient at high risk for active viral infection and progression to neoplasia.

and halt disease progression (2, 15, 36, 69, 71, 99, 109, 131, 137, 143, 172, 185, 197) (Fig. 2).

While high EBV loads are seen in nearly all patients with PTLD, less-marked elevations in viral load may be seen in healthy recipients (e.g., immunosuppressed transplant patients without PTLD or incipient PTLD), emphasizing the importance of using a quantitative rather than a qualitative assay to measure EBV DNA. The literature contains no consistent number or threshold corresponding to the term "high EBV load." Individual testing laboratories have set their own cutoffs distinguishing PTLD or impending PTLD from baseline viral loads, but it is difficult to translate these cutoff values across laboratories because of a lack of international consensus on the best calibrator, specimen type, or unit of reporting. In one study, Wagner et al. found that whole blood from PTLD patients had a median EBV load of 19,200 (range, 6,255 to 171,795) copies/μg DNA, while immunosuppressed controls without serologic evidence of active infection typically had levels of below 5,000 copies/μg DNA. Plasma from PTLD patients had a median EBV load of 3,225 (range, 1,015 to 4,745) copies/100 μl, while controls had fewer than 740 copies/100 μl (206).

In another study, Schubert et al. found six cases of PTLD among 41 pediatric heart transplant recipients (172). All tumors occurred late after transplant (1.3 to 8.6 years), and these patients had blood EBV loads of above 2,000 EBV copies/μg of DNA. However, some patients without PLTD also had levels at least as high. Receiver operator characteristic (ROC) curve analysis showed sensitivity and specificity values of 100% and 86% for PTLD using a cutoff of 2,000 copies/μg, 100% and 90% for 3,000 copies/μg, and 67% and 94% for 5,000 copies/μg. Furthermore, EBV levels were linked to the drug regimen used for immunosuppression, and changes in drug regimen or dose were correlated with changes in EBV load (172).

To predict PTLD with greater accuracy, some investigators suggest calculating a mean viral load over time instead of using a single cutoff value. A higher mean baseline EBV load correlates with heightened risk of PTLD or recurrent PTLD (7, 19). Other investigators propose combining EBV DNA measurement with a complementary test, such as EBV-specific T cell enumeration as measured by a peptide tetramer or enzyme-linked immunosorbent spot, EBV serology, reverse transcription-PCR (RT-PCR) targeting EBV transcripts, cytokine gene polymorphism, ATP release, gammopathy by serum protein electrophoresis, microarray-based expression profiles, and CD20 or CD4/CD8/NK cell counts (6, 10, 17, 25, 53, 107, 112, 117, 123, 151, 158, 166, 173, 177, 192, 207, 211, 213). Some of these tests reflect the immune system's ability to control EBV infection, while other tests reflect general immune function. At this time, evidence linking these tests to patient outcomes is anecdotal. Further research is needed to develop evidence-based strategies and algorithms predicting PTLD with greater accuracy. Caution is advised since some test results are misleading in the aftermath of transfusion or other exogenous influences (e.g., maternal antibodies in infants).

A report by Omar et al. exemplifies current medical practice and also illustrates the observational nature of investigations in this field (137). In this single-institution study, 131 consecutive

stem cell transplant recipients were divided into two groups based on prior risk factors, with high-risk patients undergoing EBV load measurement weekly during the first 3 months, while standard-risk patients underwent testing only when they were suspected to have EBV infection (which turned out to be a common scenario). Forty percent of high-risk patients had at least one positive EBV result, compared to 24% of standard-risk patients, and median values were elevated in the high-risk group. Rituximab was given when the EBV load exceeded 10,000 copies per ml of serum or when symptoms suggested EBV disease, which happened in nine high-risk and three standard-risk patients. Four patients developed biopsy-proven PTLD, three in the high-risk group (6%) and one in the standard-risk group (1%), at a median of 70 days posttransplant. None of the PTLD cases were missed by the routine monitoring strategy. Two of the four affected patients survived, and one of those survivors also received cytotoxic T cell infusion. The authors conclude that routine monitoring of EBV levels might be useful for purposes of preventing PTLD, and they recommend a large prospective study (137).

