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Measurement of EBV-IgG Anti-VCA Avidity Aids the Early and Reliable Diagnosis of Primary EBV Infection

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Current serological methods for the diagnosis of Epstein-Barr virus (EBV) infection still differentiate poorly between primary infection and reactivation. This is particularly true when IgG and IgM antibodies are present simultaneously and only a single serum sample is provided for analysis. The demonstration of the IgG avidity state has the potential to distinguish recent from past or reactivated infection. An analysis of the kinetics of avidity maturation of anti-VCA antibodies in primary EBV infection was undertaken with longitudinally collected sets of sera from 28 well-characterised EBV cases and in sera from 35 cases with previous EBV infection and recent primary infection due to HIV, CMV, or hepatitis A. Antibodies directed against the viral capsid antigen (VCA) and Epstein-Barr nuclear antigen (EBNA-1) were sought, using a commercial enzyme immunoassay (EIA). In parallel with standard IgG anti-VCA detection, serum was incubated with 8 M urea to disrupt low-avidity complexes to allow calculation of the percentage avidity. In cases with primary EBV infection, the mean avidity rose from 54% at 6 weeks to 82% by 28 weeks after the onset of symptoms, but remained lower than that of the control sera (96%). The addition of the avidity measurement improved the sensitivity of IgG and IgM anti-VCA testing in diagnosis of primary EBV infection from 93% to 100%. The specificity of IgM anti-VCA testing alone was poor, with 14 of 35 cases (49%) demonstrating false-positive results, but it improved to 97% by the demonstration of high-avidity IgG anti-VCA. The combination of negative IgG anti-EBNA and low-avidity IgG anti-VCA had a sensitivity and specificity of 100%. The routine addition of IgG anti-VCA avidity estimation to diagnostic EBV serology is recommended. ***J. Med. Virol. 70:617–623, 2003.***

KEY WORDS: infectious mononucleosis; serology; avidity; diagnostic accuracy

INTRODUCTION

Despite many years of optimisation of serological tests for primary Epstein-Barr virus (EBV) infection, these assays continue to be associated with difficulties in providing diagnostic certainty. In particular, from the clinician's perspective, the hope is for an assay that provides a high degree of sensitivity and specificity for the diagnosis of recent EBV infection from a single serum sample. From the laboratory perspective, the goals of serological testing are similar, but they have the added priorities of the potential for automation and low cost. Combining these goals remains a challenge in diagnostic microbiology.

Serological tests for EBV fall into two groups: those aimed at the detection of heterophile antibodies, and those that detect antibodies against specific EBV antigens. Heterophile antibodies, originally described by Paul and Bunnell [1932] as sheep red blood cell agglutinins, can also be conveniently detected in commercially available kits, using horse red blood cells [Evans et al., 1975]. These antibodies are reported to be detected at some time during the course of infectious mononucleosis due to EBV infection in 90% of adult cases (i.e., a sensitivity of 90%) [Evans et al., 1975]. However,

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heterophile antibodies are often negative early in infection and may remain positive for more than 1 year, precluding accurate timing of the infection [Evans et al., 1975]. In addition, heterophile antibodies have been detected in other mononucleosis illnesses, including primary infection with cytomegalovirus (CMV), hepatitis A, and human immunodeficiency virus (HIV), as well as in lymphoma, potentially leading to serious misdiagnosis [Horwitz et al., 1979; Dubois, 1988; Van Essen et al., 1998]. In primary EBV infection in young children and older adults, not only is the classical mononucleosis illness less prevalent, but up to 50% of cases may never develop heterophile antibodies [Fleischer et al., 1979].

