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MINIREVIEW

Routine Epstein-Barr Virus Diagnostics from the Laboratory Perspective: Still Challenging after 35 Years

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In 1968 Epstein-Barr virus (EBV; now human herpesvirus 4) was found to be the major cause of infectious mononucleosis (IM), a usually self-limited clinical syndrome (10). Only about 5% of adults in Western societies remain EBV uninfected; thus, antibody prevalence rates reach 95% or higher among elderly individuals (29). Although it is believed that routine laboratory diagnosis of primary EBV infection is straightforward, this minireview focuses on practice guidelines for a rational approach to the diagnosis of EBV-associated IM in immunocompetent individuals on a serological basis.

CLINICAL SYMPTOMS IN IMMUNOCOMPETENT INDIVIDUALS

Clinical symptoms and diagnostic approaches differ according to the immune status of the patients. In immunocompetent individuals primary infection with EBV is most often frequently asymptomatic (10). IM may present as a mild infectious illness of young children, but in young adults primary EBV infection can cause a type of IM known as the Pfeiffer's Drüsenfieber (glandular fever) or kissing disease (20). A variety of symptoms, such as upper respiratory tract infection, otitis media, abdominal complaints, hepatitis, enlargement of the cervical lymph nodes, tonsillitis and/or pharyngitis, and moderate to high fever are observed (31). In most cases primary EBV infection is accompanied by increases in liver enzyme levels and lymphocytosis, largely composed of atypical lymphocytes (mononuclear cells) in the peripheral blood (16). The clinical signs of IM are provoked by the massive immune response involving cytotoxic T lymphocytes directed against EBV-infected B cells (29). Mononucleosis may be caused by a variety of other pathogens, such as cytomegalovirus, human herpesvirus 6, adenovirus, rubella virus, mumps virus, human immunodeficiency virus, hepatitis A virus, influenza A and B viruses, and *Toxoplasma gondii* (11, 20). Patients with lymphoma and leukemia may present with symptoms like those of IM (20, 31). Treatment of EBV-associated mononucleosis is limited to the management of symptoms (2). EBV reactivation is not correlated to any disease so far in immunocompetent individuals, though it is a common phenomenon in such persons. Formerly, EBV was suspected to be the major cause of chronic fatigue syndrome on the basis of a "highly unusual serologic profile" (29). However, no authentic link of chronic fatigue syndrome to EBV infection has been shown (29) and additional cofactors and potential causes are being discussed (36). Therefore, the diagnosis of primary or acute infection is relevant.

CLINICAL SYMPTOMS IN IMMUNOCOMPROMISED INDIVIDUALS

In immunocompromised individuals EBV is associated with disorders with high rates of morbidity and mortality. The spectrum ranges from benign B-cell hyperplasia resembling IM to more classic malignant lymphomas (20, 21). Allograft organ transplant recipients, especially children with pretransplantation EBV seronegativity, are at particular risk for the development of posttransplantation lymphoproliferative disease (PTLD) during immunosuppressive therapy (19, 20). Anti-CD20 antibody (Rituximab) treatment (12) and a wide range of other therapeutic interventions are available for these disorders (24). However, the characteristic that these therapeutic interventions have in common is that they must be applied very early in the course of disease to be effective; thus, early diagnosis is a prerequisite.

In immunocompetent individuals EBV infection is controlled by the humoral and cellular immune responses, in cooperation with the interferon system. However, in patients with mononucleosis, cytotoxic T cells dominate over B cells. In contrast, under immunosuppression B cells dominate over T cells (29).

