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Review

Molecular Diagnosis of Epstein-Barr Virus-Related Diseases

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Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis, and it may also be found in a wide variety of benign and malignant lesions including oral hairy leukoplakia, inflammatory pseudotumor, Hodgkin's disease, non-Hodgkin's lymphoma, nasopharyngeal carcinoma, and gastric carcinoma. Molecular testing is increasingly important in the diagnosis and monitoring of patients affected by these diseases. In biopsy tissues, molecular detection of EBV-encoded RNA transcripts by *in situ* hybridization remains the gold standard for proving that a histopathological lesion is EBV-related. EBV-encoded RNA hybridization and EBV LMP1 immunostains are used routinely to detect latent EBV in tissues affected by posttransplant lymphoproliferative disorder (PTLD) or in enlarged nodes from patients with infectious mononucleosis. Traditional serology is the best test for evaluating acute *versus* remote infection in healthy individuals. High serological titers serve as a tumor marker for some EBV-related malignancies, but titers are not a dependable tumor marker in immunocompromised hosts. EBV viral load testing by quantitative DNA amplification of blood samples is a promising new laboratory test that has proven useful for early diagnosis and monitoring patients with PTLD. Recent studies suggest a role for EBV viral load testing in nasopharyngeal carcinoma, Hodgkin's disease, and AIDS patients with brain lymphoma. Further research is needed to define more fully the clinical utility of viral load tests in the full spectrum of EBV-associated diseases. Gene expression profiling is on the horizon as a means to improve subclassification of EBV-related diseases and to predict response to therapy. *(J Mol Diag 2001, 3:1–10)*

Epstein-Barr virus (EBV) was first identified using electron microscopy of Burkitt's lymphoma cell cultures in 1964.[1] In subsequent decades, EBV has been linked to a wide variety of benign and neoplastic diseases. Nasopharyngeal carcinomas and posttransplant lymphoproliferative disorders are nearly always EBV-associated, whereas several other tumors, such as Hodgkin's disease, non-Hodgkin's lymphoma, lymphoepithelioma-like carcinoma, gastric adenocarcinoma, and several types of sarcoma, are less uniformly EBV-associated.[2–7] EBV causes benign transient lymphoproliferative lesions at the time of primary infection, and it is found in a benign lesion of the tongue called oral hairy leukoplakia.[8,9] Patients affected by these benign or malignant diseases may benefit from laboratory detection of EBV to confirm their diagnosis or to monitor disease burden after the initiation of therapy.

Laboratory detection of EBV is accomplished in several ways (Table 1), and recent progress has focused on the molecular analysis of viral DNA and RNA. *In situ* hybridization has long been considered the gold standard for detecting tumor-associated viral infection, and EBV viral load assays are now being adopted for clinical evaluation of tumor burden in affected patients. This review article summarizes the pathobiology of EBV infection and describes the clinical laboratory tests that are used to assist in diagnosis and monitoring of patients with EBV-related diseases.

The Pathobiology of EBV Infection

EBV has a 173-kb DNA genome for which the nucleotide sequence and predominant transcripts are well characterized. EBV is capable of infecting B and T lymphocytes, squamous epithelial cells of the oropharynx and nasopharynx, glandular epithelium of the thyroid, stomach, and salivary gland, smooth muscle cells, and follicular dendritic cells. Healthy virus carriers harbor 1 to 50 EBV genomes per million blood mononuclear cells, with B lymphocytes representing the major cellular reservoir.[10] Beyond B cells, it is nearly impossible to find infected

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Table 1. Laboratory Tests for EBV

Name	Purpose
In situ hybridization	Identify EBER transcripts or EBV DNA in specific cell types within histologic lesions
EBV clonality assay by Southern blot analysis	Assess clonality of lesions with respect to EBV DNA structure; distinguish latent from replicative infection based on the episomal versus linear structure of the EBV genome
EBV DNA amplification	Detect viral DNA in patient tissues; disease specificity is lacking
EBV viral load	Quantitate EBV DNA in blood or body fluids to monitor disease status over time
Immunohistochemistry (LMP1, EBNA1, EBNA2, LMP2A, BZLF1)	Identify EBV protein expression in specific cell types within histologic lesions; distinguish latent from replicative infection based on expression profiles
Culture of EBV or of EBV-infected B lymphocytes	Detect and semiquantitatively measure infectious virions or latently-infected B lymphocytes; impractical for routine clinical use
Electron microscopy	Identify whole virions representing replicative viral infection; impractical for routine clinical use
Serology (VCA, EBNA, EA, heterophile antibodies)	Measure antibody response to viral proteins in serum samples; distinguish acute from remote infection; monitor disease status over time

cells of other lineages in healthy carriers, but we presume that the other cell types listed above are capable of being infected based on the identification of EBV DNA in lesions arising from them. Investigation of patients with EBV-infected tumors provides reasonable evidence that EBV was present before neoplastic transformation, raising the still unresolved question of the extent to which EBV contributes to tumorigenesis.