In terms of the detection of antibodies against specific viral antigens, it was the successful cultivation of EBV-infected lymphoid cell lines that allowed the development of the indirect immunofluorescence antibody assay (IFA) for EBV-specific antibodies [Henle and Henle, 1966]. In the IFA, immunoglobulin class-specific conjugates can be used, allowing the kinetics of development of specific IgM and IgG antibodies to be characterised. Consequently, IFA was accepted as the reference assay for detecting EBV antibodies [Henle et al., 1974]. A decade later, EBV antigens from infected cells were purified using solid-phase absorption [Milman et al., 1985], and polypeptides with immunodominant epitopes have been prepared by recombinant technology [Pearson and Luka, 1986]. These advances have enabled commercially available enzyme immunoassays (EIA) for the specific diagnosis of EBV infection to be developed [Gorgievski-Hrisoho et al., 1990; Farbor et al., 1993]. The EIA has several advantages over IFA, including the fact that the steps in the assay can be automated, allowing cost savings in labour, and the results can be read as absorbance values thereby potentially allowing easier quantitation than the endpoint titrations used in IFA [Debyser et al., 1997].

Of the antigens produced by EBV, viral capsid antigen (VCA) and nuclear antigen (EBNA-1 antigen) are most commonly used in EIAs [Henle et al., 1971, 1974]. The presence of IgM antibodies against VCA is generally designated as an indicator of recent primary infection [Evans et al., 1975], and the response to the EBNA-1 antigen is accepted as a late marker of primary infection [Henle et al., 1979]. However, several studies have shown the diversity in the serological responses to EBV infection, especially in IgM antibodies [Evans et al., 1975]. The appearance of IgM anti-VCA can be delayed and has been reported to be consistently absent in a small proportion of primary EBV infections in adults [Horwitz et al., 1983]. Elevated levels of IgM anti-VCA may persist for several months after infection [Evans et al., 1975] and may also reappear in reactivation in the context of immunosuppression, or in other mononucleoses, including infections with CMV, *Toxoplasma*, hepatitis A, and HIV [Naveau et al., 1985; Rhodes et al., 1990; Fikar and McKee, 1994; Aalto et al., 1998; Van Essen et al., 1998]. Antibodies of the IgG class directed against VCA also appear early in primary infection and may even precede the appearance of IgM, giving rise to further uncertainty in diagnosis from a single serum sample [Evans et al., 1975].

During the maturation of the primary immune response, significant changes have been recognised to occur in the avidity of specific IgG antibodies for antigen [Eisen and Siskind, 1964]. This change in avidity has been described in many infections, including rubella, toxoplasmosis, and hepatitis B [Bottiger and Jensen, 1997; Thomas, 1997; Cozon et al., 1998], and for antibodies directed against EBV VCA [Gray, 1996]. Significant differences in the reduction of optical density values measured in the presence or absence of 8 M urea in the EIA have been used to reflect this change in avidity [De Ory et al., 1993]. Generally, the kinetics of avidity maturation appear to span several weeks [De Ory et al., 1993; Andersson et al., 1994], although delays of up to 3 months after onset of symptoms have been reported [Bottiger and Jensen, 1997; Thomas, 1997; Cozon et al., 1998].

In the present study, sequential serum samples collected from patients with clinically diagnosed and serologically confirmed primary EBV infection of recent onset were available, as well as sera from a group of patients with past EBV infection who had well-characterised primary infection with agents also known to cause a mononucleosis illness, including CMV and HIV. Sera were also available from a well-defined isolated cluster of hepatitis A infections. This report describes the results of testing this unique collection of characterised sera using commercial EIA tests for IgG and IgM antibodies directed against EBV antigens (VCA and EBNA-1), as well as an assessment of the value of IgG (VCA) avidity testing as an aid to establishing the early and reliable diagnosis of primary EBV infection.

PATIENTS AND METHODS

Patients and Sera

Clinical data and sera were available from 28 adult subjects enrolled in a prospective cohort study following individuals from shortly after the onset of provisionally diagnosed acute EBV infection [Bennett et al., 1998]. In this study, subjects with recently detected IgM anti-VCA antibodies were recruited from their referring general practitioners provided that the self-reported onset of symptoms was within 6 weeks. For the purposes of this analysis, mononucleosis was defined as a consistent clinical illness (i.e., two or more of the following: rash, pharyngitis, tender lymphadenopathy, and hepatomegaly or splenomegaly on clinical examination) plus atypical lymphocytes (>5%) on a peripheral blood smear, or a positive Monospot examination.