EBV LIFE CYCLE

Knowledge of the EBV life cycle is important to better understand clinical symptoms and EBV diagnostics. The 186-kb double-stranded DNA EBV genome codes for a number of structural and nonstructural genes. The port of entry for EBV is also the port of exit, i.e., the oropharynx. After entry, EBV replicates in epithelial cells and B cells in the oropharynx and spreads through the body via infected B cells, while latent genes that either drive B cells to EBV lytic cycle entry or acquire the status of latency are differentially expressed (5, 24). Like other herpesviruses, EBV follows a productive lytic infection and establishes latent infection in the host (29). The latent infection is established by self-replicating extrachromosomal nucleic acid, the episomes (25). Three different latencies are now known and are referred to as latencies I, II, and III, due to the differential expressions of certain subsets of EBV-specific genes (for a review, see reference 29). Under circum-

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TABLE 1. Diagnostic methods for EBV detection

Method	Analyte, antigen, or substrate	Comment
Serology		
IFA	Cell lines like P3HR-1 and Raji	Classical method; gold standard; highly specific; staging of EBV infections possible with a single serum sample
Complement fixation reaction	Lysate of EBV-transformed cell lines	Less sensitive, less specific; not widely used; staging of EBV infections not possible with a single serum sample
EIA, ELISA, or chemoluminescence with coated beads	Lysate of EBV-transformed cell lines; EBV lysates; combination of lysates and recombinant proteins; recombinant proteins; synthetic peptides	Rapid, highly sensitive, suitable for automation; synthetic peptides as antigens less sensitive and less specific (due to cryptic epitopes in native molecules); with a single serum sample
Blot techniques (Western blot analysis or line blot assays)	Lysate of EBV-transformed cell lines; EBV lysates; recombinant proteins; combination of lysates and recombinant proteins	Highly specific; mostly a confirmatory method; staging of EBV infections possible with a single serum sample
IgG avidity determination, IFA, and/or ELISA or Western blot analysis	Titration of antibodies in the absence and presence of increasing amounts of urea or other chaotropic reagents	Rather special method used for confirmation of indeterminate results (Table 2) (antibodies in an acute EBV infecti on are of low avidity)
Heterophile antibody agglutination	Paul-Bunnell antigens; bovine erythrocytes	Less sensitive, less specific; 10–50% of children <4 years of age do not produce heterophile antibodies
Virus isolation	Lymphoblastoid cell lines from patient lymphocytes	Performed only in special laboratories; long-lasting test (up to 4–8 weeks)
Nucleic acid detection		
PCR	Lymphocytes, plasma, serum, cerebrospinal fluid, tissue	Method of choice if EBV-associated meningoencepahlitis (from cerebrospinal fluid) is suspected; used to detect virus load and reactivation
In situ hybridization, in situ PCR	Tumor tissue; paraffin-embedded sections	Used to detect EBV-associated tumors
Virus antigens, immunhistochemistry and immunocytology	Tumor tissue; paraffin-embedded sections	Used to detect EBV-associated tumors

stances such as B-cell homing by an antigen-driven trigger and other so far unknown triggers, EBV sporadically reactivates from latency (5, 24). The virus is intermittently shed from saliva; thus, the main route of transmission is directly from person to person; however, transmission via blood products, transplantation, and sexual transmission were shown (7, 31). Interestingly, Walling et al. (34) found that healthy individuals may be infected with multiple different EBV genotypes, in which the sequences of the open reading frames encoding EBV nuclear antigens (EBNAs) differ among the different genotypes (20). Taken together, the prevention of virus spread seems impossible.

EBV transforms B cells in vivo (in the body) and in vitro (in cell culture), thus immortalizing B cells (29). In vitro permanently growing EBV-transformed B cells provided the prerequisite for the first EBV-specific assay, the immunofluorescence assay (18).

