

Comparison of Fluorescent-Antibody-to-Membrane-Antigen Test, Indirect Immunofluorescence Assay, and a Commercial Enzyme-Linked Immunosorbent Assay for Determination of Antibody to Varicella-Zoster Virus

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The demand for sensitive and specific assays to determine immune status to varicella can be expected to increase with the anticipated availability of a varicella-zoster virus vaccine for use in nonimmune adults, especially health care personnel, and in immunosuppressed children. Although the fluorescent-antibody-to-membrane-antigen (FAMA) test remains the reference standard to which other tests are compared, simpler alternative assays are needed. In this study, the FAMA was compared with a simple indirect immunofluorescence assay (IFA) and a commercially available enzyme-linked immunosorbent assay (ELISA) for the detection of antibody to varicella-zoster virus. One hundred and twelve serum samples were screened by the FAMA test and IFA at a 1:5 dilution, and 100% agreement was found. Of these samples, 101 were available for testing by ELISA, and identical results were obtained with 97 samples (96% agreement). When the samples were screened at a 1:2 dilution, 99 of 101 results agreed. In addition, 31 spinal fluid samples were tested by all three methods. When screening was at a 1:2 dilution, there was 96.8% agreement between the FAMA test and IFA. When the cutoff value established for sera was used for the spinal fluid samples, there was 90.3% agreement between the ELISA and the FAMA test. Thus, both IFA and ELISA can be considered sensitive and specific alternatives to the FAMA test, and in addition, both use commercially available reagents.

The importance of sensitive and specific assays for the determination of immune status to varicella-zoster virus (VZV) has long been recognized. The fluorescent-antibody-to-membrane-antigen (FAMA) test was originally developed because other commonly used tests, such as the complement fixation test, were insensitive, or, in the case of the indirect immunofluorescence assay (IFA) using fixed cells, produced nonspecific reactions when low dilutions were used (13). A negative FAMA test was subsequently found to correlate well with susceptibility to VZV infection (5). In addition, detection of FAMA antibody in the spinal fluid was found to correlate with VZV-associated encephalitis (1, 3). The FAMA test is the standard by which other methods are judged. However, despite improvements (15), it remains somewhat cumbersome.

In the search for a simple but sensitive alternative to the FAMA test, the anticomplement immunofluorescence (ACIF) test has been evaluated. The ACIF test has been used successfully for measuring antibodies to other herpesviruses, since nonspecific fluorescence due to reactions of immunoglobulins with Fc receptors induced on the membranes of herpesvirus-infected cells is avoided. On the whole, correlation between the FAMA and ACIF tests appears to be good (2, 9), although in one report, ACIF detected only 4 of 43 sera positive by the FAMA test (11). More recently, the enzyme-linked immunosorbent assay (ELISA) has been used to determine susceptibility to VZV with good results (6, 8, 10, 12, 14).

In this study, we compared three methods for the detection of VZV antibody in both serum and cerebrospinal fluid (CSF): the FAMA test, IFA, and a commercially available ELISA. We chose to reevaluate the conventional IFA because of its simplicity and because, unlike cells infected by the other human herpesviruses, there is evidence that VZV-infected cells do not express receptors for the Fc portion of immunoglobulin G that are responsible for nonspecific results in indirect immunofluorescence tests (7).

MATERIALS AND METHODS

Specimen selection. The serum samples used were submitted for testing to the laboratory of one of us (W.A.A.), in which the FAMA test has been used routinely for a number of years. Most of the serum samples submitted for FAMA tests to this laboratory are obtained from either hospital employees or from immunocompromised patients, especially children, who lack a history of chicken pox. Samples were selected to ensure that adequate numbers of negative and borderline-positive sera were included for testing by the other two methods. The sera were then submitted under code to the laboratory of a second investigator (M.L.L.) for testing by IFA. All sera were then submitted under code to a third laboratory (D.R.M.), in which a commercial ELISA has been in use for the past year. Inadequate quantities remained of 11 of the original 112 sera tested by the FAMA test and IFA, and therefore they were not tested by ELISA. Information on the correlation of antibody titers with immunity was not generally available. Thirty-one spinal fluid samples previously submitted to any one of the three labo-

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TABLE 1. Correlation between detection of VZV serum antibody by FAMA test with detection by IFA and ELISA^a

| Assay and result | No. of samples with indicated result in FAMA test | |
|--------------------|---|---------------|
| | Positive (72) ^b | Negative (29) |
| IFA ^c | | |
| Positive | 72 | 0 |
| Negative | 0 | 29 |
| ELISA ^d | | |
| Positive | 70 | 2 |
| Negative | 2 | 27 |

^a Serum samples were screened at a 1:5 dilution in both the FAMA test and IFA.

