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Review

Laboratory Assays for Epstein-Barr Virus-Related Disease

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Epstein-Barr virus (EBV) infects various cell types in a wide spectrum of benign and malignant diseases. Laboratory tests for EBV have improved and are increasingly used in diagnosis, prognosis, prediction, and prevention of diseases ranging from infectious mononucleosis to selected subtypes of lymphoma, sarcoma, and carcinoma. Indeed, the presence of EBV is among the most effective tumor markers supporting clinical management of cancer patients. In biopsies, localization of *EBER* transcripts by *in situ* hybridization remains the gold standard for identifying latent infection. Other RNA- and protein-based assays detect lytic viral replication and can distinguish carcinoma-derived from lymphocyte-derived EBV in saliva or nasopharyngeal brushings. Analysis of blood using EBV viral load and serology reflects disease status and risk of progression. This review summarizes prior research in the context of basic virologic principles to provide a rational strategy for applying and interpreting EBV tests in various clinical settings. Such assays have been incorporated into standard clinical practice in selected settings such as diagnosis of primary infection and management of patients with immune dysfunction or nasopharyngeal carcinoma. As novel therapies are developed that target virus-infected cells or overcome the adverse effects of infection, laboratory testing becomes even more critical for determining when intervention is appropriate and the extent to which it has succeeded. ***(J Mol Diagn 2008, 10:279–292; DOI: 10.2353/jmoldx.2008.080023)***

Epstein-Barr virus (EBV) causes infectious mononucleosis and is also associated with a wide variety of malignancies including Hodgkin and non-Hodgkin lymphomas, post-transplant lymphoproliferative disorder (PTLD), nasopharyngeal carcinoma (NPC), and gastric carcinoma. The prevalence of EBV-related cancers, estimated to affect up to 1% of humans worldwide, warrants increased focus on laboratory assays to detect and characterize the infection. Within a given neoplasm, consistent presence of EBV implies that the virus might contribute to pathogenesis or maintenance of the clonal process. Furthermore, the physical location of viral DNA within every malignant cell of a given tumor implies that the virus is a biomarker that can be used to evaluate the extent of tumor spread and to monitor disease burden in response to therapy. Even before disease is clinically evident, high-risk individuals may benefit from screening tests that predict impending progression so that preemptive measures may be taken. Finally, improvements in EBV-directed therapy highlight the importance of laboratory detection and the potential for targeting viral gene products or their downstream pathways driving cell proliferation, inhibiting apoptosis, or evading immune response. While much research remains for understanding the full promise of EBV testing in patient care, it is clear that EBV is a broadly useful tumor marker.

Lifelong Infection in Healthy Carriers

EBV infects nearly all humans by the time they reach adulthood, after which the viral genome is retained for life in a small fraction of B lymphocytes. Healthy carriers have approximately 1 to 50 infected cells per million leukocytes,[1] consistent with an average EBV viral load in whole blood of about 7 copies (range, 1 to 30 copies) of EBV DNA per million leukocytes.[2] Any biopsy tissue may contain B lymphocytes and therefore may harbor amplifiable EBV DNA. Cell-free body fluids such as serum or plasma contain negligible amounts of EBV DNA, suggesting that EBV is detectable only in association with reactivated infection or EBV-related disease.[2,3] To maximize the utility of EBV as a marker for disease, it is important to evaluate EBV in a quantitative rather than a qualitative fashion and to localize EBV to particular cell

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types when lesional tissue is available for histopathological analysis.

Oral mucosa is a major site of replication of EBV and shedding of infectious virions.[4] Remotely infected healthy carriers have salivary EBV DNA levels varying from undetectable to over 1000 copies/ml, with periodic salivary shedding accounting for nearly universal infection of humans before adulthood.[4] Stress and immunodeficiency are postulated to trigger EBV reactivation and increased oral shedding.

Cell Types Infected by EBV

EBV is capable of infecting B lymphocytes, squamous epithelial cells, glandular epithelial cells, myoepithelial cells, smooth muscle cells, T cells, NK cells, plasma cells, and follicular dendritic cells. This wide spectrum of susceptible cell types was determined because of pathological lesions in which EBV is localized to these cells, whereas healthy carriers seem to harbor EBV almost exclusively in B lymphocytes. The importance of B cells in the life cycle of EBV is emphasized by the inability of infection to take hold in children with Bruton's agammaglobulinemia, a rare genetic disorder in which B cells are absent.[5] EBV can productively infect epithelial cells as evidenced by a tongue lesion called oral hairy leukoplakia in patients with human immunodeficiency virus and also by rare infection of healthy epithelial cells.[6] It remains unclear whether the virions that are intermittently shed in saliva of healthy carriers originate from mucosal B lymphocytes, plasma cells, or squamous epithelial cells.

Its presence in genital tract secretions of both genders, along with anecdotal reports of genital ulcers in infectious mononucleosis patients, implies that EBV could be sexually transmitted.[7,8] Breast milk of nursing mothers may contain EBV, but this appears to be an uncommon route of vertical transmission.[9]

Clonality Assays Based on EBV Genomic Structure

A single virion successfully infects any given cell, as evidenced by the unique structure of the viral terminal repeat sequences in individual cell clones. The novelty of each virion relies on the number of tandem repeat sequences (up to 20) found at the ends of the 172,000-bp linear EBV genome. When a cell is infected, the double-stranded viral DNA circularizes to form an episome that may then replicate to produce 1 to 50 clonal copies of the EBV genome, and these clonal episomes are passed along to cellular progeny. If an infected cell undergoes malignant transformation, every neoplastic daughter cell inherits the same unique viral episomal structure, making the EBV genomic structure a marker for tumor clonality. On Southern blot analysis of the viral terminal repeat fragment, a monoclonal neoplasm displays as a single band, and the size of the band differs among various patients' tumors because of differing numbers of terminal repeats in the fused episome.[10] Oral hairy leukoplakia and other lytic infections display a ladder of smaller bands corresponding to the polyclonal linear viral genomes.

