

## Detection of Antibodies to Epstein-Barr Virus Capsid Antigen by Immune Adherence Hemagglutination

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The immune adherence hemagglutination assay was found to be as sensitive and specific as the indirect immunofluorescence technique for titration of antibodies to Epstein-Barr virus capsid antigen. Satisfactory virus capsid antigen-specific and negative control antigens for the immune adherence hemagglutination assay were prepared from cell extracts of the Epstein-Barr virus producer P3HR-1 and the Epstein-Barr virus genome-negative BJAB lymphoblastoid cell lines, respectively. As the immune adherence hemagglutination assay can be used to titrate antibodies to both the heterophil antigen of the Paul-Bunnell type and to virus capsid antigen, it offers a promising alternative to the immunofluorescence methods in the serodiagnosis of Epstein-Barr virus infections which can be performed by most diagnostic laboratories.

Epstein-Barr virus (EBV) infections result in responses ranging from asymptomatic seroconversion to infectious mononucleosis (IM), a lymphoproliferative disease which can have serious central nervous system and other complications (2, 6, 11). IM-like illnesses are caused also by other infectious agents such as cytomegalovirus, adenovirus, and several other viruses or *Toxoplasma gondii*. At present, the serological method most widely used to diagnose IM is the test for heterophil antibodies of the Paul-Bunnell type. A positive heterophil antibody test is considered diagnostic for IM in patients who present with the typical clinical and hematological picture of the disease. However, in patients without heterophil antibody responses, with an atypical course of illness, or with complications, EBV-specific serological tests are required for establishing the diagnosis.

The indirect immunofluorescence assay (IFA) is presently the method most widely used to detect both EBV-specific antigens and antibodies in primary EBV infections. The fluorescence tests are specific and sensitive (5, 8). Unfortunately, EBV-specific IFA procedures are complex and thus impractical for many diagnostic laboratories. Serodiagnosis by IFA depends on the presence of immunoglobulin M (IgM) and IgG antibodies to the virus capsid antigen (VCA) and to the D (diffuse) component of the early antigen (EA) complex, and on the absence of antibodies to the EBV-associated nuclear antigen (EBNA). Although cell smears suitable for the detection of IgG antibodies to VCA are becoming commercially available, they rarely

suffice for diagnosis, because anti-VCA persists after the primary infection for life. In addition peak titers of antibodies to this antigen are often reached before the patient seeks medical attention so that diagnostically significant rises in titer are seen in fewer than 20% of the cases.

Current recommendations for the serodiagnosis of primary EBV infections involve a stepwise approach (9). The test for Paul-Bunnell heterophil antibodies is frequently performed first, as it is highly specific for IM and is widely available. EBV-specific serology is often carried out for either confirmatory or exclusionary purposes. This approach creates difficulties for both the clinician and the laboratory: for the physician because it means a delay in diagnosis, as the EBV serology can be carried out in only a few laboratories; for the laboratory because the heterophil test is positive in only a fraction of the total number of tests requested, and if it is negative, no useful information is obtained on the immune status of the patient. Ideally, the test for heterophil and EBV-specific antibodies should be performed in the same diagnostic laboratory.

As part of efforts toward purification and characterization of EBV-determined antigens, our laboratory has been evaluating immunoassays for the detection of solubilized antigens and their corresponding antibodies. As reported previously, the immune adherence hemagglutination assay (IAHA) is possibly the most sensitive and specific assay for Paul-Bunnell heterophil antibodies (12). This report shows that the IAHA technique is suitable also for the detection of antibodies to VCA and that the results obtained are closely comparable to those recorded

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TABLE 1. Interpretation of EBV-specific serodiagnostic tests by immunofluorescence techniques

Antibodies to:	Susceptible	Current primary	Recent	Past
VCA <sup>a</sup>				
IgM	<1:10	>1:10	+ or -	<1:10
IgG	<1:10	>1:10	>1:10	>1:10
EA-D <sup>b</sup>	<1:10	+ or -	+ or -	- <sup>c</sup>
EA-R <sup>b</sup>	<1:10	+ or -	+ or -	-
EBNA	<1:2	<1:2	1:2 to 1:5	>1:10

<sup>a</sup> IFA titers.