^b The number of samples tested is shown in parentheses.

^c A total of 78 positive and 34 negative serum samples were tested by IFA with 100% agreement with the FAMA test; the quantities of 11 of these were insufficient for testing with ELISA.

^d The correlation between the ELISA and the FAMA test was 96%.

ratories for VZV antibody testing were then tested under code by all three methods. These samples were obtained from patients with neurologic disease in whom a diagnosis of VZV infection was considered. Samples were received from medical facilities throughout Connecticut and the eastern United States. Complete clinical information was not generally available on these patients. In all studies, the FAMA test was considered the reference standard.

FAMA procedure. The FAMA test was performed by methods previously described (15). Briefly, seed pools of VZV-infected human foreskin fibroblasts were used to infect two 75-cm² flasks containing confluent monolayers of human foreskin fibroblasts. When cytopathic effect involved 75% of the monolayer, the cells were dispersed with trypsin and used to infect freshly seeded foreskin fibroblasts in 8 to 10 150-cm² flasks. When cytopathic effect involved 75% of the monolayers, all cells were trypsinized, washed, and then fixed in a final concentration of 0.075% glutaraldehyde for 60 s at 0°C. Fixation was terminated by the addition of glycine, and the cells were then centrifuged at 600 × *g* for 10 min at 4°C. The cells were suspended at a concentration of 2 × 10⁶ cells per ml in cold freezing medium (8% dimethyl sulfoxide and 20% fetal calf serum in basal essential medium) and frozen in 0.1-ml aliquots at -70°C until use. Uninfected cells, similarly processed, were used as negative antigen controls. Serum samples were inactivated at 56°C for 30 min and diluted 1:5 in Puck saline. The samples (50-μl aliquots) were then mixed in 15-ml conical-bottom plastic test tubes with 50 μl of VZV-infected or uninfected cells, thawed just before use. The sera and cells were then incubated for 30 min at room temperature. All further reactions and washes were done in these tubes, as previously described (15). After the last washing step, the cell pellets were suspended in 1 drop of buffered glycerol and mounted under a cover slip on a microscope slide. Spinal fluid samples were processed similarly but were screened at a 1:2 dilution.

Indirect immunofluorescence procedure. VZV-infected cells were prepared by inoculating confluent monolayers of MRC-5 cells (Whittaker M.A. Bioproducts, Walkersville, Md.) with VZV stock at an input multiplicity of 0.02 PFU per cell. When cytopathic effect involved 50% of the monolayer, the cells were trypsinized and divided into two new flasks, and trypsinized uninfected MRC-5 cells were added. After an additional 3 days of incubation at 37°C, VZV-infected cells were harvested by trypsinization, centrifuged, and washed in phosphate-buffered saline (PBS). The cell pellet

from one 75-cm² flask was suspended in 7 to 10 ml of PBS, and one-fourth volume of trypsinized, washed uninfected MRC-5 cells was added. Monolayers of uninfected MRC-5 cells were processed separately for use as uninfected-cell controls. Approximately 50% of the cells in VZV-infected cell suspensions were in fact infected. This was done to allow for an additional internal control to avoid nonspecific results. Samples (10 μl) of the appropriate cell suspension were added to individual wells on eight-well toxoplasmosis slides (Bellco Glass, Inc., Vineland, N.J.). The slides were then fixed in acetone for 10 min at 4°C.

For the IFA, as for the FAMA test, all serum samples were screened at a 1:5 dilution and spinal fluids were screened at a 1:2 dilution. A positive control serum was included on each slide. Each sample tested was added to a well containing VZV-infected cells and to a well containing uninfected cells (15 μl of sample in each). The slides were then incubated in a moist chamber for 30 min at 37°C. After incubation, the slides were rinsed in PBS, washed for 10 min with two changes of PBS, rinsed with distilled water, and then air dried. Fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin G (15 μl; Litton Bionetics, Charleston, S.C.) containing Evans blue counterstain was then added, followed by incubation for 30 min at 37°C in a moist chamber. The slides were then washed with PBS as described above, air dried, mounted with buffered glycerol, and examined under a fluorescence microscope. The immunofluorescence observed with acetone-fixed cells was somewhat less intense than that seen with glutaraldehyde-fixed cells, was also confined to the membrane of infected cells, and was absent from uninfected cells with all sera tested.