Monoclonal EBV DNA is present in infected lymphomas and carcinomas.[10,11] PTLDs are usually monoclonal, although occasional biclonal or oligoclonal examples imply multiple synchronous neoplastic transformations, and polyclonality characterizes early disease having a better prognosis.[12] Although EBV DNA typically persists as an episome, in some instances it recombines with the human genome to create one or more chromosomal integrations of varying structure with respect to viral and host breakpoints.[13] Whether integration contributes to neoplasia requires further study.

Viral Life Cycle Balances Latent and Lytic Infection

Infection of a B lymphocyte leads to two alternative outcomes supporting viral persistence and propagation.[14] These outcomes reflect physiological processes underlying humoral immunity. EBV infection mimics antigen stimulation by triggering the host B cell to divide, producing daughter cells that become either memory B cells persisting long term or plasma cells supporting lytic viral replication with virion production. Throughout the remaining life of the host, EBV lies latent in some B cells by expressing few if any immunogenic viral proteins. When an infected cell is stimulated by its natural antigen, which would typically occur on a mucosal surface where foreign antigens abound, the B cell recapitulates the process described above by dividing to create more infected memory B cells and more terminally differentiated plasma cells producing thousands of virions that could reinfect the host or be shed from the mucosal surface to infect other human hosts.

Spectrum of EBV Gene Expression in Lesional Tissue

Latent infection is characterized by limited expression of viral proteins to avoid immune recognition and destruction. One tolerated protein is EBV nuclear antigen 1 (EBNA1) that functions to propagate the viral genome to daughter cells on cell division. EBNA1 is unable to elicit an effective cytotoxic immune response, partially explaining why EBV is never eliminated from the body.[15] In immunocompromised hosts and in tissue culture systems in which immune surveillance is absent, infected B cells may express a broader range of viral proteins: LMP1, LMP2, and the EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA5). Inexplicably, Hodgkin lymphoma patients tolerate LMP2-expressing tumor cells even though their immune system seems to be competent in other regards. HLA type and human immunodeficiency virus status impact on risk of developing EBV-related Hodgkin lymphoma, emphasizing the importance of the immune system in controlling the outcome of infection.[16] Prognosis relates to EBV status in a

Table 1. Characteristic Patterns of EBV Gene Expression in Normal and Lesional Tissue

Cell or tissue type	Typical EBV gene expression*
AIDS-related plasmablastic lymphoma	Type 0 latency (*EBERs, BARTs*)
Burkitt lymphoma	Type I latency (EBNA1, LMP2, *EBERs*, *BARTs*)
Hodgkin lymphoma	Type II latency (EBNA1, LMP1, LMP2, *EBERs, BARTs*)
AIDS-related Burkitt or primary effusion lymphoma	Type II
Peripheral T cell lymphoma	Type II
NK/T cell lymphoma, nasal type	Type II
Nasopharyngeal carcinoma	Type II plus BARF1
Gastric adenocarcinoma	Type II plus BARF1
Post-transplant lymphoproliferative disorder	Type III latency (EBNA1, -2, -3A, -3B, -3C; LMP1, LMP2, *EBERs*, *BARTs)*
AIDS-related immunoblastic or brain lymphoma	Type III
Infectious mononucleosis	Type III
Chronic active EBV infection	Type III
Lymphoblastoid cell lines *in vitro*	Type III
Oral hairy leukoplakia	Lytic infection (LMP1, LMP2, BZLF1, BMRF1, BHRF1, BCRF1, and other replication factors)
Remotely infected carriers	
Circulating B cells	Type 0
Tonsil/mucosal B cells	Type II

*Viral gene expression may be focal or variable in a given lesion.

complex manner: children whose Hodgkin tumors are EBV-positive fare better, whereas older adults do better if their tumors are EBV-negative.[17,18]

Several patterns of latent viral gene expression have been described based on the spectrum of expressed proteins, transcripts, and noncoding RNAs like EBV-encoded RNAs (*EBER1* and *EBER2*) and BamH1A rightward transcripts (*BARTs*) including microRNAs (Table 1).[19–21] Even monoclonal monomorphous tumors may have heterogeneous viral gene expression among cells, so adequate sampling is required to fully appreciate the spectrum of expressed viral factors. *EBERs* are reliably expressed in virtually all latently infected cells in every benign and malignant lesion, which explains why *EBER in situ* hybridization is the assay of choice in clinical laboratories for defining a lesion as EBV-related.

Viral replication is associated with diminished *EBERs* and sequential expression of a cascade of lytic viral proteins beginning with BZLF1. Lytic proteins elicit strong humoral and cellular immune responses, explaining why viral infection is so well controlled in healthy carriers. Indeed, 5% of all circulating mononuclear cells are EBV-directed in healthy carriers.[22]

Serology

Serological tests confirm primary infection and document remote infection. The most widely used serological assay, the heterophile antibody test (colloquially called the "Monospot" test), was first introduced in 1932 well before EBV was identified as the causative agent of infectious mononucleosis. The original heterophile test was based on the discovery that serum or plasma from patients with infectious mononucleosis could agglutinate horse or sheep erythrocytes. Modern variants of the test detect serum-mediated agglutination of latex beads coated by bovine heterophile antigens. The heterophile test is used to facilitate rapid clinical decision-making in patients with infectious mononucleosis-like symptoms (fever, sore throat, lymphadenopathy, hepatosplenomegaly, malaise, headache). Complementary findings include an elevated white blood cell count and reactive lymphocytosis representing cytotoxic T cells responding to EBV infection.