DIAGNOSTIC STRATEGY

The spectrum of antibody assays comprises unspecific tests, such as the long-known test for the detection of heterophile antibodies, as well as EBV-specific assays that use different methods with different substrates, different antigens, and different interpretation criteria, in addition to molecular and immunohistochemical methods, which are summarized in Table 1. The diagnostic strategies differ between immunocompromised and immunocompetent individuals due to the distinct therapeutic interventions required. Because the time of intervention is a critical factor in immunocompromised patients, a diagnostic method must meet the following criteria: early detection of EBV replication and a high positive predictive value for the respective disease, thus enabling preemptive therapy. In addition, monitoring of therapy should be possible. Thus, direct detection methods mainly meet this profile (15). In contrast, in immunocompetent individuals the key issue of EBV diagnostics is the detection or exclusion of a primary, a past, or no EBV infection (14). Therefore, serology provides rational criteria for interpretation of the results (Table 2), although EBV serology presents a high degree of variability (3, 4). However, serological assays are preferred. Although the EBV genome encodes a number of different structural and nonstructural genes, those of most importance for serodiagnosis are the genes encoding the viral capsid antigens (VCAs), the early antigens (EAs), and the EBNAs EBNA-1 and EBNA-2 (two of the six EBNAs, now denoted EBNA-1, -2, -3A, -3B, -3C, and -LP) (24, 33). Only three serological parameters are essential for the detection of EBV-specific antibodies in immunocompetent individuals on a qualitative basis, i.e., VCA immunoglobulin G (IgG), VCA IgM, and EBNA-1 IgG (14). Serology builds upon detection of EBV-specific antibod-

TABLE 2. State-of-the-art interpretation of EBV-specific serological profiles for diagnoses

Heterophile antibodies	Atypical lymphocytes	VCA IgG	VCA IgM	EBNA-1 IgG	Interpretation
±	±	+	+	−	Acute infection
−	−	+	−	+	Past infection
−	−	−	−	−	No infection
±	±	+	−	−	Indeterminate[a]
−	−	+	+	+	Indeterminate[a]
−	−	−	+	−	Indeterminate[a]
−	−	−	−	+	Not plausible

[a] Further testing needed, such as avidity testing of VCA IgG, Western blot analysis, or PCR.

ies, which is preferably done with a single acute-phase serum sample, and allows stage-specific diagnosis (Table 2). Specific tests with VCAs and EBNA-1 are used for the detection of IgG and IgM antibodies, respectively (14). VCAs cause lifelong persistent IgG titers, while antibodies of the IgM type are produced only transiently but are not necessarily produced in all patients with primary infections (Table 3) (20, 30). Early in the course of clinical presentation, VCA IgM antibodies may not necessarily be present before VCA IgG presentation, and antibodies of both classes may appear simultaneously (3, 4).

EBNA-1 IgG antibodies, in contrast, are produced late in the course of infection, while EBNA-2 IgG antibodies appear earlier and may be present in up to 30% of individuals at the time of onset of the disease (4, 20). EBNA-1 IgG antibodies basically persist lifelong. However, not all individuals produce EBNA-1 IgG antibodies, although most individuals do, and EBNA-1 IgG antibodies may secondarily be lost under circumstances such as immunosuppression and thus do not persist lifelong (3, 4). Therefore, the presence of EBNA-1 IgG antibodies and not EBNA-2 IgG antibodies definitely excludes a primary infection. Although some investigators use the anti-EBNA-1 antibody versus anti-EBNA-2 antibody ratio for the serodiagnosis of EBV reactivation (37), that type of analysis may be restricted to specialized laboratories, because no tests for the detection of anti-EBNA-2 antibodies are commercially available. EAs are usually expressed during the early phase of lytic replication. Anti-EA antibodies of the IgG and IgA types are detectable in a number of individuals early after primary infection and individuals with past infections (4, 38). Table 3 illustrates the estimated rates of seroprevalence of diagnostically relevant analytes (4, 6, 11, 20, 21, 30, 35; unpublished observations) in single acute-phase serum samples from individuals with primary EBV infections. These prevalence rates may vary to some extent, depending on the respective methods used for their determination or even if the same method (e.g., VCA IgM enzyme-linked immunosorbent assays [ELISAs] from different manufacturers) is used (35). VCA IgG and VCA IgM antibodies in the absence of EBNA-1 IgG antibodies are typically found in patients with primary infections. In contrast, past infections are typically characterized by the presence of VCA IgG and EBNA-1 IgG antibodies in the absence of VCA IgM antibodies. However, serology is complicated by the fact that some individuals do not produce VCA IgM antibodies during primary infection and the fact that some individuals lack EBNA-1 IgG antibodies (either the individuals are EBNA-1 nonresponders or the individuals may have lost the anti-EBNA-1 antibodies under circumstances such as immunosuppression) even some months and sometimes years after the primary infection (3, 4). Moreover, in rare cases VCA IgM antibodies persist longer even during the period when EBNA-1 IgG antibodies are already produced (4, 21). Therefore, a patient with a primary infection may exhibit the same serological profile as a patient with a past infection, and vice versa. In these cases further diagnostic approaches are required, as discussed below. Other parameters such as the presence of EA IgG antibodies are dispensable for the key concern in the diagnosis of EBV infection, since the correlation of EA IgG antibodies with primary infections is very low and antibodies are also found in blood donors (4, 14, 20, 38). Transient immunosuppression of immunocompetent individuals may lead to EBV reactivation, whose detection requires molecular diagnostic methods such as PCR (15). Until now in many countries worldwide, reimbursement codes have mostly favored the use of quantitative EA serology for the diagnosis of EBV reactivation. EA antibodies are also detectable in clinically healthy individuals, however (20). Therefore, EA-specific serological parameters do not confirm any stage-specific diagnosis.