These subjects form part of a larger prospective study investigating the microbiological, immunological, and psychological determinants of delayed recovery after acute EBV infection [Bennett et al., 1998]. At enrolment, a clinical history was recorded and the outcome of the physical examination, which was conducted by the general practitioner before referral into the study was reviewed. Subjects were followed up again at 2–3 weeks

and at 4–6 weeks after enrollment. A subset of subjects (five in this sample) had additional follow-up at 3 months and at 6 months. At each time point, subjects completed self-report questionnaires to record ongoing symptoms. Blood sampling was undertaken at enrolment (during the febrile period, as soon as possible after diagnosis), then again at 2–3 weeks and at 4–6 weeks. For the purposes of this analysis, the provisional clinical/laboratory diagnosis of primary EBV infection made by the general practitioner was confirmed, either by documenting IgG seroconversion to VCA, or by detection of IgG anti-VCA in the enrollment sample and repeated detection of IgM anti-VCA in at least the first of the longitudinally collected sera (in addition to the original test conducted by the referring general practitioner, which triggered enrollment into the study).

Stored sera were also available from 35 patients with serologically confirmed infections known to be associated with false positive IgM anti-VCA antibody results: (1) primary HIV infection designated by seroconversion in HIV antibody tests, including Western blot, and also by HIV p24 antigenemia (n = 14); (2) primary CMV infection designated by detection of specific IgM and IgG seroconversion in sequential sera (n = 6); and (3) hepatitis A (n = 15; all from a point source outbreak in a local restaurant) designated by detection of anti-HAV IgM in each of two assays and biochemical evidence of acute hepatitis.

Enzyme Immunoassays for EBV Antibodies

IgM and IgG antibodies to EBV VCA and EBNA-1 were measured using commercial diagnostic assay kits (Panbio, Windsor, Australia). The antigens in these assays are VCA purified by solid-phase immunoabsorption and recombinant EBNA-1 [Milman et al., 1985; Pearson and Luka, 1986]. All assays were performed using single batches of the kits. The assays were performed according to the manufacturer's instructions, which are identical for each IgG and IgM assay, apart from the serum dilutions and conjugate utilised. For the IgG assay, the sera were diluted 1:100, whereas for the IgM assays, sera were diluted 1:10 and then a further 1:10 in diluent containing goat anti-human IgG (to reduce IgG competition and remove rheumatoid factor activity). All reagents were allowed to equilibrate to room temperature before commencing the assays. Diluted samples and kit controls (kit negative control single well, kit cutoff control in triplicate and kit positive control single well) were added to the antigen-coated microwells. After a 30-min incubation at 37°C, the microwells were washed six times with the wash solution; 100 μl of kit conjugate (horseradish peroxidase [HRP]-conjugated anti-human IgG or IgM) was added to appropriate microwells. After another 30-min incubation at 37°C, the microwells were washed six times before 100 μl kit substrate (tetramethylbenzidine [TMB]) was added to all microwells. The microwells were incubated at room temperature (21°C) for 10 min. The final colour reaction was stopped by the addition of kit Stop Solution (1 M phosphoric acid). The absorbance of each microwell was read on a dual wavelength (450 nm, reference = 650 nm) spectrophotometer. Each assay was validated by ensuring that the absorbance readings of the controls and calibrator met the specifications of the batch. The cutoff for each plate (96 microwells) was determined from the absorbance of the cutoff control. Optical density readings within 10% of the cutoff were recorded as equivocal.