A panel of sensitive and specific serological tests is applied in patients for whom the heterophile test is suspected to be false-negative (especially young children or older adults) and to document prior EBV infection (Figure 1). Indirect fluorescent antibody tests are useful, but they are also labor-intensive and subjective because they involve applying patient serum to infected cells immobilized on glass slides followed by microscopic interpretation of patterns of antibody localization to complexes of viral proteins collectively referred to as early antigen (EA), viral capsid antigen (VCA), or EBNA.[23] In recent years, more economical and objective tests for antibodies against recombinant EBV proteins have been introduced

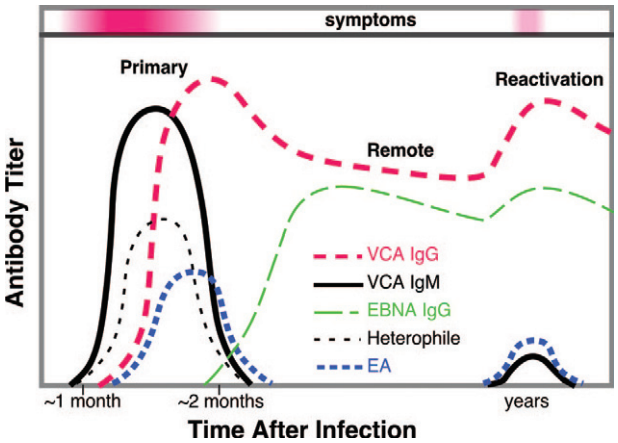

Figure 1. Serological titers distinguish primary infection from remote infection. IgG anti-VCA and IgM anti-VCA rise in concert with symptoms of primary infection and a positive heterophile test. After symptoms resolve, remote infection is characterized by EBNA and IgG anti-VCA without EA, although EA and IgM may reappear with or without symptoms on viral reactivation or EBV-related neoplasia.

using enzyme-linked immunosorbent assay technology and related methods.[23–25]

EBV-related cancer is typically associated with high serological titers against EA and IgG VCA with low EBNA titer. Results should be interpreted with caution since similar patterns are possible in autoimmune disease and other reactive conditions. Furthermore, serology is not reliable when the immune system is dysfunctional, eg, acquired immunodeficiency syndrome (AIDS) or allogeneic transplant patients including solid organ and marrow recipients. Surprisingly, certain abnormal EBV serological patterns are a risk factor for developing lymphoma that is not EBV-infected, such as chronic lymphocytic leukemia, suggesting that atypical antibody response to EBV reflects a more general deficit in the immune system.[26] A more direct role for the virus is postulated in Richter transformation of chronic lymphocytic leukemia in which EBV is localized to the higher grade lymphoma in 15% of cases.

There is one type of cancer for which EBV serology is critical: NPC patients often have high IgA titers against lytic EBV proteins in keeping with the origin of the cancer on the mucosal surface of the nasopharynx.[27] A panel of serological and molecular tests on serum or plasma can screen for NPC in high-risk populations, assess prognosis, and monitor disease status over time.[28–30]

EBV Strain Types

There are two major strains of EBV (types A and B) as well as many minor strains that are being evaluated for their impact on disease association, oncogenicity, and drug resistance. Pilot data suggest that strain variation impacts aggressiveness for both lymphomas and carcinomas.[31,32] Interestingly, healthy carriers often harbor multiple EBV strains, and strain type may differ by anatomical site and over time in a given individual.[31] The variation appears to derive from infection by multiple strains more than acquired polymorphisms in a pre-existing strain.

Strain variation may impact detectability of the virus by laboratory methods. Point mutation, deletion, or rearrangement of the EBV genome could contribute to false-negative probe hybridization results.[33] Furthermore, integration into host chromosomal DNA may result in partial loss of viral gene sequences. Strain variation emphasizes the need to target a conserved viral gene segment (or preferably multiple segments) when designing a laboratory assay to detect EBV DNA. Pilot studies of AIDS lymphoma identified interfering sequence variants in the *LMP1*, *LMP2,* and *BZLF1* genes, whereas BamH1W, *EBER1*, and *EBNA1* regions were highly conserved.[33] An advantage of targeting the reiterated BamH1W region is improved assay sensitivity at the expense of accuracy since BamH1W repeat number varies from 7 to 11 copies per EBV genome. However, accuracy may be less important than other performance characteristics for certain clinical applications such as detecting early stage NPC. Moreover, the number of viral genomes per cell varies across tumors, further emphasizing that EBV copy number is not synonymous with infected cell count.

In EBV-related cancer patients, the same EBV strain lies in the plasma as in the tumor, and the plasma EBV DNA is naked rather than encapsidated implying that it emanates from dying infected tumor cells rather than representing new virion production.[34–36] By comparison, infectious mononucleosis patients have a mixture of encapsidated virions and naked EBV DNA in their plasma.[36]

EBER in Situ *Hybridization Assay Localizes EBV in Biopsy Tissue*

The most abundant viral transcripts in latently infected cells, *EBER1* and *EBER2*, are non-polyadenylated and thus are not translated into protein; they function to inhibit interferon-mediated antiviral effects and apoptosis. These two transcripts, collectively called *EBER*, are expressed at such high levels (around a million copies per latently infected cell) that they are considered to be the best natural marker of latent infection. *In situ* hybridization targeting one or both *EBERs* is the gold standard assay for determining whether a biopsied tumor is EBV-related. Commercial systems for *EBER in situ* hybridization facilitate implementation in clinical laboratories (eg, Ventana [Tucson, AZ], Leica [Bannockburn, IL], Dako [Glostrup, Denmark], Invitrogen [Carlsbad, CA], Biogenex [San Ramon, CA]). As a control for adequate RNA preservation and for the hybridization process, a parallel assay should be done targeting endogenous RNA.[37]

Table 2 shows a list of EBV-associated diseases along with the proportion of cases harboring EBV within lesional cells. A biopsy assessed by *EBER in situ* hybridization is needed to confirm each diagnosis and its relation to EBV, with the exception of suspected infectious mononucleosis for which clinical findings and serology are usually diagnostic, whereas biopsy may be counterproductive because of histological overlap with Hodgkin and non-Hodgkin lymphoma. Another exceptional lesion is oral hairy leukoplakia in which *EBER* is down-regulated, whereas lytic proteins BZLF1 and BMRF1 are localized to ballooned cells in mid-layers of the hyperplastic stratified squamous epithelium.