TABLE 3. Estimated antibody prevalence rates early after clinical presentation in a single acute-phase serum sample from immunocompetent individuals with primary EBV infections[a]

Antigen(s)	Antibody class	Prevalence rate (%)	Method[c]
Heterophile antibodies	IgM	50–85[b]	Agglutination, rapid tests
Viral capsid antigen	IgG	98–100	IFA, EIA, WB[d]
Viral capsid antigen	IgM	70–100	IFA, EIA, WB
Nuclear antigen 1	IgG	0	IFA, EIA, WB
Early antigen	IgG	60–80	IFA, EIA, WB

[a] Data are according to references 4, 6, 11, 20, 21, 30, and 35 and unpublished observations.
[b] Depending on the age of the patient.
[c] Prevalence rates strongly depend on the method (IFA, EIA, or Western blot analysis) and on the different commercially available tests (manufacturer) used for determination.
[d] WB, western blot analysis.

THE SPECTRUM OF SEROLOGICAL METHODS

Heterophile testing. Thirty years ago, Paul and Bunnell (26) were the first to identify that heterophilic antibodies of the IgM type are associated with IM. These antibodies are cross-species reactive and are not EBV specific. They typically result from polyclonal stimulation but are not exclusively found in patients with mononucleosis. They may coincide transiently with the time course for EBV-specific IgM antibodies (16). Heterophile antibodies can also be detected in patients with diseases other than IM, and the test results can remain positive for up to 6 to 12 months (33). Commercially available agglutination test kits for the detection of heterophile antibodies use goat, horse, or bull red blood cells after preabsorption with guinea pig kidney extracts and are effective with acute-phase serum for 85 to 90% of adolescent or adult patients but are only 50% effective for children ages 2 to 5 years (20, 21). Thus, fairly high rates of false-negative results may be expected, while false-positive results were found in 2 to 3% of patients with autoimmune diseases (20). Today, EBV-specific serology

is preferred for the diagnosis of an EBV infection, but in the absence of heterophile antibodies (20, 33).

EBV-specific serological methods. Unfortunately, EBV-specific diagnostic tests are not standardized. The EBV-specific assays differ in the substrates or antigens and the technologies that they use. Even the interpretation of the results differs greatly among the various manufacturers of the commercially available tests (as discussed below). So far, three methods serve as the method of first choice in routine EBV diagnostics: the IFA, which is still the "gold standard" method; different enzyme immunoassay (EIA) techniques, including solid-phase ELISAs and related methods, such as luminescence-based detection of anti-EBV antibodies with antigen-coated beads; and Western blot analysis. While IFA or EIA is often used for screening, Western blot analysis is mainly performed for confirmation. Today, a number of manufacturers provide commercially available EBV-specific IFAs and EIAs, such as tests that use VCA for the detection of IgG and IgM antibodies, EBNA-1 for the detection of IgG antibodies, and EA for the detection of IgG and IgA antibodies.

