A Systematic Study of Epstein-Barr Virus Serologic Assays Following Acute Infection

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Abstract

We determined the presence of IgG and IgM antibody to viral capsid antigen (VCA-IgG, VCA-IgM) and IgG antibody to the Epstein-Barr virus nuclear antigen (EBNA) by indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) during the acute illness and at 1, 2, 6, and 48 months in a prospective population-based case series of 95 persons with an acute illness serologically confirmed as Epstein-Barr virus infection. The acute illness was characterized by the presence of VCA-IgG and VCA-IgM (by ELISA) and by the absence of EBNA in most, but not all, patients. During follow-up, VCA-IgG antibodies remained detectable in all patients, while the proportion with VCA-IgM declined and the number with detectable EBNA antibodies steadily increased. The primary differences between the 2 serologic test methods were the increased persistence of VCA-IgM during follow-up by ELISA and the earlier detection of EBNA by IFA. Clinicians should consider the illness stage and the laboratory technique to appropriately interpret serologic test results in suspected cases of mononucleosis caused by the Epstein-Barr virus.

Epstein-Barr virus (EBV) produces a wide spectrum of disease, from silent infection to life-threatening illness. Symptomatic infection is typically self-limited and characterized by fever, pharyngitis, and cervical lymphadenopathy.[1-3] In most adolescents and adults, the diagnosis of infectious mononucleosis can be made in the setting of characteristic clinical manifestations and a positive heterophil antibody test. However, when the manifestations or course does not distinguish the illness as acute EBV infection, clinicians may rely on serologic assays for EBV for the detection of antibodies to confirm the diagnosis and guide management. Traditionally, these serologic assays have used indirect immunofluorescence assay (IFA) techniques. More recently, enzyme-linked immunosorbent assay (ELISA) has emerged as a relatively simple, quick, and automated alternative. Yet, our knowledge of the serologic profile during the acute illness and recovery from infectious mononucleosis produced by either test has been derived largely from investigations using incomplete or nonsystematic follow-up, specialized populations, or cross-sectional or retrospective evaluations.[4-22]

We conducted a prospective, population-based study of persons with acute illness caused by infection with EBV as confirmed by the presence of heterophil antibody and EBV viral capsid antigen IgM antibody by IFA. The goal of the study was to compare the ELISA and IFA methods during the acute and convalescent stages of illness following EBV infection.

Materials and Methods

Study Setting

The setting for this study was a large health maintenance organization in the Puget Sound area that provides prepaid

health care through facilities that include 2 hospitals, 23 outpatient medical clinics, 3 specialty centers, and a progressive care facility. This plan serves a heterogeneous socioeconomic population whose age and sex composition is similar to the region as a whole.

Subject Identification and Enrollment

All subjects who met the following criteria were eligible for the study: (1) 16 years of age or older, (2) positive heterophil antibody test result, (3) no record of a previous positive heterophil antibody, (4) onset of symptoms reported within 14 days of having the heterophil test performed, (5) no chronic medical condition, (6) not undergoing treatment with corticosteroids, and (7) demonstrated serologic evidence of acute EBV infection with an IgM detected to the viral capsid antigen (VCA) by IFA. By using triweekly review of laboratory records, we prospectively identified all patients who had a positive heterophil antibody test. Final determination of eligibility occurred after enrollment and was based on information from the chart review, patient interview, and EBV serologic assays performed at the initial evaluation (see following text). The recruitment and evaluation protocols were approved by the institutional review boards of the University of Washington, Seattle, and the health maintenance organization. All subjects or their guardians provided written informed consent.

Subjects (N = 95) were evaluated at the initial visit, and at 1, 2, and 6 months thereafter. In addition, a subset of subjects (n = 50) were reexamined approximately 4 years after the index visit. Initial and follow-up visits included the administration of self-report measures, a physical examination, and laboratory tests. Of the 380 serologic assessments that were possible from the initial, 1-, 2-, and 6-month evaluations (95 subjects × 4 study visits), 363 underwent both IFA and ELISA. All 50 participants had serologic assessments by both methods at the 4-year visit. (Antibody to the Epstein-Barr virus nuclear antigen [EBNA] was not assessed at this final visit.)