Utility of EBER in Situ *Hybridization in Carcinomas*

Nearly all undifferentiated NPCs are EBV-related, whereas a lesser proportion of keratinizing NPCs harbor EBV as demonstrated by *EBER in situ* hybridization. Around half of affected patients initially present with an enlarged cervical lymph node representing metastatic spread; identification of *EBER*-expressing carcinoma in a lateral or posterior retropharyngeal cervical node should trigger endoscopic examination of the nasopharynx in search of the primary site. In a left supraclavicular lymph node, *EBER*-positive undifferentiated carcinoma or adenocarcinoma should prompt consideration of a gastric primary, since about 7% of gastric carcinomas are infected.

Table 2. EBV-Associated Diseases

Disease	EBV-related (% cases)
Benign reactive infections	
Infectious mononucleosis	>99
Oral hairy leukoplakia	>95
EBV-related hemophagocytic syndrome	100*
Chronic active EBV infection	100*
Hodgkin lymphoma	
Hodgkin lymphoma, all subtypes	40
Hodgkin lymphoma, mixed cellularity	70
Hodgkin lymphoma, nodular sclerosis	20
Hodgkin lymphoma, lymph. predominant	<5%
Hodgkin lymphoma, lymphocyte depleted	50
Hodgkin lymphoma, AIDS-related	>95
Carcinomas and soft tissue sarcomas	
Nasopharyngeal carcinoma, Asian	>95
Nasopharyngeal carcinoma, USA	75
Lymphoepithelioma-like carcinoma	Most
Gastric adenocarcinoma	7
Smooth muscle tumor in AIDS/transplant	>95
Follicular dendritic cell tumor, IP-like	Most
Non-Hodgkin lymphomas and related neoplasms	
Non-Hodgkin lymphoma, all subtypes	5
Non-Hodgkin lymphoma, diffuse large B cell subtype	15
Richter syndrome (transformed lymphoma)	15
Non-Hodgkin lymphoma, AIDS-related	40
Brain lymphoma, AIDS-related	95
Brain lymphoma, immunocompetent hosts	5
Post-transplant lymphoproliferative disorder	95
Burkitt lymphoma, African (endemic)	>95
Burkitt lymphoma, North American	20
Burkitt lymphoma, AIDS-related	30
Lymphoma, primary immunodeficiency	Most
Pyothorax-associated lymphoma	90
Lymphomatoid granulomatosis (B cell lymphoma)	90
Plasmablastic lymphoma, AIDS-related	60
Primary effusion lymphoma, AIDS-related	70
Age-related EBV-associated B cell lymphoproliferation	100*
Angioimmunoblastic T cell lymphoma (EBV+ B cells)	80
Peripheral T cell lymphoma, unspecified	40
Extranodal NK/T lymphoma, nasal type	>95
NK leukemia	Most
$\gamma\ \delta$ T cell lymphoma, mucosal	Most
T cell lymphoma in chronic active EBV infection	Most

*By definition the disease is EBV-related.

Although some investigators claim to have identified *EBER*-negative cancers that harbor EBV by other analytic methods, these claims are controversial and are not universally accepted.[38,39] DNA amplification results alone are inadequate to prove localization of the infection to malignant cells as opposed to reactive lymphocytes. Furthermore, RNA-based tests like *EBER* histochemistry are prone to false-negative results, emphasizing the need to interpret negative *EBER* stains in the context of a control assay to demonstrate that RNA is preserved and available for hybridization. In questionable cases, *in situ* hybridization to EBV DNA is the next most reliable histochemical test, and secondary support comes from Southern blot analysis of EBV clonality and other histochemical assays.

Utility of EBER in Situ *Hybridization in Lymphomas*

EBV-driven PTLD is a feared cause of morbidity and mortality in allogeneic transplant recipients. The disease is manifest when iatrogenic immunosuppression leads to diminished T cell immunity that predisposes to massive proliferation of EBV-infected B lymphocytes. *EBER in situ* hybridization is useful in the work-up of suspected PTLD as well as any lymphoproliferation arising in the setting of immunodeficiency, such as the following. 1) Immunosuppressive drugs (eg, methotrexate for rheumatoid arthritis) may lead to an EBV-driven lymphoproliferation that responds to withdrawal of the drug. 2) Impaired immunity in the elderly may cause "age-related EBV-associated B cell lymphoproliferative disorder." 3) Immunodeficiency predisposes to lymphomatoid granulomatosis, a rare neoplasm of the lung or other extranodal sites in which infected neoplastic B cells are surrounded by far more abundant reactive T cells. 4) The longstanding chronic inflammation of tuberculosis predisposes to pleural-based pyothorax-associated B cell lymphoma that is EBV-related in 90% of cases. 5) AIDS patients are predisposed to develop B cell lymphoma (diffuse large B cell, immunoblastic, Burkitt, plasmablastic, Hodgkin, or primary effusion subtypes) with 50% of cases being EBV-related. In addition, brain lymphoma arising in an AIDS patient is nearly always EBV-related.[40]

Enlarged lymph nodes from infectious mononucleosis patients harbor variable numbers of *EBER*-expressing small to large lymphocytes. In contrast, Hodgkin and non-Hodgkin lymphomas generally lack *EBER*-expressing small lymphocytes while the larger tumor cells may be uniformly *EBER*-positive, a feature that can be helpful in resolving a benign versus malignant differential diagnosis. A histological mimic of Hodgkin lymphoma, anaplastic large cell lymphoma, is virtually never *EBER*-positive, at least in Western nations. Nasal NK/T lymphoma is a unique form of lymphoma that is so tightly linked to EBV that failure to identify the virus should prompt re-evaluation of the differential diagnosis. Aggressive NK cell leukemia, which may represent systemic dissemination of nasal NK/T lymphoma, is also EBV-related.