VCA IgG Avidity EIA

Paired dilutions of each serum were added to each of two wells. After a 30-min incubation at 37°C, the microwells were washed twice with the wash solution, and 100 μl Avidity Reagent (8 M urea solution; Pan Bio, Windsor, Australia) was added to one well (designated the Avidity VCA IgG well) and 100 μl of phosphate-buffered saline (PBS) to the other (designated the Standard VCA IgG well). The microwells were incubated at room temperature (21°C) for 5 min. The microwells were then washed a further four times, and the assay was completed as described above. The Avidity index (%) was calculated using the following formula:

$$\text{Avidity \%} = (\text{absorbance of avidity VCA IgG well} / \text{absorbance of standard VCA IgG well}) \times 100$$

Serological Testing for Hepatitis A, CMV, and HIV Infections

The non-EBV infections were confirmed using standard serological assays. Hepatitis A IgM antibodies were sought in each of two assays (Abbott IMX, Abbott Laboratories, Chicago, IL; and Denka Seikan EIA, Japan). Primary CMV infection was documented with a seroconversion in both IgG EIA and detection of IgM in EIA assays for CMV antibody (Dia Sorin Laboratories, Italy). HIV seroconversion was documented between acute and convalescent specimens in Murex HIV EIA (Abbott Laboratories Murex, Chicago, IL) as well as the presence of HIV p24 antigen in serum (Innotest p24 antigen; Innogenetics Belgium). In each case seroconversion was confirmed by the development of a complete set of diagnostic bands to individual HIV antigens on the Western blot (HIV Blot 2.2, Genelabs, Singapore).

Statistical Analysis

The sensitivity of the serological testing protocol for primary EBV infection was calculated based on testing of a (putative) single serum sample using anti-VCA IgM, or anti-VCA IgM plus IgG avidity determination, or anti-EBNA IgG or plus IgG avidity determination. Values of <2 SD from the mean of the recorded IgG VCA avidity in the 35 control subjects were designated as "low avidity" thereby implying primary EBV infection. The sensitivity was calculated as the number of persons with primary EBV infection with a positive test (or combination of tests) divided by the total number of

those with confirmed primary EBV infection who were tested in that time period. The data obtained from testing of sera from the primary EBV group from the third sampling point (at 6 weeks after enrollment, equivalent to ~8–12 weeks after onset of symptoms) were included in the analysis, as well as results obtained from testing of the 35 control sera taken from subjects with hepatitis A, HIV or CMV infections. The specificity was calculated as the number of nondiseased persons (i.e., control subjects with primary infections due to hepatitis A, CMV or HIV infection) with a negative test (or combination of tests) divided by the total number of all non-diseased persons tested (i.e., n = 35).

RESULTS

In the prospective cohort, 28 patients were enrolled after presentation to the general practitioner with an illness that triggered testing for anti-VCA IgM. These subjects had a mean age of 20 years (range, 16–33), and included 11 males and 17 females. Twenty of these cases (71%) had findings on examination that met the study criteria for a clinical designation of infectious mononucleosis. Four cases had symptoms and signs suggestive of an upper respiratory tract infection only. Four cases had inadequate data recorded to designate the clinical status. The sera obtained from 35 subjects with primary infections due to hepatitis A, CMV or HIV, included 24 males and 11 females, whose ages ranged from 16 to 68 years (mean 30).

Serological testing for the non-EBV infections (hepatitis A, CMV, and HIV) was negative in all cases in the designated primary EBV cohort. Six subjects (6/28; 21%) in the primary EBV cohort had a documented seroconversion to IgG VCA between the baseline and week 3 serum samples. The remaining 22 subjects (79%) had IgG VCA detected in the baseline samples as well as IgM VCA in one or more samples of the minimum of three collected.

Examination of the kinetics of development of IgG and IgM antibodies against VCA and EBNA-1 antigens (Fig. 1) illustrated some of the typical difficulties in interpretation of IgM EBV serology: Two subjects (2/28; 7%) did not develop VCA IgM at any time point although all those who ultimately demonstrated such antibodies had done so within six weeks of onset of symptoms. Six subjects had positive IgM antibodies against VCA at enrolment (i.e., 2–6 weeks after onset of symptoms), but subsequently negative IgM VCA antibody tests were recorded 4–6 weeks later (i.e., the IgM response was relatively short-lived). The kinetics of development of EBNA IgM antibodies was unexpectedly rapid in that although 23/28 subjects (82%) ultimately developed IgM antibodies against this antigen, 21 of the 23 (91% of this subset) had done so within 6 weeks of onset of symptoms and all had done so by 12 weeks. Consistent with previous reports the kinetics of development of IgG anti-EBNA antibodies was slow with only 4 of the 28 subjects (14%) recording detectable antibodies by 12 weeks after onset of symptoms.