EBV Serologic Assays

Serologic tests for IgG and IgM antibodies to the VCA (VCA-IgG, VCA-IgM) and for IgG antibody to the EBNA were performed by both IFA and ELISA. Indirect immunofluorescence assays were performed for antibody against VCA using fixed EBV-infected P3HR1(K) cells as the antigen. An IgG secondary antibody (Kallestad, Chaska, MN) or fluorescein isothiocyanate–conjugated goat antihuman IgM was added to detect IgG and IgM, respectively. Serum samples were run at dilutions of 1:10, 1:20, and 1:40 for IgM and at serial 2-fold dilutions from 1:40 to 1:320 for IgG. Titers of 1:10 or more for VCA-IgG and VCA-IgM were considered positive. Antibodies to EBNA by anticomplement immunofluorescence were determined at a single 1:2 dilution using fixed Raji cells that were incubated with serum samples at a 1:2 dilution, then with human complement (C3), and, finally, with fluorescein isothiocyanate–conjugated antibodies to human complement (C3). Fixed EBV-uninfected BJAB cells were used as a control for nonspecific anti–lymphocyte antibody binding. Serum samples with reactivity to this control were scored as antinuclear antibody positive. EBNA antibodies are not detectable by anticomplement immunofluorescence in such serum samples. ELISA kits were purchased from Gull Laboratories, Salt Lake City, UT (now Meridian Diagnostics, Cincinnati, OH) and tests performed according to the manufacturer's instructions.

Statistical Analysis

For each visit and overall, we determined the concordance between IFA and ELISA results for VCA-IgM and EBNA. The concordance was determined by taking the sum of results for subjects either simultaneously positive or negative by both IFA and ELISA and dividing by the total number in the cohort. The McNemar test for paired dichotomous data was used to examine differences between the tests.[23]

Results

The majority of the sample were students. the average age was 21 years (range, 16-46 years). Approximately 50% were women, and 90% were white and single. Subjects averaged 12.6 years of education. All subjects were acutely ill during the initial evaluation. Characteristic symptoms were sore throat in 74% (70/95) of cases and fatigue in 77% (73/95). Physical examination findings were pharyngitis in 73% (69/95) of cases and cervical lymphadenopathy in 77% (73/95). At the initial study evaluation, lymphocytes constituted more than 50% of the WBC differential in 45 of 90 subjects (50%), while 63 of 90 subjects (70%) had more than 10% atypical lymphocytes and 38 of 90 (42%) had more than 20% atypical lymphocytes.

Antibody to VCA

VCA-IgG by IFA was detected in all subjects at illness onset and throughout the study. The ELISA produced similar results, with 98% having a detectable VCA-IgG titer; 96% during the acute illness (3 negative results), 98% at 1 month (1 negative and 1 equivocal result), 100% at 2 months, 99% at 6 months (1 equivocal result), and 100% at 4 years.

In contrast, the proportion of the sample with VCA-IgM by either IFA or ELISA steadily decreased during the study **Table 1**. Although the IFA and ELISA results concurred at the initial visit and at the 4-year follow-up (all 50 patients tested at the 4-year visit were VCA-IgM negative by both

Table 1
VCA-IgM Status by IFA and ELISA Over Time*

Result	Initial (n = 90)	1 mo (n = 94)	2 mo (n = 89)	6 mo (n = 90)	Overall (n = 363)
IFA positive	90 (100)	64 (68)	21 (24)	7 (8)	182 (50.1)
ELISA positive	87 (97)	78 (83)	37 (42)	13 (14)	215 (59.2)
IFA negative	0 (0)	30 (32)	68 (76)	83 (92)	181 (49.9)
ELISA negative	1 (1)	14 (15)	43 (48)	74 (82)	132 (36.4)
IFA and ELISA positive	87 (97)	59 (63)	11 (12)	2 (2)	159 (43.8)
IFA and ELISA negative	0 (0)	10 (11)	37 (42)	70 (78)	117 (32.2)
Concordance	87 (97)	69 (73)	48 (54)	72 (80)	276 (76.0)

ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescence assay; VCA, viral capsid antigen.
* Data are given as number (percentage). For 2, 2, 9, and 3 subjects, results were equivocal by ELISA at the initial, 1-month, 2-month, and 6-month visits, respectively. No subject had an equivocal test by IFA. All subjects were required by entry criteria to be VCA-IgM positive by IFA. All subjects were ill at the initial visit.