Peripheral T cell lymphoma frequently harbors EBV. Surprisingly, dual stains show that the infection is often localized to B lymphocytes that lie in proximity to uninfected malignant T cells.[41] An example is angioimmunoblastic T cell lymphoma (AILT) which, in 80% of cases, has *EBER* localized to B cells. These B cells may be monoclonal, although their small numbers make it difficult to demonstrate clonality in the context of the more prevalent uninfected T cell clone.[42] Regardless, B cell expansion correlates with *EBER* positivity in AILT as well as in peripheral T cell lymphomas of unspecified type.[43] Attygalle et al found that expansion of EBV-infected lympho-

cytes (defined as ≥5 EBER-expressing cells per section) was helpful in supporting a diagnosis of AILT and distinguishing it from peripheral T cell lymphomas of unspecified type that harbor far fewer *EBER*-positive cells.[42] This finding is controversial since Tan et al reported that both AILT and peripheral T cell lymphomas of unspecified type frequently contain infected cells (defined as more than three EBER-expressing cells per section).[43] Cases of peripheral T cell lymphomas of unspecified type that are EBV-related tend to have a worse prognosis.[41] Interestingly, some AILT are coinfected with HHV6 or are infected by HHV6 alone, and the histopathological appearance of AILT depends on which of these two viruses are present.[44]

Ongoing research is refining the criteria for distinguishing chronic active EBV infection or EBV-associated hemophagocytic syndrome from T cell lymphoma and NK cell neoplasia. These conditions are most prevalent in southeast Asia and may begin when an as yet uncharacterized immunodeficiency permits EBV infection of NK or T cells that then predisposes to subsequent development of NK or T cell lymphoma. The various preneoplastic and neoplastic lesions may occur separately or in combination in a given patient. In any case, *EBER in situ* hybridization, especially when combined with a dual stain for CD3 T cells, is useful in localizing the infection.[45] Still to be resolved is whether identification of *EBER*-positive T cells is always indicative of neoplasia or whether latent EBV can be found in normal T cells.

It is interesting to note how often EBV-related lymphomas were historically categorized as reactive conditions. What was first described as Hodgkin's granuloma was later termed Hodgkin's disease and is now called Hodgkin lymphoma. What was once called lethal midline granuloma is now nasal NK/T lymphoma. Angioimmunoblastic lymphadenopathy with dysproteinemia is now AILT. Many follicular dendritic cell tumors were previously termed inflammatory pseudotumor. Lymphomatoid granulomatosis, now known to be an EBV-infected B cell lymphoma, should be more appropriately renamed. It is likely that EBV draws abundant reactive inflammatory cells into the region of an infected tumor, possibly contributing to misclassification of these lesions as benign. Further refinement of the World Health Organization criteria for classifying lymphoma and for distinguishing lymphoma from reactive conditions are predicted to depend increasingly on laboratory assays targeting EBV and other viruses such as HHV6, HHV8, HIV, HTLV1, and HCV that are cofactors in lymphomagenesis. Furthermore, advances in targeted therapy that eliminate virally infected cells or thwart their oncogenic effects will make it all of the more important to reliably detect and characterize viruses in lesional tissue.

Amplification Assays Targeting the EBV Transcriptome

The sequences of all 84 EBV genes and their variant transcripts are well described, as are the virally encoded microRNAs.[20,21,46] Panels of real-time polymerase chain reactions (PCRs) characterizing the viral gene expression profile may identify novel biomarkers for subclasses of EBV-related disease.[47,48] Individual real-time PCR or nucleic acid sequence-based amplification assays appear to help distinguish tumor from normal tissue even when the normal tissue harbors "background" EBV infection.[49,50] For example, suspected NPC was confirmed by *BARF1* nucleic acid sequence-based amplification in nasopharyngeal brushings since viral *BARF1* is expressed only in carcinoma and not in normal nasopharynx nor in benign epithelial infection.[49,50] Because *BARF1* is specific to malignant epithelial cells, it should be explored as a biomarker for infected gastric carcinoma.[50,51] DNA- and protein-based assays are complementary, with EBV DNA and BARF1 protein both elevated in nasopharyngeal brushings or saliva at high sensitivity and specificity for NPC.[52,53] In a screening situation (eg, testing for local recurrence of NPC), high levels of EBV DNA, RNA, or protein in a brushing or in saliva should be followed by a biopsy, including "blind" biopsies if no lesion is seen by endoscopy. Rare malignant cells within a biopsy, especially in specimens with crush artifact, can sometimes be highlighted using *EBER in situ* hybridization.[54]

Immunohistochemical Assays Localize EBV Proteins in Biopsy Tissue

Western blot, flow cytometry, and enzyme-linked immunosorbent assay can potentially detect and measure selected viral proteins for which antibodies are available. However, the single most informative protein-based assay is immunohistochemistry, because it permits localization of protein in the context of histopathology, facilitating assessment of the medical significance of the infection. Localization is achievable in paraffin-embedded sections for latent and lytic viral factors including EBNA1, EBNA2, LMP1, LMP2, BHRF1, BZLF1, and BMRF1.[45] Interpretation of results, including defining the spectrum of expressed genes and their localization to benign or malignant-appearing cells, complements *EBER in situ* hybridization for diagnosis of EBV-related disease.[55]

LMP1 immunohistochemistry is as informative as *EBER in situ* hybridization for defining whether a given Hodgkin lymphoma case is EBV-related as defined by localization of EBV to the neoplastic Reed-Sternberg/Hodgkin cells.[37] Unfortunately, few other diseases so reliably express EBV-encoded proteins: Burkitt lymphoma, PTLD, and NPC may have diffuse, focal, or completely undetectable expression of viral proteins by immunostains. *EBER in situ* hybridization remains the most reliable histochemical assay to localize EBV in these cancer tissues.