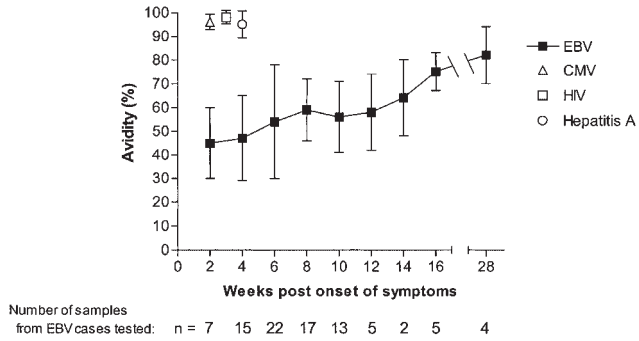

Fig. 1. Timing of the development of Epstein-Barr virus (EBV)-specific IgG and IgM antibodies in relation to onset of symptoms in 28 cases of primary EBV infection. Although the timing of enrollment (and thus baseline sampling) varied between subjects from 2–6 weeks after onset of symptoms, all data for the period 0–6 weeks are included at the 6-week time point to ensure that all 28 subjects were represented. Similarly, the 12-week time point includes the accumulated data from follow-up evaluation of all 28 subjects, whereas additional data in the time points thereafter is derived only from those subjects who completed additional follow-up beyond 12 weeks (5/28).

The kinetics of the maturation of the avidity of the VCA IgG antibody response was relatively slow (Fig. 2) with a steady increase evident over 16–28 weeks after the onset of symptoms. The mean avidity measured in subjects 6 weeks after the onset of symptoms was 54 (SD = 24); by 12 weeks, the mean was 58 (SD = 16). By the 6-week time point after the onset of symptoms, two of 28 subjects (7%) with primary EBV infection had recorded avidity measurements within 2 SD of the mean avidity recorded for the 35 control subjects (see below). The maximum recorded avidity did not correlate with the age, sex, or the presence of a typical mononucleosis illness in the 28 cases with primary EBV infection.

In contrast, only high avidity IgG anti-VCA antibodies were detected in sera from subjects with other primary infections known to be associated with the "false-positive" EBV IgM serological tests. These samples had a mean avidity of 96.3% (SD 4.4). Indeed, in the sample of 35 subjects with proven infection due to hepatitis A, CMV, or HIV infection (and prior EBV infection),

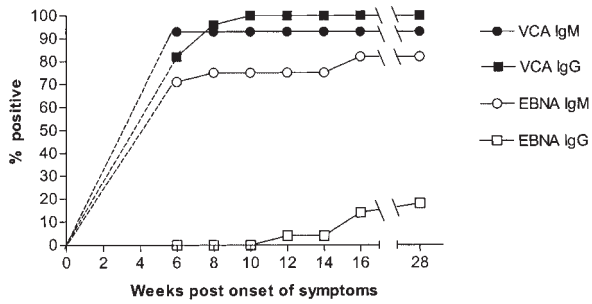

Fig. 2. Mean avidity (±SD) of IgG viral capsid antigen (VCA) antibodies after onset of symptoms in subjects with primary Epstein-Barr virus (EBV) infection (closed symbols) or other primary infections (open symbols). As the timing of samples in relation to onset of symptoms varied from subject to subject in the primary EBV group, the numbers tested at each time point are indicated below the horizontal axis.

TABLE I. Results of Serological Testing for EBV in Cases With Other Primary Viral Infections (n = 35)

		No. (%) of group with positive serology				
		VCA		EBNA		
	Sample size (n)	IgG	IgM	IgG	IgM	IgG VCA avidity (mean % and SD)
Hepatitis A	15	15 (100)	12 (80)	15 (100)	12 (80)	95 (5.7)
HIV	14	14 (100)	5 (36)	14 (100)	10 (71)	98 (2.8)
CMV	6	6 (100)	1 (16)	6 (100)	6 (100)	96 (3.2)
Combined	35	35 (100)	18 (51)	35 (100)	28 (80)	96.3 (4.4)

EBV, Epstein-Barr virus; VCA, viral capsid antigen; EBNA, Epstein-Barr nuclear antigen; HIV, human immunodeficiency virus; CMV, cytomegalovirus.