CLINICAL INDICATIONS FOR TESTING

EBV load testing is commonly used to assist in diagnosis and monitoring of transplant recipients, despite a paucity of clinical trials demonstrating the utility of EBV DNA measurement in such settings. Indications for EBV load testing in an immunosuppressed transplant recipient typically include lymphadenopathy or other mass lesion, organ dysfunction, fever, malaise, or other signs and symptoms suggestive of PTLD. In addition, routine monitoring of EBV load in high-risk patients can help identify PTLD before signs and symptoms appear (15, 89, 109, 204). Early diagnosis is critical, as it may prevent secondary genetic events that render the tumor less treatable and may permit intervention before organ dysfunction becomes irreversible (59, 191). An EBV load above the laboratory's established threshold for PTLD should be conveyed to the clinician immediately so it may trigger a search for putative sites of disease followed by biopsy, when reasonable, to establish a histopathologic diagnosis. Even in the absence of biopsy-proven PTLD, preemptive intervention may be used to resolve laboratory-detected disease (3, 60, 110, 185). Preemptive therapy may include reducing immunosuppression and infusing anti-CD20 antibody or donor T cells.(110) The threshold for preemptive treatment is reported in a number of prior studies, and the success of preemption is gauged by a drop in circulating EBV DNA by at least one log unit within the first week of intervention (2, 3, 15, 99, 109, 130, 137, 143, 185, 197).

Frequency of EBV Load Testing

Patients at high risk for PTLD (e.g., those who are intensely immunosuppressed and who were seronegative at the time of transplant) (89) tend to be monitored frequently (e.g., weekly in the first few months after transplant and then monthly) so that preemptive therapy may be considered (11, 67, 71, 91, 99, 137, 185). Preemptive therapies include reducing immunosuppression and infusing anti-CD20 antibody or donor T cells (60, 110, 124, 183).

Optimally designed trials should measure EBV load once monthly during the first year, with some patients continuing to be frequently monitored beyond the first year if they have a history of high EBV loads, if their drug regimen is particularly immunosuppressive, or in the aftermath of discontinuing antiviral prophylaxis (36, 67, 91). The European Best Practice Guidelines for Renal Transplantation recommend using EBV load to gauge intervention (11). In its practice guidelines, the "Kidney Disease: Improving Global Outcomes" Transplant Work Group recommends that high-risk renal transplant patients be tested for EBV nucleic acid once within the first week after transplant, then at least monthly for 3 to 6 months, and then every 3 months for the rest of the first year (96a). Additional EBV testing is recommended after treatment for acute rejection (96a). Renal transplant recipients lacking risk factors for PTLD forego routine monitoring in some centers (89). Reduced immunosuppression is recommended upon diagnosis of PTLD and in patients likely to develop PTLD based on rising EBV levels (96a).

The Second European Conference on Infections in Leukemia issued guidelines calling for routine EBV load testing of high-risk allogeneic stem cell transplant recipients (185). Screening should begin the day of transplantation and continue at least weekly for the first 3 months and even longer if the patient (i) is being treated for graft-versus-host disease, (ii) has a haploidentical graft, or (iii) has already experienced EBV viremia. More frequent testing is worth considering if the EBV load is rising. The threshold for intervention varies by local experience; a level of 100 g eq/ml of whole blood or plasma was suggested in one study (185). It is difficult to discern how this threshold corresponds to levels measured by another testing laboratory, further reinforcing the need for a universal calibrator.

Using EBV Levels To Predict Graft Dysfunction, Rejection, or Degree of Immunosuppression

Asymptomatic viremia implies a heightened risk for progression to PTLD and also predicts other adverse outcomes, such as graft dysfunction, acute rejection, or late-onset PTLD (5, 19, 92, 113). Among heart transplant recipients studied by Bingler et al., those with chronic EBV viremia were more likely to develop late-onset PTLD, occurring as long as 8.4 years after transplant (19). D'Antiga et al. had similar findings with pediatric liver recipients (36). However, the majority of patients with chronic high EBV levels do not go on to develop PTLD (36, 70).

Jabs et al. showed that EBV viremia occurring immediately after renal transplant was associated with subsequent rejection episodes, and they speculate that T cell responses to viral infection might cross-react with the graft (92). Li et al. likewise linked subclinical cytomegalovirus or EBV viremia with rejection episodes and also with poor long-term renal graft function or graft loss (114). Interestingly, Ahya et al. showed the opposite effect in lung transplant patients, in whom EBV PCR positivity correlated with a lower incidence of graft rejection episodes (5). They speculate that EBV load serves as a functional marker of the degree of immunosuppression and that undetectable EBV implies underimmunosuppression and associated risk of rejection (5). A link between viral load and level of immunosuppression in heart transplant patients was noted as well (43).