Table 2
EBNA-IgG Status by IFA and ELISA Over Time*

EBNA Test Pattern	Initial (n = 90)	1 mo (n = 94)	2 mo (n = 89)	6 mo (n = 90)	Overall (n = 363)
IFA positive	20 (22)	37 (39)	43 (48)	70 (78)	170 (46.8)
ELISA positive	6 (7)	6 (6)	15 (17)	59 (66)	86 (23.7)
IFA negative	61 (68)	49 (52)	38 (43)	13 (14)	161 (44.4)
ELISA negative	84 (93)	86 (91)	71 (80)	30 (33)	271 (74.7)
IFA and ELISA positive	3 (3)	4 (4)	10 (11)	52 (58)	69 (19.0)
IFA and ELISA negative	59 (66)	48 (51)	33 (37)	12 (13)	152 (41.9)
Concordance	62 (69)	52 (55)	43 (48)	64 (71)	221 (60.9)

EBNA, Epstein-Barr virus nuclear antigen; ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescence assay.
* Data are given as number (percentage). For 9, 8, 8, and 7 subjects, results were uninterpretable (antinuclear antibodies present) by IFA at the initial, 1-month, 2-month, and 6-month visits, respectively. For 0, 2, 3, and 1 subjects, results were equivocal by ELISA at the initial, 1-month, 2-month, and 6-month visits, respectively. All subjects were ill at the initial visit.

IFA and ELISA), the 2 tests demonstrated less agreement during the interim visits. From 1 month on, subjects were more likely to be VCA-IgM positive by ELISA than by IFA ($P < .01$). (All subjects were required to be VCA-IgM positive by IFA for enrollment.)

Antibody to EBNA

The proportion of subjects with IgG EBNA antibodies increased over time by both methods **Table 2**. The level of agreement between the 2 methods ranged from approximately 70% at the initial and 6-month visits to about 50% at the 1- and 2-month visits. At each visit, subjects were more likely to have a positive EBNA titer by IFA than by ELISA ($P < .01$). IFA resulted in a larger number of uninterpretable results (ie, antinuclear antibody–positive or equivocal serum sample) than ELISA (8.8% vs 1.7%; $P < .01$).

Serologic Patterns

We also evaluated the various combinations of VCA-IgM and EBNA antibody responses **Table 3**. By both methods, the majority of subjects were VCA-IgM positive and EBNA negative at the initial visit, and VCA-IgM negative and EBNA positive at the 6-month visit. However, at each follow-up visit, all 4 combinations of antibody patterns were evident. Of note, up to one third of subjects during various stages of follow-up were simultaneously negative for VCA-IgM and EBNA. The agreement between IFA and ELISA for a given VCA-IgM and EBNA pattern was generally less than 50% (data not shown), a level expected given the results for the individual VCA-IgM and EBNA tests. The ELISA method more frequently produced a VCA-IgM positive, EBNA negative result than the IFA, while the IFA more often produced a VCA-IgM negative, EBNA positive profile ($P < .01$).

Discussion

We evaluated the acute and convalescent serologic responses to EBV by the ELISA and IFA methods. As in past studies,[4-7,15,17] virtually all subjects initially were positive for VCA-IgG and remained so throughout the study. As demonstrated previously,[8,24,25] we found the VCA-IgG by the ELISA to be comparable to the IFA in acute, convalescent, and past EBV infections. Thus, a positive VCA-IgG antibody by IFA or ELISA served as a definite marker of infection, but on its own provided no information about the timing of infection.

In contrast with the VCA-IgG response, the proportion of persons who were VCA-IgM positive declined over time by both assays, similar to reports in other investigations.[4-7,15,17] In conjunction with a positive VCA-IgG titer, the presence

Table 3
Combined VCA-IgM and EBNA Antibody Status by IFA and ELISA Over Time*

Serologic Pattern	Initial (n = 90)	1 mo (n = 94)	2 mo (n = 89)	6 mo (n = 90)	Overall (n = 363)
VCA-IgM positive/EBNA negative					
IFA	61 (68)	41 (44)	11 (12)	1 (1)	114 (31.4)
ELISA	82 (91)	73 (78)	30 (34)	3 (3)	188 (51.8)
VCA-IgM negative/EBNA negative					
IFA	0 (0)	8 (9)	27 (30)	12 (13)	47 (12.9)
ELISA	0 (0)	11 (12)	32 (36)	24 (27)	67 (18.0)
VCA-IgM positive/EBNA positive					
IFA	20 (22)	19 (20)	8 (9)	5 (6)	52 (14.3)
ELISA	5 (6)	3 (3)	5 (6)	10 (11)	23 (6.3)
VCA-IgM negative/EBNA positive					
IFA	0 (0)	18 (19)	35 (39)	65 (72)	118 (32.5)
ELISA	1 (1)	3 (3)	10 (11)	49 (54)	63 (17.4)

EBNA, Epstein-Barr virus nuclear antigen; ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescence assay; VCA, viral capsid antigen.
* Data are given as number (percentage). For 9, 8, 8, and 7 subjects, results were uninterpretable (antinuclear antibodies present) by IFA at the initial, 1-month, 2-month, and 6-month visits, respectively. For 2, 4, 12, and 4 subjects, results were equivocal by ELISA at the initial, 1-month, 2-month, and 6-month visits, respectively. All subjects were ill at the initial visit.