18 sets of sera (51%) revealed EBV VCA IgM antibodies (Table I), whereas 28 samples (80%) had positive EBNA IgM antibody results.

The sensitivity of testing for EBV VCA IgM alone was 93% (i.e., 26 of 28 subjects who ultimately had confirmed EBV were correctly identified by 12 weeks), whereas the addition of detection of an IgG avidity of <2 SD from the mean of the control samples (i.e., an avidity index of 87.5%) improved the sensitivity to 100%.

The specificity analysis was based on the control subjects with hepatitis A, CMV or HIV. Testing with anti-VCA IgM alone has a specificity in the context of these non-EBV primary infections as low as 49%, whereas the addition of an IgG avidity assay would improve this figure to 97% (i.e., a single subject with hepatitis A recorded an avidity of 78%). When combined with a negative EBNA IgG result, the IgG anti-VCA avidity testing has a sensitivity and specificity of 100% in the subject group studied in the present report.

DISCUSSION

These findings support the value of measurement of the avidity of IgG anti-VCA antibodies in improving the accurate the diagnosis of primary EBV infection, and the poor specificity of detection of IgM anti-VCA in a single serum sample. The previous studies of the avidity of EBV specific IgG antibodies have generally advocated the utility of the assay, but compared single samples collected from patients with recent infection with those obtained from past infection. For example, Schubert et al. [1998] reported avidity differences between recent and past EBV infection but did not provide data with regard to the timing of onset of infection, or analysis of a control group to determine specificity. Weissbrich [1998] found 100% sensitivity and 100% specificity when an avidity index was calculated, but used the traditional titration approach rather than optical density. De Ory et al. [1993] showed that IgG avidity for EBV diagnosis had a sensitivity at least equal to those of the classical procedures for diagnosing EBV, but relied on single samples for their analysis. These authors advocated further studies to establish the specificity of this assay and the duration of the low-avidity antibodies. Similarly, Andersson et al. [1994] used endpoint titrations in an IFA to establish the value of avidity measurement in distinguishing recent from past EBV infection. Thus, in each of these studies only single samples were used and the timing of the samples in relation to the clinical illness was not specified, hence the kinetics of the changes in avidity of the IgG anti-VCA could not be established. The data from the present study highlight the fact that in previously healthy adult patients with a putative primary EBV infection that a single serum sample collected within the first 12 weeks of onset of symptoms is likely to yield relatively low avidity IgG anti-VCA antibodies and hence confirm the diagnosis of recent infection. The addition of IgG anti-VCA avidity estimation either to VCA IgM analysis or to IgG anti-EBNA results improved the sensitivity of this serological testing to 100% in our sample.

The advent of increasingly sensitive EIAs for detection of IgM antibodies has led to increasing difficulties with specificity in the diagnosis of primary infection when based on a single serum sample. This difficulty is particularly evident in the context of herpesvirus infections, as reactivation of latent infection is common in the context of other herpesviral infections in both immunocompromised and immunocompetent hosts. For instance, it has been recognised that primary infection with CMV frequently triggers an additional IgM antibody response to EBV or HHV-6 [Irving et al., 1990]. In addition, truly cross-reactive antibody responses directed against conserved epitopes are well recognised amongst the herpesviruses, such as between the 63-kDa protein of varicella-zoster virus (VZV) and herpes simplex virus (HSV) glycoprotein B [Edson et al., 1985] and a glycine-alanine epitope shared between EBV and CMV [Rhodes et al., 1990].