EBV infects nearly all humans by the time they reach adulthood. Primary infection results in transient viremia followed by rapid immune response. The virus persists for life in its human host by cleverly balancing its ability to hide from the immune system via latent infection of B lymphocytes with its ability to replicate and shed from oral mucosa. At any given time, about 20% of carriers are shedding salivary virions, leading to nearly universal propagation of the virus in human populations.

EBV infection of B lymphocytes leads to two alternate outcomes mimicking the physiological effects of antigen stimulation. One outcome culminates in the production of memory B cells that persist long-term; the other outcome results in differentiation toward plasma cells that are destined to die. These two outcomes support latent viral persistence and lytic viral replication, respectively. Lifelong infection of the human host relies on these dual phases of infection whereby the virus hides from the immune system in memory B cells, and a subset of these cells are diverted to produce thousands of virions that not only infect more of the host's own lymphocytes but also are shed in saliva to infect other individuals. Viral replication is naturally enriched in the oral mucosa where memory B cells are routinely stimulated to differentiate after exposure to foreign antigens.

Lytic viral replication is accompanied by expression of about 90 viral proteins, including BZLF1 (also known as ZEBRA), and complexes of viral proteins collectively referred to as early antigen and viral capsid antigen. These lytic antigens elicit a humoral immune response, resulting in elevated antibody titers that quell rampant lytic virus production in the healthy carrier.

Latent infection is characterized by abundant production of EBV-encoded RNA (EBER), but it is important to mention that EBER transcripts remain untranslated. EBER transcripts are thought to function in controlling translation. Also expressed in latently infected cells are EBV nuclear antigen (EBNA) 1 and latent membrane protein (LMP) 2A , neither of which elicits an effective immune response. EBNA1 functions to ensure that the viral genome is propagated to daughter cells upon cell division, whereas LMP2A keeps other viral proteins from being expressed. Limited protein expression helps avert immune destruction *in vivo*.

In vitro where immune surveillance is absent, infected cell cultures tend to express a broader spectrum of EBV proteins, such as LMP1, -2A, and -2B, and EBNA2, -3A, -3B, -3C, and -LP. LMP1 and EBNA2 are critical for the unique ability of EBV to immortalize B cells *in vitro*. In this immortalization process, EBV can be cultured by cocultivating virions with B cells from uninfected persons (usually neonatal umbilical cord lymphocytes). The resulting lymphoblastoid cell lines are capable of being propagated indefinitely in culture media. Naturally infected B lymphocytes can likewise be cultured from the blood of viral carriers. Viral culture represents an accurate and semiquantitative measure of EBV in clinical samples, but it is rarely used in clinical laboratories due to high costs and slow turnaround time.

More practical laboratory tests for EBV rely on detection of viral DNA and its gene products. In EBV-infected tissues, three different patterns of latent viral gene expression are seen. Type I latency refers to a very limited spectrum of latent viral gene expression, namely EBER transcripts along with EBNA1 and LMP2A proteins. This pattern is found in circulating lymphocytes of healthy viral carriers, and it is also characteristic of Burkitt's lymphoma and gastric carcinoma. Type II latency, characterized additionally by LMP1 and LMP2B coexpression, is seen in Hodgkin's disease, T cell lymphoma, and nasopharyngeal carcinoma, all of which tend to occur in immunocompetent hosts. Type III latency refers to the full spectrum of latent viral gene expression, as found transiently in acute infectious mononucleosis, and as seen in EBV-driven lymphoproliferations arising in immunocompromised hosts. Viral genes expressed in Type III latency include all of the EBNAs (1, 2, 3A, 3B, 3C, LP), the LMPs (1, 2A, 2B), and EBER.

Although the patterns of gene expression described above are useful for characterizing various histopathological entities, in practice there is heterogeneity of expression among different tumors of the same histological type, and even among cells within a given tumor. For example, lymphoid tumors arising in AIDS or transplant patients tend to express more viral products than do their histological look-alikes arising in immunocompetent hosts. Therefore, the typical expression patterns described here provide only rough guidelines to assist in the clinicopathological diagnosis of each entity.

EBER in Situ *Hybridization*

EBER *in situ* hybridization is considered the gold standard for detecting and localizing latent EBV in tissue samples.[11] After all, EBER transcripts are consistently expressed in virtually every EBV-infected tumor, and they are likewise expressed in lymphoid tissues taken from patients with infectious mononucleosis, and in the rare infected cell representing normal flora in healthy virus carriers. The only EBV-related lesion that lacks EBER is oral hairy leukoplakia, a purely lytic infection of oral epithelial cells.[12]

EBER actually represents two RNA species, EBER1 and EBER2, encoded from two separate but homologous viral genes. EBER transcripts are expressed in latently infected cells at levels approaching a million copies per cell.[13] Because EBER transcripts are naturally amplified, they represent a reliable target for detecting and localizing EBV in tissue sections by *in situ* hybridization. The literature is replete with EBER hybridization protocols that rely on either oligonucleotide DNA probes, RNA probes (riboprobes), or peptide nucleic acid (PNA) probes.[14–20] Commercially available EBER probes are labeled with biotin, digoxigenin, or fluorescein (Dako, Glostrup, Denmark; Enzo Diagnostics, Farmingdale, NY; Kreatech Diagnostics, Amsterdam, The Netherlands; Novocastra Laboratories Ltd., Newcastle, UK; Shandon Lipshaw, Pittsburgh, PA; Innogenex, San Ramon, CA; Ventana Medical Systems, Tucson, AZ).