<sup>b</sup> Anti-complement immunofluorescence titers.

<sup>c</sup> May be positive in immunosuppressed patients.

in IFA tests. Parallel titration of antibodies to VCA and heterophil antigen in the IAHA test offers a promising alternative to the IFA procedure.

#### MATERIALS AND METHODS

**Sera.** Sera were selected from a collection of frozen sera from patients with suspected primary EBV infections. They had been submitted for routine EBV-specific serodiagnostic tests which included titration of IgM and IgG antibodies to VCA, IgG antibodies to the D and R (restricted) components of the EA complex by IFA (5, 8), and of antibodies to EBNA by anti-complement immunofluorescence (7). On the basis of criteria established in our laboratory, the sera were subdivided into three major groups (Table 1) according to the antibody spectra and titers to each of the antigens: (i) sera from *susceptible* individuals if VCA antibodies were absent; (ii) sera from individuals with *past infections* if IgG anti-VCA and anti-EBNA titers were both  $\geq 1:10$ ; and (iii) sera from patients with *current IM* if IgM and IgG anti-VCA were present while anti-EBNA was absent ( $\leq 1:2$ ) or with *recent IM* if IgG anti-VCA was present (with or without IgM anti-VCA) and anti-EBNA was of low titer (1:2 to 1:5).

**Cell extracts.** The P3HR1 culture is an EBV producer line derived from a Burkitt's lymphoma patient (10). VCA is expressed in about 10 to 15% of the cells as detected by IFA. P3HR1 cells served as the source of EBV VCA test antigen for the IAHA assay. BJAB cells, an EBV genome-negative B-lymphoblastoid line, were the source of negative control antigen. Both lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Each week, cells were seeded at a concentration of  $3 \times 10^5$  cells per ml of fresh medium and grown at 32°C. For IAHA antigen extraction, cells from 12-day-old cultures were collected after centrifugation at  $10,000 \times g$  for 20 min; they were washed twice in phosphate-buffered saline of pH 7.2 and frozen at -20°C as a suspension of  $2 \times 10^8$  cells per ml in phosphate-buffered saline. When needed, the frozen cell suspensions were thawed, sonicated in a Branson Sonifer 185, equipped with microtip, at a power output of 10 W with five bursts of 30 s each. The sonicated suspension was then centrifuged at  $7,000 \times g$  for 20 min. After addition of 0.2% sodium azide, the supernatant fluid, or crude extract, was stored at -70°C until use. For the IAHA assay, the optimal antigen concentration was determined by

block titration against standard reference anti-VCA-positive and antigen-negative sera as measured by IFA assays.

**IAHA.** Procedures to detect Paul-Bunnell heterophil antibodies by IAHA have been described (12). Heterophil antibody titers of  $\geq 1:40$  were considered diagnostic of IM. Assays using soluble EBV antigens were identical to those reported previously for other viral antigens (13). Briefly, duplicate dilutions of inactivated sera were made in twofold steps in polystyrene V-bottom plates with Veronal buffer containing 1 mg of bovine serum albumin per ml. Optimal concentrations of the test antigen, as determined in a block titration, were added to one set of dilutions, and negative control antigen at equivalent concentration was added to the other set of dilutions. After incubation of the plates at 37°C for 30 min, guinea pig complement diluted 1:100, as determined by block titration, was added and the plates were further incubated for another 40 min at 37°C. A mixture of EDTA (0.04 M in Veronal buffer) and 3 mg of dithiothreitol per ml was added immediately prior to the addition of 0.8% human type O erythrocytes which had been prescreened for IAHA reactivity. All reagents were added in 0.025-ml drops followed by shaking of the plate on a vibrator for 10 s. Wells with 50% or greater hemagglutination were considered positive. If the hemagglutination titers observed with the positive antigen were at least fourfold greater than those obtained with the negative control antigen, they were considered specific. Appropriate antibody-positive and -negative reference sera were included with each test as controls.

