Detection of the Carrier State for Classic Hemophilia Using an Enzyme-Linked Immunosorbent Assay (ELISA)

By David J. Fishman, Paul K. Jones, Jay E. Menitove, Oscar D. Ratnoff, and Barbara Everson

A high proportion of carriers of classic hemophilia can be identified in the laboratory because, in comparison to normal women, the concentration of antigens related to antihemophilic factor (AHF, factor VIII) that are detected in their plasma by heterologous antisera (factor VIII:Ag) is relatively higher than the titer of AHF that is measured in clotting assays (factor VIII:C). Enzyme-linked immunosorbent assay (ELISA) appears to overcome some of the technical difficulties associated with measurement of AHF-like antigens. The results of ELISA correlated closely with those obtained by semiquantitative immunoelectrophoresis, except in patients with von Willebrand's disease, in which ELISA appeared to provide a more quantitative estimate of AHF-like antigen. Utilizing the ELISA technique and a revised method of logarithmic discriminant analysis, we were able to distinguish all of 37 obligate carriers of hemophilia at the level of certainty that would have misclassified 5% of normal women as carriers. The relative simplicity of ELISA suggests its utility in the diagnosis of the carrier state in the female relatives of hemophiliacs.

CARRIERS OF classic hemophilia can be identified in a high proportion of cases because the procoagulant (factor VIII:C) titer of antihemophilic factor (AHF, factor VIII) is relatively lower than the concentration of AHF-like antigen that is detected by specific heterologous antisera (factor VIII:Ag). Several techniques have been used for the measurement of AHF-like antigen, among them, semiquantitative immunoelectrophoresis, hemagglutination inhibition, and radioimmunoassay. Each of these techniques has its drawbacks. Semiquantitative immunoelectrophoresis and hemagglutination inhibition methods are cumbersome and technically difficult. Radioimmunoassay, although more accurate, utilizes expensive reagents and equipment, requires training in the use of radioisotopes, poses potential health risks, and necessitates storage and disposal of the radioisotope.

More recently, enzyme-linked immunosorbent assay (ELISA) procedures have been introduced to measure AHF-like antigens (factor VIII:Ag). The basic principles of the ELISA are similar to those of radioimmunoassay. Briefly, heterologous antiserum against AHF is adsorbed onto the surface of the wells of a microtiter plate. Excess antiserum is washed away and a dilution of the test plasma is then added. AHF present in the sample adheres specifically to the solid-phase antibody adsorbed to the surface. After incubation of the test sample, the wells are again washed and a solution containing heterologous antiserum against AHF, labeled with alkaline phosphatase, is added. After a period of incubation, the wells are again washed and a dilution of a substrate of alkaline phosphatase is added. Alkaline phosphatase activity, measured colorimetrically, is a function of the concentration of AHF in the test sample.

Using a minor modification of the technique of Bartlett et al., we have been able to identify the carrier state for classic hemophilia in all of 37 obligate carriers of this disease at a level that misidentified 5% of the normal control subjects (i.e., the "95% level of certainty"). ELISA has proved to be technically easy and readily reproducible, and its use should widen the number of laboratories capable of conducting studies for the carrier state of hemophilia.

MATERIALS AND METHODS

Citrate plasma was separated from venous blood to which 1/50 volume of 0.5 M sodium citrate buffer (pH 5.0) had been added, as described previously. One aliquot of fresh plasma was used for immediate determination of procoagulant activity and a second aliquot was quick-frozen and stored at −70°C in polyethylene vials coated with silicone oil for later determination of AHF-related antigen activity by the two methods described below.

A standard pool of 24 normal male plasmas was prepared and stored as described earlier. The standard was said to contain 1.0 U/ml of procoagulant AHF (VIII:C) and 1.0 U/ml of AHF-related antigen (VIII:Ag).

AHF-related antigen was measured in 137 individuals by both the modified Laurell technique of semiquantitative immunoelectrophoresis and the ELISA method. These subjects included 44 normal adult nonpregnant women, 5 normal men, 8 classic hemophiliacs, 34 obligate carriers of classic hemophilia, 28 other female relatives of hemophiliacs, 2 individuals with acquired circulating anticoagulants directed against AHF, 14 patients with von Willebrand's disease, and 2 individuals with hereditary angioneurotic edema. Blood was

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drawn with the subjects' consent after approval by the Committee on Human Experimentation of Case Western Reserve University.

To detect the carrier state, plasma samples of 37 obligate carriers were tested by ELISA and 110 obligate carriers by the Laurell technique; 34 obligate carriers were tested simultaneously by both techniques. Obligate carriers fell into one of three categories: mothers of more than one hemophiliac, mothers of one hemophiliac who had other affected relatives, or daughters of individuals with classic hemophilia.