EBER *in situ* hybridization can be accomplished on paraffin sections or on cytology preparations. A typical 1-day procedure begins with removal of any paraffin followed by treatment with proteinase K and detergent to enhance probe entry into the nucleus where EBER transcripts are located. Any unbound probe is washed away, and then colorization and counterstaining are performed. Interpretation of EBER stains relies on microscopic visualization of the nuclear EBER signal in latently infected cells. Evaluation of cell type and distribution is helpful in evaluating the clinical significance of the result (Figure 1).

Even though EBER transcripts are usually abundantly produced in latently infected cells, users are cautioned about the possibility of false negative EBER hybridization results as a consequence of RNA degradation. A control hybridization must be run in parallel to ensure that RNA is preserved and available for probe binding. This control might target the polyA mRNA tail using a polyT probe, or it might target a ubiquitous cell-derived transcript such as U6 RNA. U6 RNA is a particularly appropriate control because it is similar to EBER in size, abundance, and intranuclear localization, but it is encoded by a cellular gene that is constitutively transcribed. With such a control, the likelihood of false negative EBER interpretation is markedly diminished. Accurate interpretation of results relies on the ability of the morphologist to distinguish tumor cells from background lymphocytes or artifact. When proper attention is paid to these quality control issues, EBER *in situ* hybridization is the most reliable method for determining if a lesion is EBV-associated.

The primary advantage of EBER *in situ* hybridization is its ability to localize EBV in the context of cytological and histopathological features of the tissue. Enlarged lymph nodes from infectious mononucleosis patients typically contain EBER in a high fraction of lymphoid cells, including small and large lymphocytes and immunoblasts.[21,22] In contrast, lymphoid tissues from remotely infected virus carriers harbor EBV in only rare (<0.1%) scattered small to medium lymphoid cells.[21] In EBV-related Hodgkin's disease, EBER is localized to the malignant Reed-Sternberg/Hodgkin's cells, whereas the background small lymphocytes are almost completely negative (<0.1%). Likewise, the remaining EBV-associated malignancies, including carcinomas, sarcomas, and lymphomas, exhibit EBER signal in virtually all of the tumor cells, whereas EBER is absent from the adjacent normal tissue, except perhaps for rare scattered lymphoid cells. Premalignant lesions of the gastric epithelium and nasopharyngeal epithelium have also been shown to harbor EBV, suggesting the EBV infection occurs early during carcinogenesis.

EBER hybridizations are used diagnostically in several specific clinical situations. They are used routinely for confirming a diagnosis of EBV-driven posttransplant lymphoproliferative disorder (PTLD).[3,23] PTLD is a potentially fatal complication of allogeneic transplantation that requires prompt diagnosis and therapy. About 95% of all PTLDs are EBV-associated, as shown by EBER expression by tissue-infiltrating lymphocytes and/or immunoblasts. Treatment involves cutting back or withdrawing immunosuppressives so that natural immunity is allowed to destroy virally infected tumor cells. In recent years, therapeutic success has been reported following infusion of EBV-specific T cells. The occasional EBV-negative PTLD occurs later (usually >2 years) after transplant and does not respond as well to withdrawal of immunosuppression.[24,25]

PTLD-like tumors occasionally occur in patients who have not undergone transplant but who are immunosuppressed for other reasons, such as rheumatoid arthritis patients on methotrexate therapy.[26] As with PTLD, these tumors are often EBER-positive and respond favorably to immune reconstitution.

In biopsies where the differential diagnosis includes infectious mononucleosis, Hodgkin's disease, and/or non-Hodgkin's lymphoma, EBER hybridization is often helpful in making the correct diagnosis. In EBV-related Hodgkin's disease, EBER is largely restricted to Reed-Sternberg cells and mononuclear variants, whereas infectious mononucleosis is characterized by a mixture of