Modern drug regimens have evolved to strike a balance between too little and too much immunosuppression, given the side effects of specific agents combined with the risks of opportunistic infection, neoplasia, and organ rejection. Further research is needed to explore the role of EBV load assays in monitoring the success of immunosuppressive strategies.

ASSESSING THERAPEUTIC EFFICACY

Clinical management of PTLD typically involves reducing iatrogenic immunosuppression so that natural immunity against EBV and the neoplastic clone is restored (32, 83, 96a, 185). The power of the immune system is remarkable, given that monoclonal tumors may disappear once immunity is reconstituted (67, 100). However, restoring immunity alone may be insufficient, and the risk of graft rejection calls for careful consideration of all available therapies. Complementary interventions include infusing donor lymphocytes (typical healthy donor mononuclear cells are comprised of about 5% EBV-directed cells) (17), infusing EBV-specific cytotoxic T cells that are grown *ex vivo* by exposing HLA-matched T cells to EBV antigens (37, 64, 79, 85, 168), and infusing anti-CD20 monoclonal antibody (e.g., rituximab) (33, 60, 71, 72, 171, 197). If initial intervention is insufficient, more traditional cancer treatment with radiation and multidrug chemotherapy is used (135, 190).

Traditional dogma is that antiviral agents such as ganciclovir have limited utility since they target replicating but not latent viral infection (140, 145, 185, 201). However, there are two lines of evidence suggesting that ganciclovir might have a role, in concert with other therapeutic strategies. First, PTLD often includes scattered cells expressing markers of lytic viral replication among the more predominant latently infected tumor cell population, and animal studies show that such replicative capability enhances tumor growth (87). Second, intriguing data suggest that ganciclovir is rendered a more potent killer of infected cells when combined with replication inducers (58, 65, 97, 147). This is because the EBV lytic enzyme thymidine kinase phosphorylates ganciclovir to launch the process by which activated ganciclovir carries out its cytotoxic effect (57). Activated ganciclovir is a purine analog that competes with dGTP for incorporation into nucleic acid by DNA polymerase, where it stops strand elongation and triggers cell death. More work is required to identify the best replication-inducing agents to synergize with ganciclovir. Pilot studies suggest that traditional chemotherapy or radiation could be considered (56, 97), as could demethylating agents such as azacytdine (30), histone deacetylase inhibitors such as valproate (58, 157), or cell-differentiating agents such as arginine butyrate (147).

Kinetics of EBV DNA Levels in Serial Specimens

Therapeutic efficacy is measured using EBV load as a marker of tumor burden (67, 68, 71, 193, 196, 197, 206). Funk and colleagues have mined the literature to describe the dynamics of how quickly EBV levels rise in PTLD patients and how quickly levels fall during therapy (63). The kinetics seem to depend on which organ was transplanted, which interventions are used, and the specimen type that is being tested. On average, it is estimated that transplant recipients have an EBV DNA doubling time of about 2.6 days (standard deviation, 1.2 days), which corresponds to the doubling time of lymphocytes undergoing cell division. The EBV DNA half-life can be as short as 4 h on rituximab therapy, 16 h with drugs that shut down lytic replication, or 1 day with adoptive immunotherapy using infused cytotoxic T cells (63). Further study is needed to determine whether relative changes in serial viral loads versus achieving absolute cutoffs in blood or plasma viral load would better predict disease progression or resolution.

TECHNICAL ASPECTS OF EBV LOAD ASSAYS

Blood and Plasma Are Suitable Specimen Types

Specimen types that can be obtained by noninvasive means, such as blood and plasma, are suitable for EBV load measurement. In healthy individuals, EBV DNA is usually not measurable in plasma or serum, whereas it is usually amplifiable from whole blood because the viral genome is retained after primary infection in about 0.0001% of circulating leukocytes (2, 12, 90). In healthy transplant recipients, baseline viral loads tend to be higher than in normal immunocompetent hosts, both in whole blood and in plasma, and viral loads over time reflect the progression and resolution of PTLD (Fig. 2).