Procoagulant AHF activity was measured by a modification of the kaolin-activated partial thromboplastin time described previously.¹⁴,¹⁵ The coefficient of variation of this method, based on the mean of duplicate determinations, is ± 8%.¹⁶

Precipitating AHF-related antigens (factor VIIIIR:Ag) were measured by a modification¹⁷ of Laurell's method for semiquantitative immunoelectrophoresis and by enzyme-linked immunosorbent assay. The Laurell method is based on the immunoelectrophoretic migration of plasma AHF-like antigens into agarose impregnated with heterologous anti-AHF antibody. The height of the precipitin arcs that form is a function of the concentration of AHF-related antigen in the test plasma; the lower limit of sensitivity is 0.15 U/ml. The coefficient of variation of this technique is approximately ± 12%.¹⁷

ELISA was performed essentially as discussed by Bartlett et al. A partially purified immunoglobulin fraction of a commercially available rabbit antiserum to human AHF (Calbiochem-Behring Corp., La Jolla, Calif.) was separated by extraction with octanol acid, as previously described.¹⁸ Typically, the fraction prepared from 3 ml of crude antiserum contained at least 6 mg protein/ml (as measured by Lowry's method)¹⁹ in barbitonal-saline buffer (0.025 M barbitonal-sodium barbital, 0.125 M NaCl, pH 7.5). Alkaline phosphatase (type VII, bovine, specific activity 1500 U/mg, Sigma Chemical Co., St. Louis, Mo.) was conjugated to partially purified rabbit anti-AHF immunoglobulin with 0.2% glutaraldehyde by reported methods.²⁰,²¹ Unconjugated rabbit anti-AHF immunoglobulin was diluted in 0.05 M sodium carbonate buffer (pH 9.6) to a final working concentration of 100 μg protein/ml. Of this antiserum, 0.2 ml was pipetted into each well of a microhemagglutination plate (rigid-polystyrene, V-type, Dynatech Labs, Alexandria, Va.) and incubated for 2 hr at room temperature in a humidified atmosphere. The plates were then washed 3 times, for 3 min each, with 0.2 ml of phosphate-buffered saline (PBS) (pH 7.4, 0.15 M NaCl, 0.002 M K2HPO4, 0.008 M Na2HPO4, 0.003 M KCl, 0.02% NaN3) containing 0.05% polysorbate-20 (polyoxyethylene sorbitan mono laurate, Sigma Chemical Co., St. Louis, Mo.) (PBS-20). After removal of the third wash solution, 0.2 ml of suitable dilutions in PBS-20 of the plasmas under test were added to each well and incubated for 3 hr at room temperature in a humidified atmosphere. Two dilutions (1/40 and 1/80) of each plasma were tested in triplicate. A standard curve was generated, using dilutions of pooled normal plasma in PBS-20 diluted by twofold steps, 1/10-1/320. After incubation of the test samples, the plates were washed as before, after which 0.2 ml of a dilution of conjugated anti-AHF in PBS-20 was added to each well. The dilution of the conjugated anti-AHF in PBS-20 was determined empirically and was generally in the 1/200-1/400 range. The plates were incubated overnight at 4°C in a humidified atmosphere and then washed as before. 0.2 ml of "phosphatase substrate" (40 mg tablets, disodium p-nitrophenyl phosphate, Sigma) in 40 ml diethanolamine (Fisher Scientific Co., Fair Lawn, N.J.) buffer (97 ml diethanolamine, 903 ml H2O, 0.2 g NaN3, 100 mg MgCl2, H2O, pH 9.8) was added to each well and the plates were incubated for exactly 15 min at room temperature. The enzymatic reaction was stopped by addition of 0.05 ml 3 M NaOH; the mixture was transferred with a Pasteur pipette to a 10-mm cuvette (capacity, 0.3 ml) and absorbence of the test solution at 405 nm was measured in a modified Beckman DU spectrophotometer against a blank consisting of 0.2 ml "phosphatase substrate" and 0.05 ml 3 M NaOH. Values obtained with the two test dilutions were averaged. A linear standard curve was obtained graphically by plotting the logarithm of the optical density against the logarithms of the concentrations of the pooled normal plasma.

In an earlier study, differentiation between normal individuals and obligate carriers was conducted by discriminant analysis, using logarithms of the procoagulant AHF titer and AHF-like antigen to minimize skewing of the data.²² For subjects studied more than once only the first set of determinations were used. The logarithms of procoagulant AHF and AHF-like antigen were assumed to follow a bivariate normal distribution. The group means and covariance matrices were used to estimate the bivariate density functions for normal individuals and obligate carriers.²³

Examination of data obtained in normal women prompted questioning the bivariate normality assumption. Some subjects had unusually high titers of AHF activity and antigen that were not age-related. Although the precise reasons could not be ascertained, estrogen use and unreported or unrecognized stress or disease possibly accounted for some of the high titers.