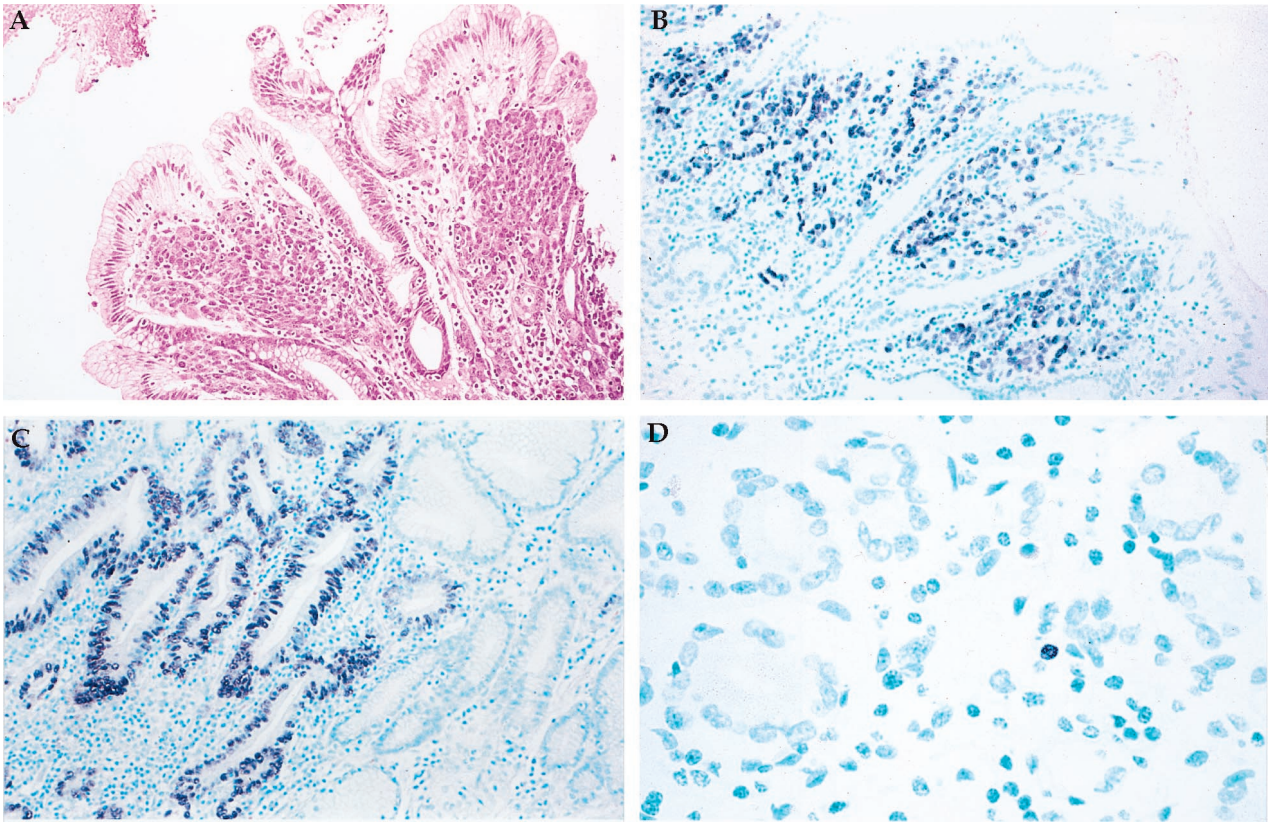

Figure 1. A: H&E stain of invasive gastric adenocarcinoma surrounded by normal surface epithelium. **B:** EBER *in situ* hybridization reveals EBER transcripts in the nucleus of the carcinoma cells, but not in the overlying normal surface epithelium, nor in the surrounding benign stromal cells. **C:** EBER is localized to dysplastic gastric epithelium but not to adjacent normal-appearing glands, implying that EBV infection is an early event in gastric carcinogenesis. **D:** EBER is localized to the nucleus of a single small lymphoid cell, representing the rare infected lymphocyte that might be found in any previously infected individual. Original magnifications, ×50 (**A** and **B**), ×80 (**C**), and ×150 (**D**).

small and large EBER-positive cells including immunoblasts rimming necrotic zones.[22] EBER is not expressed in Kikuchi's lymphadenitis, a lesion that shares some clinical and histological features with infectious mononucleosis.[27]

Nearly half of all classical Hodgkin's disease and T cell lymphomas have EBER-positive tumor cells, whereas only 5% of diffuse large B cell or anaplastic large cell lymphomas express EBER.[4,5,28] Certain subsets of these lymphomas are more likely than others to harbor EBV, such as nasal T/NK lymphoma (Table 2). In classes of tumors that are only fractionally associated with EBV, further investigation of the prognostic value of EBV testing is warranted.

Selected subtypes of carcinoma express EBER, notably nasopharyngeal carcinomas and lymphoepithelioma-like carcinomas of the thymus, thyroid, salivary gland, lung, or stomach.[2,6,29] The majority of nasopharyngeal carcinoma patients initially present with enlarged lymph nodes containing metastatic undifferentiated carcinoma of unknown primary, and EBER expression is touted as an indicator of nasopharyngeal origin.

EBER is usually expressed uniformly in all of the tumor cells comprising an EBV-associated malignancy, although occasional tumors have only focal EBER expression. Lack of uniform expression could be a technical artifact related to focal preservation of RNA, or it could represent true biological variability in EBER levels. John Sixbey and colleagues have proposed a "hit-and-run" hypothesis whereby the virus is lost from some or all cells within a tumor.[30] Further research on this topic is warranted. In the meantime, it is prudent to interpret focal EBER hybridization results in conjunction with control assays for RNA preservation and in conjunction with other tests for EBV.