IFA. IFA is generally performed with human EBV-transformed B-cell lines derived from Burkitt's lymphoma patients, such as the P3HR-1 cell line (ATCC HTB-62, derived from subclone Jiyoye [ATCC CCL-87]) or the Raji cell line (ATCC CCL-86), which served as the first substrates for IFA (3, 18, 20, 28). While P3HR-1 cells express EBNA-1, approximately 5 to 20% of the cells additionally express VCA in the nucleus. The EBV-specific protein pattern of the Raji cell line is restricted to EBNAs, especially EBNA-1 and EBNA-2 expression in the nucleus. The Raji cell line does not produce VCA. Iododesoxyuridine treatment of Raji cells induces EA expression to a small extent. In order to test for non-EBV-specific cellular cross-reactivity, a third cell line, BJAB (ATCC HB-136), an EBV-negative continuously growing human lymphoblastoid cell line, can be used as an option. An amplified method, that is, the anticomplement immunofluorescence procedure, is required to detect anti-EBNA-1 antibodies by IFA. By this procedure EBV-specific antibodies bind to P3HR-1 cells and complement-fixing antibodies against EBNA-1 are stained by adding complement and, subsequently, anticomplement fluorescein conjugate. Thus, the complement-fixing reactions of antibodies are based on the detection of the EBV-specific immunoglobulin class and subclass antibodies (the IgM subclass and some IgG subclasses) to EBNA-1. As an alternative, stably EBNA-1-transfected cell lines can be used as substrates for regular indirect immunofluorescence techniques (14). Lennette (20) describes helpful details on the IFA technique.

EIA. VCA antigens are serologically defined antigens, because traditionally, the first EBV-specific assays are IFAs. Therefore, most EIAs manufactured are referenced against IFAs. As a consequence, for the detection of antibodies to VCA, various antigens are commonly used with EIA for binding to the solid phase. Either native purified or recombinant proteins, fusion proteins, or synthetic peptides that represent either the full-length VCA-encoded gene or only fragments of the VCA-encoded gene are used (14, 33). The same holds true for EBNA-1. However, while most manufacturers today use recombinant full-length EBNA-1 proteins, only one manufacturer uses synthetic peptides and one manufacturer uses the full-length EBNA-1 protein sequence devoid of the amino-terminal glycine-alanine stretch without the glycine-alanine copolymer (14, 33). Using the amino-terminal glycine-alanine stretch of the EBNA-1 protein, Linde et al. (22) were able to detect antibodies as early as 7 days after primary infection, while traditional IFA may show EBNA-1 IgG antibodies 4 to 6 weeks after clinical presentation (20, 21). Manufacturers of the EBNA-1 EIAs try to adjust the cutoff according to the IFA cutoff (and, thus, modulate sensitivity), since the IFA has always been used as the reference method. The EBNA-1 EIA principally could be manufactured to be more sensitive than IFA (in terms of the earlier detection of anti-EBNA-1 antibodies), while the sensitivities of the VCA EIAs (for IgG and IgM) may either reach or exceed those of IFAs. Only one manufacturer uses a controlled mixture of EBV-specific antigens simultaneously (i.e., EA, VCA, and EBNA) in an EIA to screen for either IgG or IgM antibodies to EBV proteins. This assay may be useful for determination of the general seroprevalence of EBV (and, e.g., detection of EBV-specific antibodies in cerebrospinal fluid), but the valuable use of the assay for stage-specific diagnosis is lost (8, 22). In addition, this assay proved to detect primary infections with an acceptable sensitivity when sera with immunological interference were used, such as sera from patients with rheumatoid disorders or cytomegalovirus cross-reactive sera, but its specificity is problematic (B. Gärtner, unpublished results).