#### RESULTS

**Crude cell extracts as antigens in the IAHA test.** Four successive lots of extracts from P3HR1 and BJAB cells, prepared as described in Materials and Methods, were tested in block titrations against reference sera with known positive and negative IFA titers to VCA. Specific antigen-antibody reactions were observed with all four lots. Optimal antigen titers of the four different preparations of P3HR1 cell extracts were 1:80 (twice), 1:160, and 1:320. No reactivity was observed with the BJAB cell extracts when used at comparable dilutions. For subsequent antibody titrations, between one and two times the optimal concentration of each antigen lot was used.

The antigenic activity of the four antigen lots was tested against a panel of 12 sera which were positive (8 sera) or negative (4 sera) in the IFA test for anti-VCA to determine the lot-to-lot reproducibility of the reactions. Maximal deviations of the antibody titers obtained with the four lots of P3HR1 antigen were twofold or less for every serum tested.

The activity of the cell extracts was stable at refrigerator temperature. For long-term storage, the antigen was kept frozen at -70°C. Repeated cycles of freezing and thawing tended to decrease the optimal titer. The decreases in titers were probably due to aggregation because they

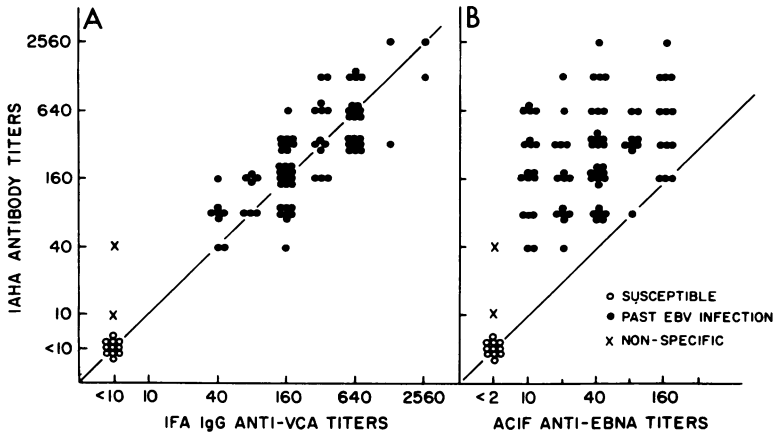


FIG. 1. Comparisons between IAHA titers with P3HR1 antigen and IgG anti-VCA titers obtained by IFA (A) and anti-EBNA titers obtained by anti-complement immunofluorescence (ACIF) (B). Symbols: ×, sera with IAHA activity in the absence of added antigens; ○, sera from susceptible individuals with no antibodies to EBV; ●, sera from individuals with past EBV infections.

were reversible by brief sonication (10 to 15 s), restoring the original titer in each instance.

**Reactive antigenic component of P3HR1 cell extracts.** To determine the identity of the antigen(s) reactive in the P3HR1 cell extracts, a panel of 97 sera was tested by IAHA: 84 sera from individuals with known past exposure to EBV and 13 sera from susceptible individuals. The IAHA antibody titers were plotted against the IFA antibody titers to various EBV antigens. No correlation was found between IAHA titers and anti-EBNA titers (Fig. 1B). As most of the sera contained neither anti-D nor anti-R, the reactions observed in the IAHA test were obviously unrelated to the EA components. In contrast, there was an excellent correlation between the IAHA titers and the IgG anti-VCA titers obtained by IFA (Fig. 1A). The geometric mean titers (GMT) of antibodies measured by the two procedures were 1:256 and 1:234, respectively, indicating comparable sensitivities of the two methods. The IAHA and IAF titers of 89 of the 97 sera (92%) agreed within a twofold and of 6 sera within a fourfold dilution step. Two sera showed nonspecific reactivities in both tests.