EBV DNA normally lies inside circulating B lymphocytes, whereas patients with active EBV infection or EBV-related PTLD also have detectable viral DNA in plasma. This extracellular EBV DNA either is encapsidated, signifying that virions are being produced, or, more frequently, is naked EBV DNA that is partially degraded and probably emanates from dying cells (31). Nucleases may contribute to the degradation.

There is currently no consensus on whether plasma or whole blood is the better specimen type for EBV load measurement in transplant patients; both specimen types appear to be informative (16, 49, 98, 99, 121, 180, 193, 198, 202, 203, 205, 206). Some investigators have applied the assay to isolated blood mononuclear cells, but the added effort of leukocyte isolation seems to render this method less desirable in clinical settings, especially since results with whole blood and blood mononuclear cells are similar (78).

Several investigators recommend whole blood over plasma (16, 180, 202, 203). While whole blood more frequently contains amplifiable EBV DNA than does plasma, it would be inappropriate to conclude that whole blood is more informative than plasma for assessing PTLD status. Indeed, healthy transplant recipients often have low-level EBV DNA in whole blood with or without EBV DNA in the plasma fraction, and only a small proportion of these patients progress to PTLD (16, 43, 78, 99, 193). A practical advantage of whole blood is that it saves labor compared to preparing plasma by centrifugation.

Two studies comparing blood or blood cells to plasma showed greater clinical specificity when plasma was used (193, 206). The first study of pediatric solid organ transplant recipients, by Wagner et al., showed some overlap between patients with or without PTLD when blood cells were tested for EBV levels but perfect sensitivity and specificity when plasma was examined using a cutoff of 1,000 copies/100 μl (206). A study design flaw was that the transplant control group differed from the PTLD group with respect to the spectrum of organs transplanted. The second study, by Tsai et al., evaluated 35 adult solid organ and stem cell transplant patients who had signs and

symptoms of PTLD (193). After clinical evaluation, 13 were diagnosed with EBV-related PTLD and the rest served as a control group. When applied to whole blood, the EBV PCR assay had some false-positive results and was considered inferior for ruling out PTLD compared to the plasma assay, which was 100% specific. Both blood and plasma had similar false-negative rates that might be attributable to location of the tumor (e.g., brain) or timing of specimen collection (193). Several studies showed that plasma is also more informative than blood or blood cells for monitoring therapeutic efficacy (134, 144, 193, 196, 206, 212).

Plasma has some practical advantages over whole blood. When testing is delayed or when residual specimen is needed for use in downstream quality assurance, plasma is more reliably storable than whole blood. Automated instruments to extract nucleic acid may accommodate some specimen types better than others (34), and the volume of specimen required as well as the efficacy of extraction may weigh in the decision of whether to use blood or plasma (125). For either specimen type, it is important to perform analytic and clinical validation studies to define assay performance characteristics and to demonstrate that test results are helpful in diagnosis and clinical management.

In nasopharyngeal carcinoma patients, plasma is clearly the specimen of choice (98), and so laboratories evaluating both transplant and nasopharyngeal carcinoma populations may benefit from choosing plasma as their specimen type. In nasopharyngeal carcinoma, the infected malignant epithelial cells are not seen in circulating blood, and the EBV that is measurable in plasma is naked DNA emanating from dying tumor cells (31, 162). PTLD, on the other hand, is a neoplasm comprised of lymphocytes that tend to circulate and turn over rapidly. However, PTLD pathology is complex: some PTLD cases may be localized, with relatively few circulating tumor cells; some PTLD cases express antiapoptotic factors that could render them resistant to cell death and release of naked DNA; and some PTLD cases produce virions, potentially leading to varying proportions of EBV DNA in the cellular and plasma fractions of whole blood. This biologic variability could complicate attempts to set a threshold for diagnosing EBV-related disease.

Specimen Collection, Handling, and Storage

The preanalytic factors influencing stability of EBV DNA in stored whole blood or plasma have not been studied in a systematic fashion. To obtain accurate viral load results with plasma, it is wise to promptly separate plasma from cells so that any subsequent cell lysis does not contaminate the plasma with intracellular viral genomes (29). Blood collection in a "plasma preparation tube" promotes specimen integrity by creating a gel barrier between cells and plasma during centrifugation, thus permitting plasma storage in the original collection tube until testing (47). (Serum is also an informative specimen type but is not considered ideal for EBV DNA testing, in part because it is prone to contamination by EBV released from cells during clotting [2, 133, 180].)