Western blot analysis. Different Western blot techniques have been established as methods that can be used to confirm the results of screening tests (32). Examples of these include classical lysate blots assays (with EBV-transformed cells) and line blot assays with recombinant antigens, such as p72 (EBNA-1), p18 (VCA), p23 (VCA), p54 (EA), and p138 (EA). VCA antigen p18 is considered a marker that substitutes for the lack of EBNA-1 IgG, since p18 IgG is mostly produced late in the course of infection (4). The various recombinantly expressed EBV-specific antigens proved to be superior to lysate blots since potential anticellular material-reactive antibodies (often present in patients with mononucleosis) do not influence the result (4, 13). However, Western blot techniques are not standardized in terms of the buffer conditions (such as ionic strength, which is critical for the elimination of potential autoantibody cross-reactions), the lysates from cell lines, and the combination of recombinant antigens used. Western blot analysis provides the advantage of detecting EBV-specific antibodies to multiple EBV-specific antigens simultaneously, and this makes the results of stage-specific diagnostic assays comfortable to interpret and justifies the use of this technology as a confirmation method.

Avidity testing. As an additional method, the avidity testing of VCA IgG may differentiate between primary infection and past infection in anti-EBNA-1-negative cases and may also resolve those cases in which VCA IgM persists long term (1, 4, 17, 32, 39). During the course of infection only antibodies with high avidities are selected; thus, maturation of IgG in vivo can be "measured" in vitro by determination of avidity. B cells switch from the IgM to the IgG isotype in vivo. The first IgG antibodies produced are of low avidity. Over time, IgG antibodies mature through somatic hypermutation in the IgG DNA-encoded region and B-cell clones end up producing IgG antibodies of relative higher avidities or binding strengths (compared to early those of IgG antibodies). The kinetics of

the IgG maturation process may vary from individual to individual, although the maturation process may be complete within a few weeks after primary EBV infection (1, 39). Measurements are obtained by EIA with a VCA-specific substrate, IFA, or Western blot techniques (1, 4, 32). The serum samples are split into two, and tests are performed in parallel with titrated samples. While the test with one aliquot of serum is performed as usual, the other aliquot is treated with different concentrations of urea after the first incubation step. Urea dissociates antibodies from antigens, which is reflected by the loss of titer. The ratio between urea-treated and non-urea-treated samples is used to define the avidity index. VCA IgG avidity determination may be of help in the diagnosis primary EBV infections, especially for VCA IgM-negative cases and cases with long-term persisting VCA IgM, and, additionally, supports the occurrence of past infections in the absence of EBNA-1 IgG, if the avidity index is high. Avidity testing of specific IgG was shown to be helpful for the serodiagnosis of a number of infectious diseases caused by other pathogens (39).

Which method should be used? Some investigators found EIA to be more sensitive than IFA, particularly the anticomplement immunofluorescence technique (22); others found the EIA technology to be as sensitive as IFA or even more sensitive than EIA (23, 27). Already in the mid-1980s the results of assays performed with the first generations of EIAs correlated nicely with those of IFA with purified VCA and EBNA proteins as antigens (9). EIA performance characteristics strongly depend on the nature of the antigens and the preparation and the selection of antigens used (14). As a consequence, the differences in performance characteristics observed between IFA and EIA (i.e., relative sensitivity, relative specificity, and predictive values) are due to the use of various different forms and different selections of antigens with the EIA and to the different IFA substrates used (i.e., different prototype-derived EBV-transformed cell lines [e.g., Raji cells instead of P3HR-1 cells] for detection of anti-EBNA-1 antibodies), the different fixation techniques used, and different interpretations of assay results. Basically, EIA is more sensitive than IFA, but IFA is more specific than EIA, because nonspecific reactions, such as anticellular reactivity, are detected unambiguously (14). IFA is laborious and requires highly skilled and experienced personnel to read the fluorescence images, in contrast to EIA, which is easy to perform.