There is intriguing geographic variability in the incidence of EBV-related tumors. For example, Burkitt's lymphoma is the most common pediatric cancer in tropical Africa, where it is almost always EBV-related, whereas it is 50-fold less common in the United States and only 20% EBV-related. As another example, EBV-related nasopharyngeal carcinoma is the most common cancer in parts of Southern Asia, where it is nearly always EBV-related, whereas the tumor is 50-fold less common in the United States and only 75% EBV-related. There appears to be an inverse correlation between the incidence of gastric cancer and its EBV relatedness, unlike what is observed with nasopharyngeal carcinoma. These striking geographic variations have yet to be fully explained, but preliminary studies implicate environmental cofactors over genetic predisposition or oncogenic viral strains.

Once identified in a patient's tumor, EBER can be used as a marker of recurrent disease. For example, when looking for recurrence in patients treated for nasopharyn-

Table 2. EBV-Associated Diseases

Disease	Proportion of cases EBV-related	Reference
Benign, reactive infections		
Infectious mononucleosis	>99	22
Oral hairy leukoplakia	>95	8
Inflammatory pseudotumor	40	60
Non-Hodgkin's lymphomas and immunodeficiency-related neoplasms		
Non-Hodgkin's lymphoma, all subtypes	5	5
Non-Hodgkin's lymphoma, AIDS-related	40	61
Brain lymphoma, AIDS-related	95	62
Brain lymphoma, immunocompetent hosts	5	63
Post transplant lymphoproliferative disorder (PTLD)	95	3
Burkitt's lymphoma, African	>95	64
Burkitt's lymphoma, North American	20	64
Burkitt's lymphoma, AIDS-related	30	65
Lymphoma, primary immunodeficiency	most	66
Lymphomatoid granulomatosis (B cell)	most	67
Peripheral T cell lymphoma	40	28
Nasal T/NK cell lymphoma	>95	68
Smooth muscle tumors in AIDS or transplant patients	>95	69
Hodgkin's disease		
Hodgkin's disease, all subtypes	40	4
Hodgkin's disease, mixed cellularity	70	4
Hodgkin's disease, nodular sclerosis	20	4
Hodgkin's disease, lymphocyte predominant	<5%	70
Hodgkin's disease, lymphocyte depleted	50	4
Hodgkin's disease, AIDS-related	>95	71
Carcinomas		
Nasopharyngeal carcinoma, Asian	>95	2
Nasopharyngeal carcinoma, North American	75	29
Lymphoepithelioma-like carcinoma, foregut derived	most	6
Gastric adenocarcinoma	7	7

geal carcinoma, EBER hybridizations can be used to complement microscopic examination of nasopharyngeal biopsies. As described below, blood tests for EBV viral load are also useful markers of tumor burden after therapy.

In Situ *Hybridization to EBV DNA*

Probes targeting the *Bam*HIW internal repeat sequence, which is reiterated up to 11 times in each EBV genome, can be used to detect and localize EBV DNA in tissue sections.[16] Single-copy viral sequences could also be targeted, but assay sensitivity is relatively diminished. In a practical sense, there is little reason to target EBV DNA rather than EBER RNA, except perhaps in samples where the RNA has been selectively destroyed. In clinical situations, EBER transcripts remain the more common target for *in situ* detection of EBV.

LMP1 Immunohistochemistry

The relative merits of immunohistochemistry *versus* EBER *in situ* hybridization deserve attention. In fact, LMP1 immunostains are nearly as effective as EBER *in situ* hybridization for identifying EBV in PTLD cases, in Hodgkin's disease, and in infectious mononucleosis.[31] Such is not the case for non-Hodgkin's lymphomas or carcinomas, however, in which LMP1 is often undetectable even when EBER is clearly positive.

Some important differences are seen in the distribution of EBER *versus* LMP1 expression in tumor samples. In PTLD samples, LMP1 is typically expressed in about 5% of lesional immunoblasts (range, 0–100%). When the same PTLD samples are stained for EBER, it becomes apparent that many more lymphoid cells are EBV-infected, but only a fraction of those cells coexpress LMP1. Immunoblasts are often the subtype of lymphocyte that coexpress LMP1, whereas small lymphocytes are more likely to express EBER alone. Occasional PTLDs lack LMP1 entirely, even though EBER is clearly positive, implying that EBER is a more reliable target than is LMP1. Nevertheless, LMP1 immunostains are economical and rapid; therefore, they retain a role in clinical evaluation of suspected PTLD cases.

LMP1 stains reliably identify EBV in Reed-Sternberg/Hodgkin's cells, although sometimes only a fraction of the EBER-positive tumor cells coexpress LMP1. LMP1 is reliably expressed in lymph nodes from infectious mononucleosis patients, with some EBER-positive small lymphocytes failing to coexpress LMP1, but immunoblasts coexpressing both markers. Therefore, EBER and LMP1 stains appear to be equally informative in confirming a diagnosis of infectious mononucleosis.[22,32]

LMP1 immunostains can be performed on paraffin sections using commercially available antibodies (CS1–4 monoclonal cocktail, Dako, or S12 monoclonal, Organon-Teknika, Boxtel, The Netherlands).[33] True LMP1 signal is granular in character and is localized to the cytoplasm

and surface membrane. Results should be interpreted by a morphologist who is confident in discerning tumor cells from other cells in which a false positive signal has been described, namely eosinophils, plasma cells, cells of the nervous system, and poorly fixed cells.