Prior to extraction of DNA, it is recommended that plasma be spiked with an exogenous control sequence that can later be amplified as a control to demonstrate that extraction and amplification were successful (26). For whole blood, it is reasonable to target an endogenous human gene. The advantage of an endogenous control is that it goes through all of the same steps as EBV DNA with respect to collection and processing, while the disadvantage is that the control assay can detect only gross failures in extraction or amplification since its concentration varies from person to person (largely by white cell count). In reality, any control, whether it is endogenous or spiked, only partially reflects EBV DNA preservation and stability, given that there are four forms of naturally occurring EBV DNA: intranuclear episomes (circular viral genomes), linear viral genome integrated into host chromosomal DNA, linear viral genome encapsidated in virions, and naked extracellular DNA. Each form of EBV DNA is likely to be present in variable proportions across patient specimens and is likely to degrade at different rates *ex vivo*.

Real-Time Measurement of EBV DNA

Real-time PCR is an accurate and sensitive laboratory procedure for measuring viral DNA (178). While EBV transcripts could theoretically be measured by reverse transcription-PCR or nucleic acid sequence-based amplification (NASBA), there is little evidence to support using RNA-based assays in the diagnosis or monitoring of PTLD (82, 182). Therefore, this text focuses on measuring viral load by targeting a segment of the EBV genome in extracted DNA.

A typical real-time PCR assay utilizes a set of primers to amplify an ~80- to 100-bp conserved viral genomic sequence combined with either an intercalating dye, a labeled primer system (41), or a labeled internal probe (such as a TaqMan, peptide nucleic acid, or minor groove binding probe or a pair of fluorescence resonance energy transfer [FRET] hybridization probes) to quantify accumulating products against a series of standards (38, 48, 94, 115, 161, 181) (Fig. 3).

Real-time detection is more rapid and precise than traditional endpoint PCR detection, and it also minimizes the risk of amplicon contamination since the reaction vessel may remain sealed after amplification. Controls are run to confirm that negative and weakly positive specimens perform as expected. Periodic calibration of the test system is done to maintain accuracy and linearity (103).

Automated instruments and commercial reagents are available (Table 2). Laboratory-developed tests were recently reviewed by Espy et al. (48). While comparative effectiveness studies have not been done, proficiency surveys (described below) demonstrate that a wide variety of analytic systems can achieve good performance. Of note, commercial reagents and systems performed similarly to laboratory-developed tests in a sample exchange study involving 30 EBV quantitative PCR assays done in laboratories across North America and at two sites in Europe (150).

Interpretation by a laboratorian combines technical knowledge of relevant test systems with medical knowledge of relevant clinical conditions. Interpretation typically involves examining exogenous and endogenous control assays and any standards that were included in the run to ensure that the assays performed as expected and then examining the analytic findings for the patient to generate a reportable result. Example report wording is displayed in Fig. 4. Clinical interpretation requires knowing the clinical setting in which the test was performed and evaluating the test result in the context of pertinent