While Germany and other European countries previously had technique-dependent reimbursement codes (i.e., IFA versus EIA), nowadays in many countries reimbursement is based on the analyte, irrespective of the method used, except Western blot analysis, which is still mostly favored as a confirmation method. Changes in the basis of reimbursement codes from technique to analyte may reflect the progress made by various EBV EIA manufacturers to reach the gold standard performance characteristics of the IFA. Additionally, avidity testing for EBV-specific IgGs mostly is not reimbursed, in contrast to, e.g., *T. gondii* IgG avidity testing. There is no doubt that the reimbursement codes of the respective countries will drive the use of different EBV-specific tests and that cuts in reimbursements will promote automation and technologies that allow high-throughput analysis, such as the EIA techniques. Since EBV-specific assays are not standardized, one is left with participating in national or international proficiency programs (e.g., the College of American Pathologists). As an alternative or as an additional quality control instrument, one may use serologically highly precharacterized and commercially available EBV reference panels (the anti-EBV mixed titer performance panel [Boston Biomedica Inc., West Bridgewater, Mass.] is the single commercial product available worldwide) for in-house validation of the assays (14). These 25 EBV panel members in the commercial EBV reference panel are characterized by use of a "profile analysis," which is based on serological findings by different techniques (EIA, IFA, and latex and hemagglutination assays) from, overall, seven different manufacturers.

In immunosuppressed individuals serological assays are discouraged for many reasons, such as dysfunctions in the production and maintenance of antibodies. Therapeutic immunoglobulin preparation, the dynamics of the disease, and antibody production affect the interpretation of assay results. Even the quantitative EBV serology patterns in patients with PTLD were shown to be highly variable and therefore of limited use in determination of the diagnosis or prognosis of PTLD (15). To date, only the detection of viral load by PCR is an established marker for immunosuppressed patients (19). It has been shown that determination of the EBV viral load is a good tool for the identification of patients at risk for developing EBV-related disorders (19). However, due to the pathogenesis of EBV-related disorders, some patients replicate EBV even at high titers without progressing to disease, and in contrast to other patients, may suffer diseases not related to EBV at all (e.g., EBV-negative PTLD). Therefore, neither does a high viral load indicate an EBV-related disorder nor does an EBV-negative PCR result exclude it in some cases. At present it is still a matter of debate which material should be used for viral load testing: either cell-free samples (plasma, serum) or leukocytes. Again, reimbursement codes favor anti-EA antibody detection instead of PCR for the diagnosis of EBV reactivation. Members of the German Society of Virology and members of the German Association for the Control of Virus Diseases recently (March 2004) came together to revise the guidelines on EBV diagnostics and now recommend the use of VCA for IgG and IgM detection and the use of EBNA-1 for IgG detection in routine EBV diagnostics, while determination of EBV reactivation should be done by molecular biology-based methods (B. Gärtner, D. Huzly, and R. Braun, personal communication).

Interpretation. Interpretation of serological findings should allow EBV infection stage-specific diagnoses (Table 2). Thus, diagnoses should correlate with clinical equivalence. In immunocompetent individuals only three diagnoses are relevant: primary or acute infection as a cause of mononucleosis, a past infection that excludes mononucleosis, and the absence of EBV-specific antibodies, which indicates EBV susceptibility (14).