Measuring EBV Gene Expression by Immunohistochemistry, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), and Nucleic Acid Sequence-Based Amplification (NASBA)

Detection of viral proteins can be achieved by immunohistochemical stains of paraffin sections. Common targets include EBNA1, EBNA2, LMP2A, and BZLF1.[34–36] Of these, BZLF1, also called ZEBRA, is the only factor that is characteristic of lytic viral replication. In fact, BZLF1 immunostains are quite useful in confirming a diagnosis of oral hairy leukoplakia in tongue biopsies from AIDS patients using commercially available antibody (clone BZ.1, Dako, Carpinteria, CA). Interpreting pathologists are cautioned that BZLF1 staining is localized to the nucleus of ballooned epithelial cells of oral hairy leukoplakia, whereas cytoplasmic cross-reactivity of the antibody should be disregarded.

Alternative approaches to detecting these viral gene products are RT-PCR and NASBA.[37] Though not yet used routinely in clinical laboratories, there is much to recommend them as disease-specific markers, especially if they can be applied in multiplex or array format. In theory, this should facilitate diagnosis of each class of EBV-associated disease based on the unique expression profile of viral and cellular genes that characterizes each disease.

Progress continues to be made in profiling the expression pattern of each EBV-associated disease. For example, a recent study used NASBA to identify EBV BARF1 transcripts in gastric carcinomas.[38] BARF1 is likewise expressed in nasopharyngeal carcinomas but apparently not in lymphocytes, implying that BARF1 might serve as a marker of these epithelial malignancies without concern for interference from the occasional bystander lymphocyte that might be infected.[39] If validation studies pan out, it is feasible that quantitative measurement of this and other viral transcripts will prove useful for diagnosis and monitoring of affected patients.

Southern Blot Analysis of EBV DNA

Southern blot analysis can be used to determine the clonality of EBV-infected tissues with respect to the structure of EBV DNA. This assay, first described by Raab-Traub and Flynn in 1986,[40] is based on the presence of variable numbers of terminal repeat sequences at the ends of each EBV DNA molecule. A given cell is apparently infected only once, and each infecting genome contains up to 20 terminal repeat sequences. The relatively unique terminal repeat structure that is present in a given cell is passed along to cellular progeny upon cell division. Analysis of clinical samples has provided interesting results. Oral hairy leukoplakia, representing an infectious process, produces polyclonal viral genomes indicative of lytic viral replication. On the other hand, EBV-associated tumors harbor monoclonal EBV DNA.

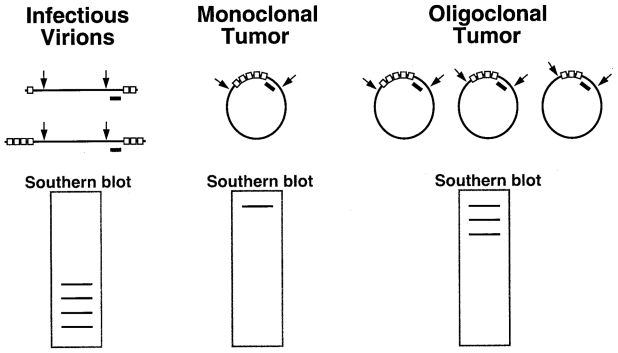

Figure 2. The EBV clonality assay evaluates clonality with respect to the structure of the EBV genome. The assay is based on the presence of variable numbers of tandem repeat sequences (shown as **open boxes**) at the ends of the linear viral genome. On infection of a cell, these ends join to form an episome by fusing up to 20 terminal repeat sequences. When an infected cell undergoes malignant transformation, the same fused terminal repeat structure is inherited by all progeny of the malignant clone. The clonality assay is accomplished by Southern blot analysis of DNA extracted from patient tissue and digested with *Bam*HI restriction endonuclease (shown by **arrows**) to cut the EBV genome at sites flanking the terminal repeats. This results in restriction fragments that are recognized by a DNA probe (**black bar**). Examination of the band pattern on Southern blots reveals that infectious virions produce a ladder array of small bands. In contrast, a monoclonal tumor exhibits a single band of high molecular weight, and an oligoclonal tumor has several such bands.

To perform the EBV clonality assay, lesional DNA is first subjected to digestion by *Bam*HI restriction enzyme, which cuts at sequences flanking the region where the terminal repeats are located. After electrophoresis and transfer, a labeled internal probe is applied to detect the fragment(s) containing the terminal repeats.[14] Analysis of the band pattern distinguishes monoclonal from oligoclonal, polyclonal, and uninfected tumors, and also reveals whether the sample contains substantial amounts of linear EBV genomes as a consequence of active viral replication (Figure 2).