ELISA procedure. Immunoglobulin G antibodies to VZV were measured with a commercially available ELISA (Varicelisa; Whittaker M.A. Bioproducts). A 1:26 dilution of sample (10 μl of sample plus 250 μl of diluent) was prepared in both VZV antigen-coated and control antigen-coated microwells. After the plates were incubated and washed, alkaline phosphatase-conjugated rabbit anti-human immunoglobulin G was added for additional incubation. After the plates were washed, *p*-nitrophenylphosphate was added as substrate. Three sera with known ELISA values (calibrators) and two additional controls with known values were included. Optical density was measured at 405 nm, and the ELISA value for each sample was calculated from a calibration curve.

RESULTS

Comparison of the FAMA test with IFA and ELISA for detection of VZV antibody in serum. Of a total of 112 serum samples originally tested by the FAMA procedure at a 1:5 dilution, 78 contained VZV antibody and 34 did not. These 112 samples were then tested under code by IFA, and complete (100%) agreement was obtained. All sera except 11 (6 FAMA positive and 5 FAMA negative) which were present in insufficient quantities were then tested under code by ELISA. Of these remaining 101 samples, 70 of 72 positive by the FAMA test and IFA were positive by ELISA and 27 of 29 negative by the FAMA test and IFA were negative by ELISA (Table 1). Of a total of 101 sera tested by ELISA, there was agreement with the FAMA test and IFA for 97 samples (96% agreement).

To further investigate the reasons for the discrepancies, titers were obtained for the four serum samples by IFA and the results were compared with the ELISA values (Table 2). The two sera (no. 258 and 280) negative by the FAMA test

and IFA at a 1:5 dilution but positive by ELISA were found to be positive at a 1:2 dilution by IFA. The two sera (no. 463 and 475) found positive by the FAMA test and IFA but negative or equivocal by ELISA both had titers of 1:5 by IFA. Sample 463 had a high background in the IFA but was still considered positive. A high background was also detected in the control well by ELISA with this sample, and when the absorbance value was corrected for this, the final reading was in the equivocal-to-negative range. Sample 475 was weakly positive by both the FAMA test and IFA at 1:5 and repeatedly tested in the equivocal range by ELISA.

Comparison of the FAMA test with IFA and ELISA for detection of VZV antibody in CSF. Of 31 spinal fluid samples tested by the FAMA procedure at a 1:2 dilution, 9 contained VZV antibody and 22 did not (Table 3). Of the 22 samples negative by the FAMA test, one was weakly positive by IFA. However, 3 of 11 samples positive by both the FAMA test and IFA were negative by ELISA when the cutoff value established for sera was used. Results obtained with these four discrepant CSF samples are presented in Table 4. The three samples positive by the FAMA test and IFA and negative by ELISA all had ELISA values in the range of 0.08 to 0.12 (in serum, a value of 0.15 is considered positive and 0.12 or less is considered negative). Corresponding FAMA and IFA titers were low (1:2 to 1:5). All samples negative by the FAMA test and IFA had ELISA values of <0.05 (data not shown). One spinal fluid sample (no. 273) was negative (<1:2) by the FAMA test, weakly positive at 1:2 by IFA, and had an ELISA value of 0.01. This spinal fluid was obtained from a patient with thoracic zoster who developed transverse myelitis.

DISCUSSION

It is important to determine immune status to VZV in immunosuppressed individuals, especially children, and in hospital personnel exposed to a patient with VZV infection. Health care workers not only can become ill themselves but can unwittingly spread varicella within the hospital in the day(s) just before eruption of the rash. An effective live attenuated VZV vaccine is being used in immunosuppressed children and will presumably become available for use in nonimmune adults, in whom primary infections with VZV can be severe and even fatal (4). The demand for sensitive and specific assays to determine immune status to VZV will undoubtedly increase in the near future.

The FAMA test remains the "gold standard" for the detection of low levels of VZV antibody. Serum samples can be screened at a 1:2 dilution, as has been done in other studies. However, in some instances a prozone phenomenon has been observed at very low serum dilutions. Furthermore, the consequences of a false-positive titer are much graver in a hospital setting than those of a false-negative titer. Therefore, a screening dilution of 1:5 has been used

TABLE 2. IFA and ELISA results for discrepant serum samples

| Specimen | IFA titer ^a | ELISA value(s) ^b |
|------------------|------------------------|-----------------------------|
| 258 | 2 | 0.22 |
| 280 | 2 | 0.21 |
| 463 ^c | 5 | 0.13, 0.04, 0.05, 0.10 |
| 475 | 5 | 0.07, 0.13, 0.13, 0.14 |

^a The titer is expressed as the reciprocal of the highest positive dilution.

^b A value of 0.15 is considered positive, 0.14 to 0.13 is equivocal, and 0.12 or less is negative. If samples were tested more than once, all values are given.

^c A high background was noted in this specimen, but it was still considered positive by IFA and the FAMA test.