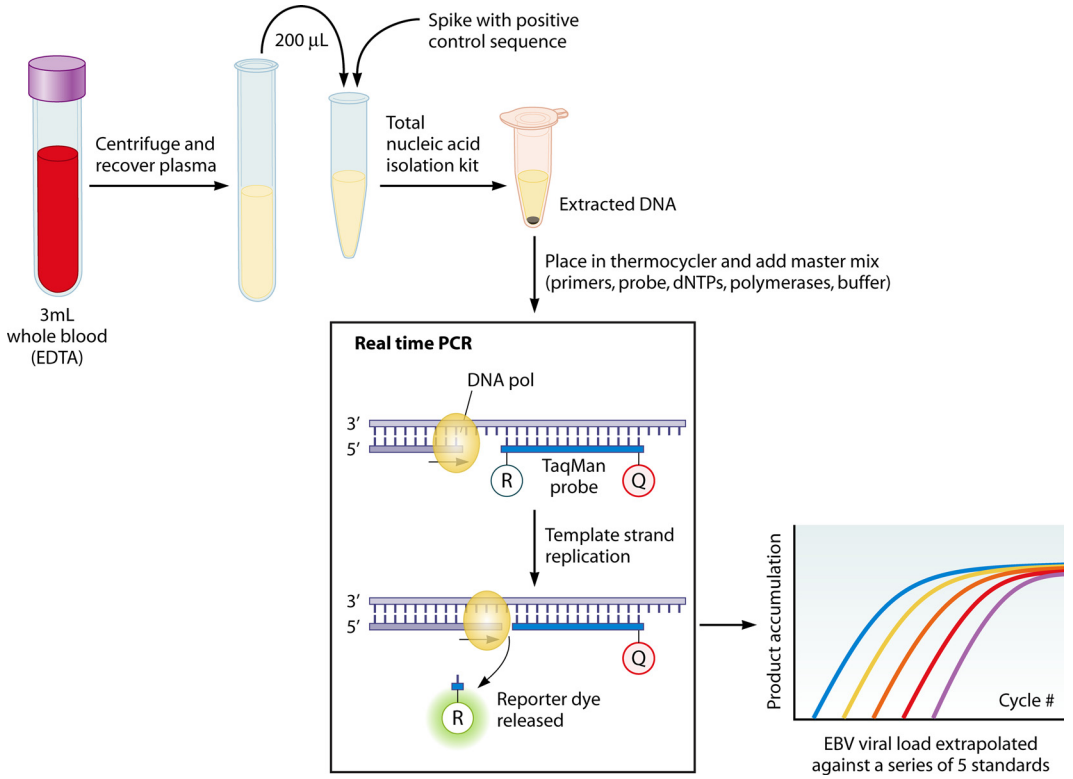

FIG. 3. EBV load is measured by real-time PCR. Extracted DNA is placed in a thermocycler with the reagents for real-time PCR to replicate a segment of the EBV genome in a cyclical fashion using DNA polymerase (DNA pol). To detect accumulating PCR products, the assay depicted here uses a TaqMan probe labeled with reporter (R) and quencher (Q) dyes. As DNA pol replicates the template strand, hydrolysis of the probe releases the reporter dye, which, when separated from the quencher, results in a measurable fluorochrome signal. As each PCR cycle ensues, product accumulation is measured over time in the patient sample and in each of a series of standards, allowing extrapolation of the EBV load in the patient sample. Spiking a nonhuman, nonpathogen DNA into the specimen before extraction and then amplifying the spiked DNA ensures adequacy of extraction and lack of PCR inhibition in each patient sample.

clinicopathologic findings. Evaluating serial test results requires knowing the coefficient of variation of the assay so that rising or falling levels can be interpreted as being significant versus lying within the technical variability of the assay.

QUALITY ASSURANCE

Prior to implementing an EBV load assay, validation studies are needed to demonstrate assay sensitivity, specificity, precision, and linearity (75, 81). In addition to determining assay performance characteristics, the laboratory must ensure that EBV load assays perform well in clinical settings (95). "Analytic validity" relates to reliability for measuring EBV DNA, and "clinical validity" relates to reliability for diagnosing and managing PTLD. Guidelines for validating molecular assays were recently published by the College of American Pathologists (95).

TABLE 2. Commercial reagents and systems for real-time measurement of EBV load

Manufacturer	Country	Gene target	Reference(s)
Roche	USA	LMP2	55, 75, 86, 102, 160
Qiagen (Artus)	Germany	BKRF1	4, 106, 153
Argene	France	BXLF1	50
Amplimedical (Nanogen)	Italy	BKRF1	146
Nanogen (Epoch, Fisher)	USA	BNRF1	
Attostar	USA	EBNA1	
TIB Molbiol	Germany	EBNA1	
EraGen	USA	LMP2	
Sacace	Italy	LMP1	
PrimeraDx	USA	Not specified	
Shanghai ZJ Bio-Tech	China	Not specified	

Optimal assay design is an important first step in developing an analytic procedure. To overcome the impact of EBV strain variants, conserved portions of the genome should be identified. Prior studies in which several alternative EBV load assays were compared revealed major variations in analytic and clinical sensitivity and specificity when applied to transplant patient specimens (161, 193). Targeting two independent segments of the viral genome is somewhat more informative than targeting a single viral gene (161, 164, 193).

Validation studies should establish a list of indications for testing and define cutoffs distinguishing normal from abnormal results in relevant clinical settings. This is typically achieved by comparing viral loads in patients with PTLD to those in healthy transplant recipients and in recipients with other diseases. Results in the weeks or months leading up to a diagnosis of PTLD can help define a threshold beyond which PTLD is a likely diagnosis, implying that a search for the site of disease should ensue.