Technical problems can foil interpretation of immunohistochemical results. For example, EBNA1 is thought to be expressed in virtually all latently infected tumors, yet EBNA1 immunohistochemistry is not sensitive enough to reliably substitute for *EBER in situ* hybridization. Furthermore, the 2B4 clone of EBNA1 antibody cross-reacts with human MAGEA4, potentially causing false-positive interpretations.[56] Problems such as this emphasize the need

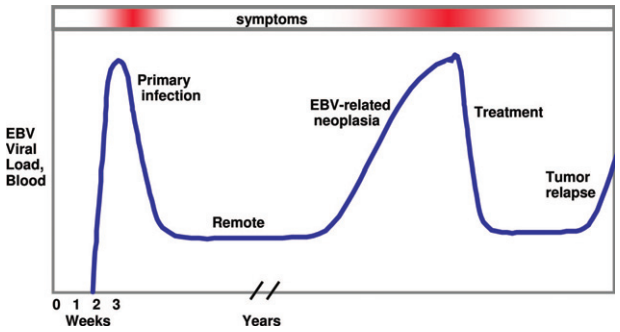

Figure 2. EBV viral load in whole blood reflects clinical status in patients with infectious mononucleosis, allogeneic transplant, and nasopharyngeal carcinoma. EBV DNA levels begin to rise within 2 weeks of primary infection and are already falling by the time the patient becomes symptomatic (due to interferon γ and other immune responses). Plasma or serum EBV DNA is undetectable in most remotely infected individuals; however, whole blood is low positive for the duration of life. If an EBV-related malignancy develops, levels may rise before clinical diagnosis, implying that high-risk patients benefit from routine monitoring. Successful therapy is marked by a decline to baseline, and rising levels may serve as a harbinger of relapse.

to validate laboratory assays before they are used in clinical investigations.

EBV Viral Load Assays

Quantitative EBV DNA measurement is essential for differentiating the low-level infection of healthy carriers from the high levels characteristic of EBV-related disease. Patients with active infection or EBV-related cancer tend to have high levels of EBV DNA in the cell-free fraction of blood (plasma or serum), whereas in healthy carriers the virus is restricted to the intracellular compartment of the blood (Figure 2).

Real-time PCR is the principal technology used for modern EBV viral load measurement. It relies on amplification of a conserved sequence (typically ~100 bp) using either an intercalating dye or a fluorescent probe (eg, TaqMan, MGB) to quantify the products against a series of standards representing serial dilutions of known EBV DNA content.[33,57–59] Since the reaction vessel remains sealed, the risk of amplicon contamination is minimized. Published procedures target any of several viral sequences.[60] Commercial primers and probes are available: Roche Molecular Diagnostics (Pleasanton, CA) targets *LMP2*, Qiagen (Valencia, CA) (Artus) targets *BKRF1 (EBNA1)*, Nanogen (San Diego, CA) (Epoch) targets a major tegument protein (*BNRF1*), Argene (Varilhes, France) targets thymidine kinase (BXLF1), Bioactiva Diagnostica (Bad Homburg, Germany), and Amplimedical (Milan, Italy) targets *BKRF1 (EBNA1)*.[3,61–64]

There is no consensus on the optimal methods for performing or reporting EBV viral load results, so it is the responsibility of each testing laboratory to validate their own procedures.[65] There is concern that prolonged or improper storage of whole blood may allow EBV DNA to escape from the intracellular compartment, potentially causing false-positive EBV results in plasma or serum specimens that are subsequently separated from the cellular fraction. False-negative findings due to nucleases are also a concern. EBV DNA in plasma is known to be partially degraded, although this may reflect apoptotic pathways in the cell from which it emanated.[35]

Automated extraction instruments promote reproducibility of DNA isolation procedures. The efficiency of extraction and amplification (as judged by amplification of an endogenous or spiked control sequence) should be considered. Units of measurement vary from copies per milliliter, to copies per microgram of DNA, to copies per 100,000 leukocytes.[65] Commercial sources of quantified EBV genomic DNA are now available that could potentially be used for assay calibration or for interlaboratory comparisons (eg, Advanced Biotechnologies Inc. [Columbia, MD], Accrometrix [Benicia, CA], Affigene [Bromma, Sweden], and ATCC's Namalwa Burkitt lymphoma cell line containing two integrated EBV genomes per cell [American Type Culture Collection, Manassas, VA]). In the future, international agreement on a reference standard and availability of FDA-approved kits will facilitate standardization of procedures across laboratories.

Despite wide variability in methods and the potential pitfalls of laboratory testing, multiple publications as well as proficiency survey data demonstrate that EBV viral load assays are sensitive, specific, precise, linear across a wide dynamic range, rapid, reasonably inexpensive, and useful in patient care. Prospective studies are needed to address methodologic concerns and also to refine the indications for testing and the use of test results in patient management.

EBV Viral Load in Infectious Mononucleosis

Circulating EBV DNA levels (in whole blood, plasma, serum, or memory B cells) spike within 2 weeks after primary EBV infection and then drop to low or undetectable over a period of several weeks to months.[36,66–70] Interestingly, the kinetics vary from patient to patient and may be accompanied by a rebound after an initial decline.[4] It may take a year or more to reach steady state,[71] after which a healthy carrier's whole blood contains about 1 to 50 copies of EBV DNA per million white blood cells,[1] whereas plasma or serum EBV DNA is nearly always undetectable.[2,3,25,33,67]

Laboratory confirmation of infectious mononucleosis should begin with the "Monospot" test followed, when necessary, by the more definitive serological assays described above. EBV DNA testing is reserved for atypical cases and for immunosuppressed patients.

EBV Viral Load in Chronic Active EBV Infection and Related Lymphoid Neoplasms

Chronic active EBV infection is a life-threatening disease that is more common in Asia than in western countries. It is characterized by persistent or recurrent infectious mononucleosis-like symptoms for at least 6 months along with atypical serology (high titers against several latent and lytic antigens with unexpected absence of EBNA antibody) and high EBV viral load in the absence of immunosuppression.[72] Levels of EBV DNA in blood

mononuclear cells correlate with disease severity.[68] Interestingly, clonal EBV genomes are often found in histologically abnormal organs (liver, spleen, lymph nodes, marrow), even though multiple lineages of lymphocytes may be infected (B cells, helper and cytotoxic T cells, NK cells), leading to the hypothesis that neoplastic transformation of an infected lymphoid progenitor cell gave rise to cells of various lineages.[73,74] Mutation in an immune-modulating gene such as perforin may be responsible for faulty control of EBV infection, resulting in chronic infection and subsequent clonal outgrowth of infected T and/or NK cells.[75] Affected patients seem to lack LMP2-specific cytotoxic T cells, and replacement by infused EBV-specific T cells is being evaluated as a potential therapy.