In the normal clinical routine, about 70% of the serum samples originate from patients with past EBV infection (since the seroprevalence rate is as high as 95 to 100% among elderly individuals). In the case of positive results for VCA IgG and EBNA-1 IgG and in the absence of VCA IgM, a past infection is confirmed. If the results for VCA IgG, VCA IgM, and EBNA-1 IgG are negative, the patient is considered EBV susceptible. If tests for VCA IgM and VCA IgG are positive

and those for EBNA-1 IgG are negative, a patient can be considered to have a primary or acute infection. In cases of positive results for VCA IgG and negative results for VCA IgM and, simultaneously, negative results for EBNA-1 IgG or if the results of tests for all three analytes (VCA IgG and IgM and EBNA-1 IgG) are positive simultaneously, only further diagnostic approaches such as avidity testing (by IFA, EIA, or Western blotting), Western blotting, or PCR may resolve the diagnosis (Table 2). Interpretations of serological findings from manufacturers of commercially available EIAs for EBV diagnostics differ greatly with respect to (i) "isolated" VCA IgG results and (ii) the simultaneous presence of VCA IgG, VCA IgM, and EBNA-1 IgG. For isolated VCA IgG results, most manufacturers interpret "primary" or "acute infection" or "primary infection, convalescence." For the simultaneous presence of all three analytes, the diagnosis of "recent infections," "primary infections, transient phase, or convalescence," or "past infections, persisting IgM" can be found. These diagnoses are not necessarily correlated with IM, and they do not meet the state-of-the-art interpretation criteria (Table 2). In addition, the serological constellation of possible diagnoses when all three analytes are present is not appreciated, because it is difficult to interpret. Gärtner at al. (14) suggested that all three analytes can be found in patients with primary EBV infections when VCA IgM persists while EBNA-1 IgG is already produced or during EBV reactivation, when VCA IgM levels are increased and EBNA-1 IgG is not yet lost. However, these cases need further testing, such as avidity testing of VCA IgG, Western blot analysis, or PCR or even low-cost heterophile testing and assays for detection of atypical lymphocytes. In addition, IFA manufacturers that use Raji cell lines for EBNA determination should be aware of the fact that Raji cells produce EBNA-1 and EBNA-2, which cannot be discriminated by the IFA. EBNA-2 antibodies of the IgG type (in contrast to EBNA-1 antibodies) are usually seen early during infection. Other IFA manufacturers use EBV-transformed cell lines and base their interpretation on the findings of assays for the presence of either IgG or IgM antibodies against nonspecified EBV proteins. None of these assays is helpful for stage-specific diagnosis. The stepwise detection of anti-EBV antibodies requires reliable assays for the diagnosis of an EBV infection, preferably with a single serum specimen.

SUMMARY

To specifically diagnose EBV-associated IM in immunocompetent individuals by use of a single acute-phase serum sample, tests for the three analytes VCA IgG, VCA IgM, and EBNA-1 IgG are sufficient. Only a few samples with indeterminate results require further diagnostic approaches, such as avidity testing of VCA IgG, Western blotting, or PCR. Heterophile antibody determination and the detection of atypical lymphocytes may support the laboratory diagnosis. The preparation of antigens, the selection of antigens and substrates, the different techniques used, and even the interpretation of the results vary remarkably among different EIA and IFA manufacturers. Comparisons of the relative performance characteristics of the commercially available tests should carefully consider in detail the antigens and the substrates used. IFA may still be considered the gold standard for the serodiagnosis of primary EBV infections, although EIA technologies nowadays provide sensitive and specific alternatives. In immunosuppressed individuals, EBV viral load determination by PCR is the method of choice. However, the use of viral load is limited by the role of EBV in the pathogenesis of related disorders.

Standardization of serological assays and assays for EBV viral load detection must be the major goal in the future. Last, but not least, the intended use, as given in the package inserts of the respective commercially available EBV-specific tests, indicates that the test used only supports the diagnosis, which is still found on the basis of clinical symptoms in combination with anamnestic aspects.

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I assure that this minireview is meant to reflect the problems that laboratories face in setting up or interpreting EBV diagnostics, and I declare that there is no conflict of interest, although HiSS Diagnostics manufactures EBV-specific ELISAs. I apologize for not being able to consider additional valued contributions of many investigators from all parts of the world on the field of EBV serology.

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