Sera from 58 patients with IM showed a fair correlation between the titers obtained by the IAHA and IFA methods (Fig. 2). However, unlike the results obtained with the sera from individuals with past infections, the GMT of IM sera obtained by IAHA (1:328) was nearly twofold higher than the GMT obtained by the IFA technique (1:192). This difference was not unexpected as the IAHA test detects both IgG and IgM antibodies. Of 31 sera with IAHA titers exceeding the IFA titers by a factor of two or more, 24 had IgM anti-VCA titers of >1:160 in the IFA test. One serum that was negative for

IgG anti-VCA but positive for IgM anti-VCA as measured by IFA was indeed positive also in the IAHA test.

Two types of nonspecific activity were observed with 6 of the total of 155 sera tested. The first was noted with 4 of the 58 IM sera which showed hemagglutination in the absence of any antigen and only in the presence of complement. However, these sera were unequivocally positive, as their EBV-specific IAHA titers were more than fourfold higher than the nonspecific titers in the serum control. The second type of nonspecific reactivity was seen with two sera which gave titers of 1:10 and 1:40 with both the positive (P3HR1) and the negative control

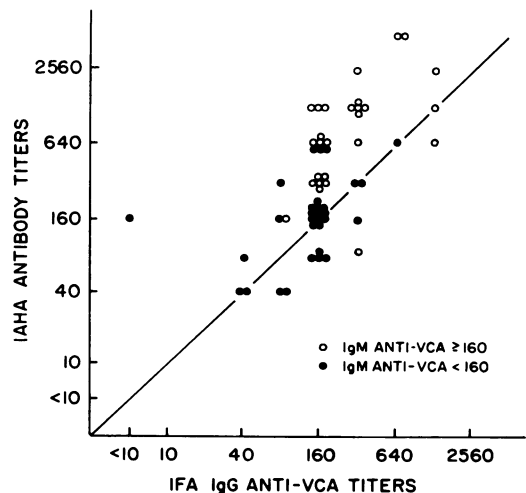


FIG. 2. Comparisons between IAHA and IFA IgG anti-VCA titers in sera from IM patients. Sera with IgM anti-VCA titers of  $\geq 160$  (○) or  $< 160$  (●).

(BJAB) antigens. The anti-BJAB antibodies were detected at identical serum dilutions also by IFA using fixed BJAB cell smears.

**Serodiagnosis of IM using IAHA procedures.** As IgM cannot be differentiated from IgG in the IAHA assay without fractionation procedures, an alternative to tests for EBV-specific IgM antibodies would be to measure heterophil antibodies by IAHA. An analysis was made of the 58 IM sera described in the previous section to see how heterophil antibody measurements by IAHA compared with the conventional IgM anti-VCA test by IFA. The results of the two tests agreed in 53 of the 58 sera (91%), 47 sera being positive and 6 negative in both tests. Disagreement was observed with 5 sera; 3 were negative for IgM anti-VCA by the IFA test but positive for heterophil antibodies as measured by IAHA, and in 2 the reverse was noted. With the 6 sera negative by both tests, the diagnosis was based upon anti-EBNA titers of  $\leq 1:5$ , whereas the IgG anti-VCA titers were  $> 1:160$ , suggesting that the sera were collected during early convalescence when the IgM anti-VCA and heterophil antibody titers had already waned.

## DISCUSSION

EBV-specific serological profiles covering antibodies of different immunoglobulin classes to VCA, D, R, and EBNA can currently be determined only in a few research laboratories as a result of the inavailability of commercial reagents and the complexities of the procedures. Some laboratories test only for IgM and IgG antibodies to VCA in an acute-phase serum for the serodiagnosis of IM. Follow-up sera would be needed only if confirmation is necessary. This approach would be useful, but cell smears suitable for IgM anti-VCA tests are not yet commercially available.