EBV Viral Load in X-Linked Lymphoproliferative Disorder

An EBV-specific immunodeficiency, X-linked lymphoproliferative disorder, is caused by heritable mutation of *SH2D1A* rendering a child susceptible to fatal primary EBV infection or EBV-related lymphoma. Both innate (NK cell) and antigen-driven (T cell) immune responses appear to be deranged, resulting in failure to kill B lymphocytes appropriately and high EBV viral load. Clinical recognition of this rare syndrome is difficult but important given the divergent therapy required for sepsis, which has similar clinical features in young children.[76]

EBV Viral Load in Nasopharyngeal Carcinoma

Undifferentiated NPC is strongly associated with EBV in southeast Asia, where NPC is endemic, and to a lesser extent in western nations, where NPC is rare.[77–79] Infection precedes malignant transformation as shown by clonal viral terminal repeat sequences as well as *EBER* expression in all neoplastic cells at the primary site and in distant metastases. Indeed, EBV infection serves as a marker of the neoplastic clone that can be capitalized on for diagnosis and management of affected patients.

The level of circulating EBV DNA in plasma or serum tracks with tumor stage at initial diagnosis and likewise serves as a marker of tumor burden during therapy.[28,80–84] Early stage patients can be further subdivided into those at low versus high risk of relapse by evaluating their plasma EBV load.[85] High pretreatment levels portend poor survival.[59,80,82,85] In contrast, low or undetectable EBV DNA in plasma implies localized NPC, assuming that *EBER in situ* hybridization reveals that the primary carcinoma is EBV-related. High risk individuals from endemic areas (eg, adult family members of NPC patients from southeastern China) could potentially be screened for early NPC using EBV-directed laboratory methods (serology, Q-PCR or *BARF1* gene products in nasopharyngeal brushings).[52,86,87]

Kinetic studies show that plasma EBV DNA becomes undetectable about 2 hours after surgical resection.[88] When radiotherapy is used, the initial rise in plasma EBV DNA may reflect release of EBV DNA on tumor lysis, followed by declining EBV with a median half-life of 3.8 days.[89] Patients with residual disease retained measurable EBV at 6 to 8 weeks, whereas those with undetectable plasma EBV maintained stable remission.[82,89,90] Serial testing can predict relapse before it becomes symptomatic.[28,81,82]

EBV viral load is a better test of recurrence than is serology.[28,59] This is not surprising given that serology provides indirect evidence of prior infection, whereas Q-PCR measures active disease. While distant relapse is usually marked by EBV DNA in plasma,[91] local relapse is better assessed endoscopically using biopsy or perhaps less invasive tests like *BARF1* RNA in nasopharyngeal brushings, BARF1 protein in saliva, or promoter hypermethylation that distinguishes carcinoma from "background lymphocyte" infection.[49,52,53] Prospective clinical trials are needed to examine the utility of various laboratory approaches for diagnosing and managing NPC.

EBV Viral Load in Gastric Adenocarcinoma

EBV DNA is detectable within the malignant epithelial cells of about 10% of gastric adenocarcinomas, especially in those having a lymphoepithelioma-like appearance (ie, undifferentiated with abundant tumor-infiltrating lymphocytes) and in those arising in the stump after surgical gastrectromy.[11,92] Surprisingly, little has been done to evaluate the role of EBV testing in diagnosis and monitoring of gastric cancer. A pilot study has indicated that serum EBV DNA levels are often elevated in patients whose cancer harbors EBV.[93]

EBV Viral Load in Transplant Recipients

Blood represents a convenient and noninvasive specimen type in which to evaluate EBV as a biomarker for PTLD. Affected patients have high levels of EBV DNA in both the cellular fraction of blood and in cell-free (serum or plasma) fractions.[94] EBV DNA levels often rise before a mass lesion or symptoms become evident, implying that routine monitoring of high-risk patients may predict incipient PTLD.[95–98] Early warning permits preemptive therapy to reverse disease progression.

Despite the high mortality rate of PTLD, it is costly to prospectively monitor EBV viral load in all allogeneic transplant recipients. Typically, only patients at highest risk of PTLD are screened on a regular basis. Risk factors include the degree of T-cell depletion (eg, anti-thymocyte globulin or fludarabine use), prior immunity as demonstrated by EBV serology before transplant, an HLA-mismatched or unrelated donor, and reduced intensity conditioning.[99,100] High-risk patients are monitored as frequently as once per week in the first few months after transplant.[100] Since low-level viremia does not correlate with progression to PTLD, some investigators have set cutoff levels beyond which they intervene.[96,100–104] An alternative approach is to complement EBV DNA mea-

surement with another predictive assay, and the following assays have been proposed in limited studies: absolute number of CD8 or CD4 T cells, EBV-specific T cell response as measured by a peptide tetramer assay or by ELISPOT, ATP release, EA serology, cytokine gene polymorphism, and viral gene expression pattern by real-time PCR.[22,68,105–111] Successful preemptive intervention is accompanied by a drop in circulating EBV DNA.

Beyond its role in prevention of PTLD, EBV viral load testing is also indicated in any transplant recipient who presents with lymphadenopathy, fever, or other signs and symptoms suggestive of PTLD. A high EBV load should trigger the search for mass lesions or organ dysfunction pinpointing putative sites of disease, followed by biopsy to diagnose the lesion. Importantly, one should not diagnose infectious mononucleosis in an immunosuppressed transplant recipient since that diagnosis implies a self-limited process not requiring active intervention.