There is often overlap between EBV levels in healthy recipients and in those who are destined to progress to PTLD, so the threshold for initiating preemptive therapy depends on

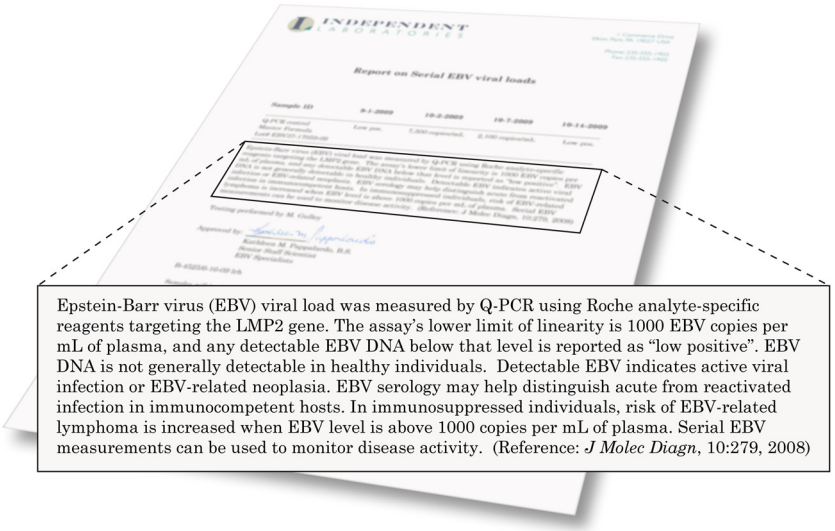

FIG. 4. Generic laboratory report content that might accompany an EBV load result following testing and analytic interpretation by a laboratory scientist.

clinicopathologic factors beyond EBV load. These factors include the many risk factors for PTLD (see Risk Factors for PTLD above) and other variables such as concurrent disease and prior response to therapy. While the laboratory should provide guidance in the form of data supporting thresholds for clinical intervention, the ultimate decision of when and how to intervene is the responsibility of the attending clinician. A clinical consultant in the laboratory must be prepared to interpret test results and discuss the risks and benefits of testing as well as use of the test in diagnosis and patient management (95).

When an FDA-approved assay for EBV load measurement becomes available, the process described above becomes one of "verification" rather than validation, assuming that the testing laboratory does not modify the manufacturer's procedure. "Verification" involves demonstrating that the assay works well in your own hands by replicating analytic performance characteristics and by vetting that the medical claims made by the manufacturer are reasonable (95).

Proficiency Testing and Sources of Interlaboratory Variability

Proficiency testing helps ensure that performance of an assay is maintained over time, and it also permits access to compiled data on methods and results used by other testing laboratories. There are two major purveyors of surveys for EBV load, and their specimens and reports are available by subscribing to the surveys (www.cap.org and www.qcmd.org [accessed on 28 August 2009]). Frozen or lyophilized plasma was chosen over whole blood for distribution in these proficiency surveys. Proficiency testing is one of many strategies to promote high-quality laboratory services. Ultimate responsibility lies with the laboratory director who oversees validation work and routine testing to ensure that assays perform adequately for their intended use.

Published surveys reveal a wide range of analytic test methods (81, 150). A survey of eight laboratories in the United States found seven different EBV genomic targets and five different instrument platforms, suggesting that most laboratories are using unique test systems (81). Nevertheless, sample exchange work demonstrates good performance across laboratories with respect to ranking results as high to low or undetectable EBV DNA (81, 150).

Research demonstrates variable lower limits of detection across test systems (38, 75, 81, 150, 161). Exquisite analytic sensitivity may not be as critical for PTLD as it is for nasopharyngeal carcinoma diagnosis and management (52, 75). After all, many healthy transplant recipients have low-level EBV viremia. Emerging evidence linking EBV loads to other aspects of transplant patient management, such as predicting graft dysfunction, rejection, or degree of immunosuppression, may affect the minimal required performance characteristics of an EBV load assay.