Application of this clonality assay reveals monoclonal EBV DNA in nearly all infected carcinomas, sarcomas, and Hodgkin's and non-Hodgkin lymphomas.[40–43] A subset of immunocompromised patients have either oligoclonal or polyclonal lymphoid proliferations, and these patients apparently have a better prognosis.[3,44] Even so, monoclonal tumors may respond to immune reconstitution, leading many clinicians to treat their patients the same regardless of clonality status.

Amplification of EBV DNA

Amplification methods have been used by many clinical laboratories for detecting EBV in blood, body fluid, or tissue samples. For example, detection of EBV in biopsies of metastatic undifferentiated carcinoma of unknown primary narrows the differential diagnosis and focuses attention on the nasopharynx. As another example, a study of HIV-infected patients with persistent generalized lymphadenopathy showed that amplifiable EBV DNA was

associated with a heightened risk of developing lymphoma.[45] Most remarkably, amplification of EBV DNA from the cerebrospinal fluid of AIDS patients is nearly always indicative of a brain lymphoma, leading oncologists to proceed with lymphoma treatment without the need for brain biopsy (assuming an appropriate clinical setting and radiographic support for the diagnosis).[46] After treatment, disappearance of EBV DNA from the cerebrospinal fluid is associated with better outcomes.[47]

From a technical standpoint, PCR amplification of EBV DNA is accomplished using primers spanning conserved EBV sequences, whereas strain typing relies on amplification of polymorphic regions of the viral genome. Strain typing will not be discussed in any detail, since there are no solid clinical indications for such testing. Even qualitative amplification assays are difficult to justify because of their inability to distinguish lesion-specific EBV from that representing normal flora. After all, EBV DNA is present in a small fraction of lymphoid cells from every healthy virus carriers, which means that nearly every adult and a substantial fraction of all children harbor amplifiable EBV DNA. The inability to distinguish EBV disease from background infection led many laboratory scientists to abandon PCR in favor of EBER *in situ* hybridization for the reliable detection of lesion-associated EBV in biopsy specimens. Indeed, EBER studies remain a mainstay of diagnostic surgical pathology. But improvements in quantitative amplification technology are stimulating a resurgence of interest in amplification strategies for detecting EBV in patient samples.

EBV Viral Load Measurement by Quantitative DNA Amplification

EBV viral load testing involves quantitative measurement of EBV DNA in patient samples. A typical viral load assay employs PCR to coamplify EBV DNA and a spiked control sequence in nucleic acid extracted from blood samples.[14] The amount of amplification product, measured either at the end point of the assay or in real time, can be used to calculate the EBV viral load in copies per milliliter of blood.

The EBV viral load assay has several technical and clinical advantages over other methods of viral detection. First, the test is rapid, with with a turnaround time of only 1 to 2 days. Second, it appears that patients with several subsets of EBV-related diseases are massively and systemically infected by EBV, allowing us to screen for these diseases by viral load assays of blood or body fluid, potentially alleviating the need for invasive tissue biopsy. And finally, recent clinical studies reveal that several EBV-related diseases can be monitored by sequential measurement of EBV viral load.

EBV viral load testing appears to be more reliable than serology for evaluating the EBV status of immunocompromised hosts. In fact, recent studies of transplant patients showed that those affected by EBV-driven PTLD have extremely high EBV viral loads, sometimes exceeding 1 million copies per milliliter of blood.[48,49] Furthermore, viral load rises as early as several months before the clinical onset of PTLD, suggesting that the assay might be used to screen high risk populations for purposes of early intervention.[50–53] And finally, EBV viral load decreases on successful therapy, suggesting that the assay should be used to monitor therapeutic efficacy.[52,54]

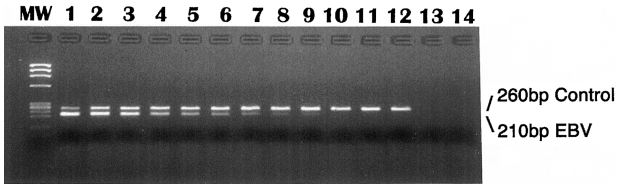

Figure 3. The EBV viral load assay is accomplished by coamplification of EBV DNA and a control sequence that is spiked into the sample before DNA extraction. In the experiment shown here, assay linearity was tested on serial twofold dilutions of EBV DNA. PCR products at the endpoint of amplification were evaluated by agarose gel electrophoresis. In **lanes 1–8**, the EBV product is seen as a 210-bp band at template levels as low as 5 copies. The control product, visible at 260 bp, ensures that no inhibitors are present, and it also serves as a gauge by which to extrapolate the amount of EBV template in each sample. A molecular weight (MW) marker is shown on the left, and **lanes 13** and **14** represent control reactions to which no template was added.