of VCA-IgM by either method typically represented an illness of less than 2 months and, since infection generally predates symptoms by 6 to 8 weeks,[2] infection with EBV for less than 4 months. VCA-IgM, however, was still present in a few patients at 6 months, though it was undetectable in all subjects by 4 years after infection. In comparing the 2 methods for VCA-IgM, the ELISA produced results similar to the IFA at the initial visit and the 4-year follow-up, findings consistent with other reports of the acute phase [8,24-28] or past infection.[8,24,25,27,29] However, detectable VCA-IgM declined more slowly by the ELISA than by the IFA and was consistently more likely to be positive by the ELISA during the first 6 months of follow-up, producing discordant results between the 2 methods 20% to 50% of the time during this period. As a consequence, the ELISA more frequently detected "recent" infection (illness for 6 months or less). Thus, the ELISA VCA-IgM may be the preferred test if clinicians are attempting to determine the cause of persistent symptoms that have features of EBV infection.

Antibodies to EBNA are classically considered a marker of infection resolution that is not present initially but develops weeks or months later.[11,12,18,20] In our study, in which all subjects had acute symptoms and were enrolled within 2 weeks of illness onset, 22% (20/90) and 7% (6/90) were initially positive for EBNA antibody by IFA and ELISA, respectively. Because some subjects had been symptomatic for up to 2 weeks, the brief delay in serologic testing may, in part, explain the relatively frequent appearance of EBNA (and infrequent appearance of classic hematologic measures of acute infectious mononucleosis[1]) at the index visit. However, some investigations have revealed that 3% to 65% of patients manifest EBNA early in the illness.[7,10,15,16,21,27] Indeed, as originally noted by Henle and colleagues,[13] "the presence of antibody to EBNA in an acute-phase serum specimen does not preclude a current primary EBV infection." During follow-up, the proportion of patients with EBNA increased, although 14% (13/90) by IFA and 33% (30/90) by ELISA remained seronegative up to 6 months after the acute illness. Subjects were less likely to have a detectable EBNA by ELISA than by IFA during the acute and convalescent stages of the illness; consequently, the EBNA by ELISA, as with VCA-IgM, was more often consistent with "recent" infection than the EBNA by IFA.

A VCA-IgM positive, EBNA negative serologic pattern is considered a marker of acute infection, while a VCA-IgM negative, EBNA positive pattern represents a long-standing infection.[5,7,11,15,20,28] The majority of our sample was VCA-IgM positive and EBNA negative at the initial visit and VCA-IgM negative and EBNA positive by the 6-month visit. However, other combinations of VCA-IgM and EBNA results were observed by IFA and ELISA at the initial visit and during follow-up. For example, a substantial minority of patients were negative for both IgM and EBNA, representing a "window" in the VCA-IgM and EBNA serologic response, a circumstance that underscores the importance of the VCA-IgG result. Given the increased persistence of VCA-IgM during follow-up by ELISA and the earlier detection of EBNA by the IFA, the ELISA was more likely to demonstrate the recent infection profile, while the IFA more frequently demonstrated the convalescent pattern.

Our study has limitations. First, we were unable to evaluate the serologic diagnostic characteristics of the ELISA in illnesses not caused by EBV (its specificity) since the sample included only subjects infected with EBV. Second, we evaluated only one of the ELISA assays available to test for EBV infection. Although there are differences among the various ELISA assays,[30] our findings may highlight the potential

issues that should be considered when using ELISA as opposed to IFA. Since the study evaluated only persons seeking medical care, the results are applicable only in clinical settings. Finally, our results pertain only to individuals 16 years of age or older.

In this population-based, prospective study with standardized follow-up, acute illness caused by EBV infection was characterized by the presence of VCA-IgG and IgM (by ELISA) and by the absence of EBNA in most but not all patients. During follow-up, VCA-IgG antibodies remained detectable in all patients, while the proportion with a positive VCA-IgM titer declined and the number with detectable EBNA antibodies steadily increased. Nevertheless, some patients remained VCA-IgM positive or EBNA negative at 6 months. The ELISA more frequently produced an acute infection result, positive VCA-IgM and/or negative EBNA antibody titers, than the IFA. Given its relative ease of operation, the ELISA is an attractive alternative to the IFA; however, clinicians must consider the illness stage to appropriately interpret the results of a specific assay in suspected cases of mononucleosis caused by EBV.

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