These false-positive IgM anti-VCA antibody results are also recognised in the context of other acute viral infections [Naveau et al., 1985; Rhodes et al., 1990; Fikar and McKee, 1994; Aalto et al., 1998; Van Essen et al., 1998]. This is particularly noteworthy in the setting of primary HIV infection, which may be remarkably similar to primary EBV infection in clinical presentation [Schlacker et al., 1996] and may also be associated with a positive Monospot test [Dubois, 1988]. A mistaken diagnosis in this circumstance has potentially serious public health consequences as well as ramifications for missed early treatment with anti-retroviral therapies and the recognition of long term disease complications.

As the clinical findings in primary EBV infection in older age groups commonly includes protracted fever and biochemical hepatitis, diagnostic uncertainty on clinical grounds between EBV and hepatitis A is not uncommon. The group of sera available for this study provided a unique opportunity to evaluate EBV serology in a well-defined cluster of hepatitis A infections, rather than relying on the results of sporadic cases in which there may be doubt as to which of the two infections is, in fact, the genuine cause of the illness [Fikar and McKee, 1994]. Twelve of 15 subjects with hepatitis A (80%) were found to have IgM antibodies against VCA, while 80% demonstrated EBNA IgM positivity. This rate is somewhat higher than that reported by Naveau et al. [1985] (9/15; 60%) who attributed the finding either to EBV reactivation or activation of EBV antibody producing B lymphocytes. This phenomenon has also been attributed to rheumatoid factor activity [Freymuth et al., 1986; Tsai et al., 1996]. In addition, in subjects with acute hepatitis A infection, IgM antibodies against the triosephosphate isomerase enzyme of EBV have been demonstrated [Ritter et al., 1996]. As these antibodies have been associated with reactivation of latent EBV infection, these findings suggest that the false-positive EBV IgM (both EBNA and VCA) in hepatitis A may be due to reactivation of latent EBV rather than antigenic cross-reaction or nonspecific anamnestic antibody responses. A high frequency of serological reactivation of EBV IgM antibodies in heterologous herpesvirus infections has also been previously reported. The induction of reactivation appears to occur only in one direction, in that previous studies have shown that EBV infections do not induce serological reactivation of CMV IgM antibodies [Aalto et al., 1998]. Consistent with this previous report, none of the 28 EBV patients in this study had detectable CMV IgM antibodies.

Given this background, the finding in the present study that over half of the control subjects (18/35; 51%) with non-EBV infections (CMV, HIV, or hepatitis A), had demonstrable VCA IgM antibodies should raise concerns about reliance upon this assay alone for diagnosis. By contrast, the addition of IgG VCA avidity analysis to the diagnostic approach, improved the specificity of the EBV serological testing in these comparable clinical illnesses to 97%, and with negative EBNA IgG to 100%. It should be noted however that a small percentage of healthy subjects who develop primary EBV infection and immunocompromised patients may not develop or maintain anti-EBNA IgG antibodies [Vetter et al., 1994; Bauer, 2001], hence the added specificity provided by this component of the analysis should be interpreted with caution.

The evidence from this study confirms previous reports [Henle et al., 1974] that the kinetics of development of EBNA IgG antibodies in primary EBV infection are slow with only four of 28 subjects (14%) developing antibodies by 12 weeks after onset of symptoms In addition, the high rate of positivity of EBNA IgM in the three groups of control subjects confirms this antibody as a potentially useful marker of EBV reactivation [Obel et al., 1996]. Thus, the combination of negative IgG and positive IgM antibodies against EBNA, in addition to high-avidity anti-VCA antibodies detected within 3 months of an acute illness should reliably indicate reactivation, rather than primary EBV infection.

The diagnosis of uncomplicated primary EBV infection may not require the full panel of assays described here. However, as the misdiagnosis of a mononucleosis illness may have serious consequences both for patient care and public health, it is appropriate to advocate the use of the four assays and VCA IgG avidity in order to stage the timing of the infection and identify primary EBV episodes unequivocally. If the aim of testing is to only confirm primary EBV infection in previously healthy adults, then the combination of IgG VCA avidity and IgG EBNA assays alone may be sufficient.

On the basis of these findings, the inclusion of IgG anti-VCA avidity analysis in the serological testing for primary EBV infection has been adopted in our diagnostic laboratory.

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