TABLE 3. Correlation between detection of VZV antibody in spinal fluid by FAMA test with detection by IFA and ELISA^a

| Assay and result | No. of samples with indicated result in FAMA test | |
|-----------------------|---|---------------|
| | Positive (9) ^b | Negative (22) |
| IFA ^c | | |
| Positive | 9 | 1 |
| Negative | 0 | 21 |
| ELISA ^d | | |
| Positive ^e | 6 | 0 |
| Negative | 3 | 22 |

^a Spinal fluid samples were screened at a 1:2 dilution in both the FAMA test and IFA.

^b The number of samples tested is shown in parentheses.

^c The correlation between the IFA and the FAMA test was 96.8%.

^d The correlation between the ELISA and the FAMA test was 90.3%.

^e Positive criteria for spinal fluid have not been established. Criteria established for serum were used.

routinely in the laboratory of one of us (W.A.A.) in this study.

The IFA was criticized in an early study for producing nonspecific results (13). Starting dilutions of 1:10 have been used to avoid this problem, but this results in a loss of sensitivity (14). Because of the simplicity of the IFA, which eliminates the laborious washing and cell transfer steps necessary in the FAMA assay, and because VZV is unique among the herpesviruses in that it does not induce Fc receptors on infected cells (7), the IFA was reevaluated in this study. Interestingly, for the 112 serum samples tested, there was 100% agreement between the FAMA test and IFA when the sera were screened at a 1:5 dilution.

Initial reports on the use of ELISA for the determination of VZV immune status have been very promising (6, 8, 10, 12, 14). In this study, the M.A. Bioproducts ELISA was compared with the FAMA test and IFA, and identical results were obtained with 97 of 101 serum samples tested. In another study, similar results were noted for the M.A. Bioproducts ELISA and the FAMA test (8). Of the four discrepant samples, two found positive by ELISA and negative by the FAMA test and IFA when tested at a 1:5 dilution were subsequently found to be positive by IFA when a 1:2 dilution was tested. The two remaining samples were positive at low dilution (1:5) by fluorescence and were equivocal to negative by ELISA; one of these samples was found to have a high background reading. It is not known which test correlates best with immunity in these or similar patients. In general, there is complete agreement between FAMA titers of greater than 2 and protection from chicken pox (15).

In addition to determination of immune status, the FAMA

TABLE 4. Results of FAMA test, IFA, and ELISA for discrepant spinal fluid samples

| Specimen | Titer ^a | | ELISA value ^b |
|----------|--------------------|-----|--------------------------|
| | FAMA test | IFA | |
| 273 | <2 | 2 | 0.01 |
| 545 | 5 | 4 | 0.08 |
| 594 | 2 | 4 | 0.09 |
| 2316 | 2 | 4 | 0.12 |

^a Expressed as the reciprocal of the highest positive dilution.

^b For serum, a value of 0.15 is considered positive, 0.14 to 0.13 is equivocal, and 0.12 or less is negative.

test has been used to detect VZV antibody in spinal fluid and thus establish a diagnosis of VZV-associated neurologic disease. To our knowledge, the present study is the first to examine a commercial ELISA or an IFA for this use. Of 31 spinal fluid samples tested, there was 96.8% correlation between the FAMA test and IFA. The M.A. Bioproducts ELISA has been standardized, and criteria for positivity have been established for serum but not for spinal fluid. When these criteria were applied to CSF, 3 of 11 FAMA-positive samples were negative by ELISA, for an overall 90.3% correlation between the two tests. In this study, all FAMA-positive samples had ELISA values of 0.08 or greater, whereas all FAMA-negative samples had ELISA values of 0.04 or less. Due to fewer nonspecific reactions encountered with CSF samples than with sera, it may be reasonable to accept a lower value as the positive cutoff. However, additional specimens should be tested in this range by ELISA and the FAMA test and the data preferably should be correlated with clinical information to determine whether this alternative cutoff is appropriate.

In summary, agreement between the three methods, the FAMA test, IFA, and ELISA, for detection of VZV antibody in serum samples was excellent. The simple IFA appears to be an acceptable alternative to the FAMA test. Prepared slides can be kept at -20°C until use, and even a single serum sample can be tested rapidly. The ELISA is available commercially and, because of its objective endpoints and automation, is particularly suitable for screening large numbers of samples for immunity to VZV; it can also be used to detect recent infection (12). The correlation between the FAMA test, IFA, and ELISA for detection of VZV antibody in spinal fluid was also excellent, but the importance of establishing a positive ELISA cutoff value for CSF was apparent. This information should prove useful in the evaluation of VZV-associated neurologic diseases.

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