We have been using the IAHA assay to monitor various EBV-specific antigens during fractionation and purification procedures with encouraging results. It appears that IAHA offers a practical alternative to IFA for EBV-specific serology. Its similarity to standard complement fixation procedures in terms of manipulations and equipment makes the IAHA technique an economical addition to current routine serological tests in many diagnostic laboratories. For laboratories which are already using IAHA as a general-purpose serological method, the EBV serology as well as tests for heterophil antibodies can readily be added to the repertoire.

The antigen preparations described in this report can be obtained as high-titered stock in large quantity by most laboratories with minimal tissue culture facilities. This antigen is stable at freezer or refrigerator temperatures. No compli-

cated fractionation procedures are needed for use in the IAHA test.

Using extracts of P3HR1 cells as antigen, the IAHA assay appears to be as sensitive and specific as the IFA techniques for the detection of antibodies to VCA. The agreement between the antibody titers obtained by IAHA and by IFA was excellent for sera collected from individuals after long past primary EBV infections, and the GMTs were nearly identical. The relatively high GMT with this panel of sera should not be taken as representative of individuals with past EBV infections, as the sera were selected to include a wide range of IFA titers (from  $< 1:10$  to  $1:2,560$ ) for the comparison with the IAHA results.

For sera with serological profiles indicative of primary EBV infections, the IAHA titers were overall higher than those obtained by IFA. As IAHA detects both IgM and IgG antibodies, the titers observed reflected the sum of complement-fixing antibodies of both immunoglobulin classes. These results highlight the previously not fully appreciated advantage of the IAHA over the standard complement fixation procedure. Whereas IgM antibodies yield variable and often low titers in the complement fixation assay (1), they are nevertheless capable of fixing complement, leading to hemagglutination in the IAHA test. This is shown strikingly by one of the IM sera which was positive in the IAHA and IgM anti-VCA tests but negative for IgG anti-VCA. Also, the Paul-Bunnell heterophil antibodies which are almost exclusively of the IgM class react well in the IAHA test (3) as do IgM antibodies to certain other viruses (4).

Two types of nonspecific reactions were observed with the IAHA procedures. The first was shown to be due to complement-fixing factors inherent in the sera. Nonspecific reactions of this type are most frequently seen in acute-phase sera of patients with primary EBV and cytomegalovirus infections (unpublished data) and are most likely due to endogenous antibody-antigen complexes, e.g., rheumatoid factors. Nonspecific reactions of the second type were due to antibodies directed to non-EBV-related cellular components. In our experience, neither type of reaction caused difficulties in interpretations. With the appropriate controls, nonspecific reactivity is readily identifiable in the IAHA assay, as in IFA procedures.

As IgM and IgG antibodies are not distinguishable in the IAHA test, the use of the test for heterophil antibodies might be an alternative to the determination of EBV-specific IgM antibodies. From our results, these two tests agreed in 91% of the sera in attempts at identifying cases of IM. Both assays failed to detect 15% of the IM cases. These IgM anti-VCA- and hetero-

phil antibody-negative cases could be diagnosed as recent primary EBV infections only with inclusion of a test for anti-EBNA. The data attest to the usefulness of the anti-EBNA assay. Sera submitted to the laboratory are at times collected too late during convalescence when IgM anti-VCA or heterophil antibodies, or both, have already declined to nondetectable or insignificant levels. Preliminary tests with purified EBNA preparations, kindly furnished by George Klein, have shown that anti-EBNA can also be measured by the IAHA technique.

The preparations of antigen used in the IAHA test undoubtedly contained a multitude of other EBV-induced antigens such as the D and R components of the EA complex and EBNA. However, these did not interfere with the determination of anti-VCA titers, judging from the excellent correlation between the antibody titers obtained by IFA and IAHA. This is not unexpected as antibodies to D, R, and EBNA are nearly always of lower titer than anti-VCA when measured by IFA. The presence of heterophil antigen in the fetal calf serum component of the tissue culture medium could also have presented a problem. However, any residual traces of heterophil antigen that might have remained after washing of the cells before preparation of the cell extracts did not interfere, as is evident from the failure of heterophil antibody-positive sera to react with the control BJAB cell extracts.

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