Early diagnosis is critical for successful clinical management of PTLD. Management is aimed primarily at restoring natural immune recognition and destruction of infected cells by reducing the degree of iatrogenic immunosuppression. Even monoclonal tumors may respond. Other therapies include CD20 antibody (eg, rituximab), donor lymphocyte infusion, infusion of EBV-specific cytotoxic T cells that were expanded *ex vivo* by exposing T cells to EBV antigens,[112–114] radiation, and multidrug chemotherapy. Successful therapy for frank PTLD is accompanied by a rapid drop in plasma EBV levels reflecting diminished disease burden.[94,98,115]

CD20 antibody therapy may fail due to outgrowth of a neoplastic subclone lacking CD20.[116,117] Indeed, EBV infection contributes to survival of defective B cells by rescuing them from programmed cell death despite their aberrant lack of CD20 or lack of immunoglobulin production.[118,119] Crippled B lymphocytes have been identified in some cases of PTLD, Hodgkin lymphoma, AIDS lymphoma, pyothorax-associated lymphoma, and AILT.[120,121]

Rare PTLDs of T or NK cell lineage are less likely to be EBV-related compared to the more typical B lineage cases.[122] EBV-negative PTLD tends to occur late after transplant, whereas nearly all PTLD occurring within the first year are EBV-related.[122] While some EBV-negative tumors undoubtedly represent lymphoma of the conventional type, anecdotal reports of response to withdrawal of immunosuppression support using PTLD terminology for all lymphoid neoplasms occurring in immunosuppressed transplant recipients.[98]

There is controversy over whether plasma, whole blood, or blood mononuclear cells is the preferred specimen type, and all three appear to be informative to some extent.[94,100,123–127] Plasma was more specific for lymphoproliferative disease in one study, and was more informative for monitoring efficacy of therapy in another study.[94,128] Plasma is the specimen type evaluated in NPC patients and in the two commercial proficiency surveys (College of American Pathologists [Northfield, IL] and Quality Control for Molecular Diagnostics [Glasgow, Scotland]).

Guidelines for EBV testing in solid organ transplant recipients in clinical trials call for frequent monitoring (at least once a month) in the first year after transplantation when the risk of PTLD is highest.[98,129] Afterward, continued monitoring should be considered for patients with a history of persistently high EBV loads or who are particularly immunosuppressed.[129] The European Best Practice Guideline for Renal Transplantation calls for serological testing of EBV immune status before transplant, antiviral prophylaxis, pathologist diagnosis of PTLD, and use of blood levels to gauge treatment.[130] Primary EBV infection is a contraindication to renal transplantation, while remote EBV infection is protective against PTLD.[130] After transplant, those renal and lung recipients having asymptomatic EBV viremia are at risk for other adverse outcomes such as graft dysfunction, acute rejection, and late onset PTLD.[131–133] Surprisingly, there was no correlation between EBV viral load and complications caused by other pathogens.[131]

EBV Viral Load in Hodgkin and Non-Hodgkin Lymphomas

EBV viral load in plasma serves as a marker of tumor burden in patients with sporadic EBV-related lymphoma including B cell, T cell, NK cell and Hodgkin subtypes.[35,134–136] EBV DNA is detectable before cancer diagnosis, and the level of EBV DNA at diagnosis may predict outcome and efficacy of therapy.[135,136] The findings argue in favor of routine plasma EBV viral load testing on initial diagnosis of Hodgkin lymphoma as well as any of the non-Hodgkin lymphomas that are likely to be EBV-related.

EBV Viral Load in Human Immunodeficiency Virus-Infected Patients

Immunocompromised hosts have higher baseline levels of circulating EBV than do healthy individuals.[137,138] AIDS patients who subsequently develop EBV-related lymphoma have high blood and plasma EBV levels that fall on initiation of therapy.[137,139] An exception is primary brain lymphoma; although EBV is nearly always present within the malignant cells, blood and plasma levels are not elevated because the blood-brain barrier limits dispersion.[137,140] Instead, cerebrospinal fluid contains EBV DNA,[40,141] and it has been suggested that cerebrospinal fluid EBV substitutes for brain biopsy in making a diagnosis of lymphoma in an AIDS patient who has clinical and radiographic evidence supporting the diagnosis.[142] Serial EBV DNA levels in cerebrospinal fluid reflect therapeutic efficacy.[143] Gancyclovir alone can lower EBV levels in cerebrospinal fluid,[141] and gancyclovir may synergize with traditional antineoplastic therapy in managing a wide spectrum of EBV-related malignancies.[144]

Measuring EBV Viral Load in Biopsy Tissues

EBV DNA can be quantified by real-time PCR in paraffin-embedded tissue or in cytologic specimens.[33] Advan-

tages over *EBER in situ* hybridization are PCR's relatively low cost, its applicability to specimens with poor quality RNA, and its ability to detect (pure lytic) infection lacking *EBER* transcripts. It is important that the PCR assay be quantitative and that parallel Q-PCR of an endogenous human gene be used to normalize for the number of nucleated cells represented in the reaction.[33]

EBV load in NK/T lymphoma tissue is touted as a prognostic indicator.[145] Likewise, a biopsy or fine needle aspirate that is suspicious for NPC contains high levels EBV if the cancer is indeed present and is EBV-related.[33,146] On the other hand, low to undetectable EBV DNA by Q-PCR is consistent with scant to absent EBV-related neoplastic cells, or rare infected B lymphocytes that might be present in any viral carrier, emphasizing the need to interpret EBV Q-PCR results in the context of histopathological findings.

Future Perspectives

EBV is one of the best tumor markers yet discovered. EBV viral load testing has been incorporated into routine care of patients with PTLD, nasopharyngeal carcinoma, and AIDS lymphoma of the brain. An international effort is underway to establish a standard by which to calibrate EBV DNA measurement. Further evidence and consensus building is needed on clinical indications for testing, optimum specimen types and handling procedures, assay design and scope, units for reporting, thresholds for intervention, and management strategies. Serology is the best way to confirm a diagnosis of infectious mononucleosis, and *EBER in situ* hybridization is the single best histochemical assay for defining EBV-related neoplasia. It is likely that emerging technologies such as gene expression profiling and proteomics will identify patterns of viral and human gene expression correlating with diagnosis, prognosis, and outcome in response to therapy. A coordinated effort by basic scientists and clinical investigators will improve our arsenal of laboratory methods and better define their clinical utility.

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