Surveys show that test results are quite reproducible within a given laboratory; however, absolute values are not comparable from laboratory to laboratory, since there is no agreed-upon calibrator (150). An effort is under way to establish consensus on assay calibration through the International Working Group for the Standardization of Genome Amplification Techniques (62). Commercial reagents that could be helpful in standardizing quantitative EBV measurement include products from Advanced Biotechnologies Inc., Acrometrix, and Zeptometrix and ATCC's Namalwa Burkitt lymphoma cell line containing two integrated EBV genomes per cell. Other cell lines, such as Raji, tend to have variable EBV copy numbers per cell because episomal viral genomes may be gained or lost over time in cells grown in culture media.

Another source of variation across testing laboratories is the units of measurement for reporting EBV load results. For plasma specimens, loads are typically reported in copies per ml, whereas whole blood results may be reported in copies per μg of DNA, copies per 100,000 leukocytes, or copies per ml (81).

TABLE 3. Common laboratory tests to diagnose and manage EBV-related PTLD

Procedure	Specimen types[a]	Analyte	Indications for testing
Real-time PCR or similar EBV load assay	Blood, plasma	DNA	Predict current or impending PTLD or assess efficacy of therapy
	Biopsy, aspirate	DNA	Detect and semiquantify EBV
EBER *in situ* hybridization	Biopsy, aspirate	RNA	Detect and localize latent EBV
Immunohistochemistry			
LMP1, EBNA1	Biopsy, aspirate	Protein	Detect and localize latent EBV
CD20	Biopsy, aspirate	Protein	Classify PTLD and predict response to anti-CD20 immunotherapy

[a] Biopsy or cell aspirate is typically paraffin embedded and sectioned onto glass slides prior to testing.

QUANTIFYING EBV DNA IN BIOPSY SPECIMENS

Biopsy tissue and cytologic specimens can be tested for EBV DNA levels using protocols similar to those used with whole blood (161). Fresh, frozen, or paraffin-embedded tissue is a suitable specimen type. Because cellularity varies and because inhibitors of amplification are common in bloody or fixed tissue, it is wise to normalize EBV load to the total number of cells evaluated using a parallel real-time PCR assay for an endogenous human gene (161).

Normalized EBV loads are very high in tissues representing EBER-positive PTLD, whereas a low to undetectable EBV load characterizes EBER-negative tissues (161, 164). Results should be interpreted in the context of histopathologic findings. While negative PCR results imply the absence of EBV-related disease, questionable or high viral loads should be followed up with a histochemical assay to localize the virus, such as EBER *in situ* hybridization (Table 3). Real-time PCR is an advantageous screening test because (i) it is a low-cost test compared to EBER *in situ* hybridization, (ii) DNA is more stable than RNA, and (iii) detecting the viral genome renders the test independent of which viral genes might be expressed. Disadvantages of real-time PCR are the inability to localize EBV to particular cell types within the lesion and the potential for mutation or partial deletion of the viral genome to interfere with DNA amplification.

EBV-NEGATIVE PTLD

EBV load assays are not expected to help detect or prevent EBV-negative PTLD. Fortunately, EBV-negative PTLD is rather uncommon, although some studies have reported rates of up to 23%, especially in cases occurring late after transplantation (128). These late-occurring EBV-negative cases may represent more traditional forms of non-Hodgkin lymphoma. While most PTLD cases are B cell lineage, the rare cases that are T or NK cell lineage are more likely to be EBV negative, and there are conflicting data on whether they are more aggressive (44, 45, 155, 186). Interestingly, some of the EBV-negative cases respond to cutting back immunosuppressive drugs, implying that the test for EBV was false negative or that the immune system can control certain lymphoproliferations even when EBV is not the driving force (67). To improve detection of EBV-related cases, it is probably worth measuring blood or plasma EBV load in addition to performing EBER *in situ* hybridization for any patient with biopsy-proven PTLD.

ON THE HORIZON

EBV load assays have rapidly been incorporated into routine medical practice because of their value in early diagnosis of PTLD. Furthermore, EBV DNA levels reflect tumor burden, so serial EBV load measurement is used to monitor the efficacy of therapy. Further studies are needed to refine the indications for testing and to recommended frequency of routine monitoring in high- and low-risk groups of immunosuppressed hosts.

The increasing availability of commercial systems facilitates implementation of viral load assays. Efforts are under way to make available stable reference standards so that EBV load assays can be calibrated and made comparable across testing laboratories. Further work is needed to optimize specimen processing and storage procedures. Panels of assays and array-based approaches are on the horizon as a way to possibly improve clinical sensitivity and specificity by testing multiple viral and human factors.

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