From a technical standpoint, EBV viral load assays have been shown to be sensitive, specific, and quantitative across a wide dynamic range. A commercial kit (BioSource International, Camarillo, CA) is available to facilitate PCR amplification of EBV EBER genomic sequences. After coamplification of EBV and a spiked competitor using biotinylated primers, products are detected in an automated enzyme-linked immunosorbent assay plate system. Comparison between the amount of EBV product and the amount of control product permits calculation of EBV viral load in the patient specimen (Figure 3).[14]

An alternative procedure for EBV viral load measurement involves real-time measurement of PCR products, a procedure that has the potential to reduce labor costs, diminish the risk of amplicon contamination, and reduce turnaround time.[49,51,55] Additional laboratory strategies will undoubtedly be developed as molecular technology continues to advance.

In nasopharyngeal carcinoma patients, EBV viral load shows promise as a marker of tumor burden that will facilitate monitoring of patients after therapy.[56] Because about half of all affected patients are destined to relapse, further investigation of the impact of EBV viral load assays is important to distinguish those patients in long-term remission from those destined to relapse.

In patients with EBV-related Hodgkin's disease, a recent study suggests that EBV viral load might likewise serve as a marker of tumor burden.[57] More research is needed on this and other EBV-related diseases to define more fully the clinical utility of EBV viral load assays.

EBV Serology

No article about laboratory testing for EBV would be complete without a discussion of serological testing, which is the gold standard for confirming acute *versus* remote EBV infection in immunocompetent hosts. The heterophile test (also known by a commercial trade name, the Monospot test) was introduced in 1932 as a

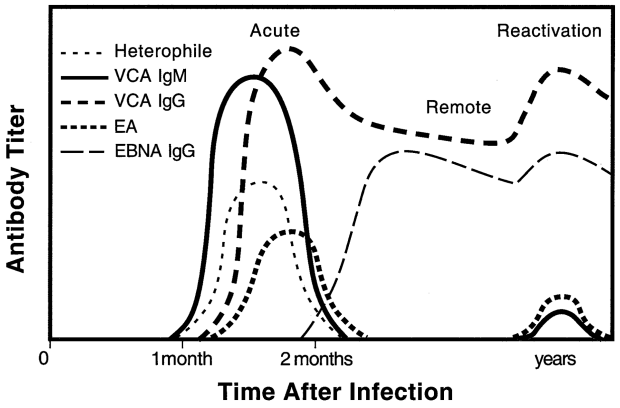

Figure 4. Primary EBV infection in healthy hosts is accompanied by an orchestrated serological response. IgM antibody against viral capsid antigen (VCA) rises first. Antibodies against EBNA appear at least 1 month after primary infection and are measured, along with IgG anti-VCA, as markers of prior infection and as indicators of EBV reactivation. Titers against early antigen (EA) rise on primary infection and again in pathological states of EBV reactivation.

marker for infectious mononucleosis, even though it would be several decades before EBV was discovered as the causative agent. Heterophile tests are still used today, often in the form of a 2-minute horse red cell agglutination test (Seradyn Color Slide II, Seradyn, Indianapolis, IN). EBV-specific serological assays by enzyme-linked immunosorbent assay or by immunofluorescent assay are used for more accurate confirmation of acute or convalescent EBV infection.[9] Figure 4 displays a typical serological response to EBV infection.

EBV-associated tumors are often characterized by abnormally high titers against early antigen and IgG viral capsid antigen with diminished EBNA titers. However, this pattern is not specific for malignancy and can be seen in patients with autoimmune diseases or other immune dysfunction, implying that serology alone is inadequate for diagnosis of EBV-related malignancy.

Nasopharyngeal carcinoma patients usually have elevated titers against multiple viral antigens, particularly IgA antibodies against lytic antigens, reflecting the tumor's origin in the mucosa of the nasopharynx.[58] In fact, a panel of serological tests is used fairly successfully to screen for nasopharyngeal carcinoma in high risk populations, to assign prognosis in those patients who are affected, and to detect early relapse after therapy.[59] Analogous studies are underway in gastric carcinoma patients who likewise harbor high serological titers against EBV.[7]

Immunosuppressed patients have inconsistent humoral responses against EBV; therefore, serology is not as reliable a marker of clinical status. In these patients, direct detection of viral nucleic acid or protein is more reliable for identifying clinically relevant EBV infection.

Summary

Molecular diagnostics is increasingly important for diagnosis and monitoring of patients affected by EBV-related diseases. These diseases represent a wide spectrum of clinical manifestations, from transient benign infection to aggressive malignancies. As virus-specific treatments continue to be investigated, it becomes even more important to recognize these EBV-associated diseases so that proper clinical management decisions can be made.

New molecular tests combined with traditional serological or histochemical assays are helpful for diagnosis and monitoring of EBV-related diseases, depending on the clinical setting and the types of samples available for testing. EBER *in situ* hybridization on biopsy samples and, more recently, EBV viral load testing of blood samples provide an accurate measure of clinical status in PTLD patients. Investigations are underway to better define the utility of these assays across the full spectrum of EBV-associated diseases. On the horizon are gene expression profiling and array technology, which likely will improve our ability to subclassify these diseases and predict responses to therapy.

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