Rather than arbitrarily exclude values that did not conform to the bivariate normal assumption, the method of discriminant analysis was modified (Jones PK, to be published). In principle, the shape of the bivariate distribution was not specified and a nonparametric rule was employed.²⁴ In constructing the nonparametric rule, the density function (f(x)) for normals was estimated as

\[
\hat{f}(x) = \frac{1}{2\pi n_1 h^2} \sum_{i=1}^{n_1} \exp \left\{ \frac{-1}{2} \left( \frac{x - \bar{x}_i)^T S^{-1}(x - \bar{x}_i)}{h^2} \right) \right\}
\]

where \( x \) was a vector with components consisting of the logarithms of AHF activity and AHF-like antigen, \( n_1 \) was the number of normals, \( \bar{x}_1, \bar{x}_2, \ldots, \bar{x}_{n_1} \) were the observed AHF activity and AHF-like antigen values for normals, \( h \) was a smoothing constant, and \( S \) was the covariance matrix for normal subjects. A corresponding formula for the carrier-estimated density is based on \( n_2 \) carrier values \( x_1, x_2, \ldots, x_{n_2} \):

\[
\hat{f}(x) = \frac{1}{2\pi n_2 h^2} \sum_{i=1}^{n_2} \exp \left\{ \frac{-1}{2} \left( \frac{x - \bar{x}_i)^T S^{-1}(x - \bar{x}_i)}{h^2} \right) \right\}
\]

Classification of \( x \) as a typical normal or carrier vector is based on the ratio of densities \( f(x)/\hat{f}(x) \), just as in the usual discriminant analysis.

Asymptotic value of the smoothing constant, \( h \), was determined theoretically.²⁵ Too small a value of \( h \) resulted in irregularly behaved density estimates, especially in regions where few normals or few carriers were observed. Too large a value of \( h \) resulted in too much smoothing, thereby neglecting the purpose of the procedure. Generally speaking, the larger the number of subjects, the smaller the optimal \( h \).²⁵ Here, \( h = 0.36 \).

The advantage of this nonparametric technique lies in its ability to recognize patterns differentiating normal and abnormal individuals.

**RESULTS**

**Reproducibility of the ELISA Method**

Based on two replicate determinations of 118 samples tested with the ELISA, the coefficient of variation was ± 7.6% with 95% confidence limits of 6.5%-8.7%.

**Correlation of ELISA With the Modified Laurell Technique (Fig. 1)**

In 44 normal women, geometric mean values (± SD) of 1.01 U/ml (+33%, -25%) were obtained as deter-
DETECTION OF CLASSIC HEMOPHILIA CARRIERS

Table 1. Correlation of ELISA Values With Laurell Values

<table>
<thead>
<tr>
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<th>Normal Females (n = 44)</th>
<th>Obligate Carriers (n = 34)</th>
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<tr>
<td>ELISA geometric mean</td>
<td>1.01 (±33%, 25%–4%)</td>
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<td>1.13 (±58%, 37%–7%)</td>
<td>1.12 (±52%, 34%–4%)</td>
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<td>Laurell geometric mean</td>
<td>1.11 (±40%, 28%–5%)</td>
<td>1.29 (±67%, 40%–9%)</td>
<td>1.22 (±72%, 42%–8%)</td>
<td>1.20 (±60%, 37%–4%)</td>
</tr>
<tr>
<td>p*</td>
<td>0.001</td>
<td>0.60</td>
<td>0.02</td>
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<td>(95% confidence limits)</td>
<td>0.820 (0.691–0.899)</td>
<td>0.879 (0.771–0.939)</td>
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*Individuals with von Willebrand’s disease excluded.
†Student’s paired t test, Laurell geometric mean versus ELISA geometric mean, one-tailed comparison.
‡Ninety-five percent confidence limits calculated using Fisher’s r to z transformation for calculation of population correlation coefficients.

Fig. 1. Correlation of AHF-related antigen values determined by the modified Laurell technique and the enzyme-linked immunosorbent assay. The regression line does not include the data obtained in von Willebrand’s disease.

The geometric mean antigen values (±SD) of the total group of 123 subjects (excluding these with von Willebrand’s disease, as explained below) using the ELISA was 1.12 U/ml (+52%, −34%), while that obtained with the modified Laurell technique was 1.20 U/ml (+60%, −37%). The geometric mean values obtained by both techniques differed significantly, with the Laurell method producing values that averaged 6.7% higher than that produced by the ELISA for any given subject (p < 0.001). The correlation coefficient of the individual values (n = 123) was 0.871 (95% confidence limits 0.820–0.908).

The range of AHF-related antigen titers in all individuals (n = 137) tested with the ELISA was 0.03–5.90 U/ml, while that obtained with the modified Laurell technique was <0.15–4.63 U/ml. This lower limit of 0.15 U/ml was arbitrarily assigned to modified Laurell assays of AHF-related antigen in individuals with von Willebrand’s disease in whom no value could be obtained.

In individuals with von Willebrand’s disease, the ELISA of AHF-related antigen yielded measurable values in the range below the sensitivity of the Laurell technique; the coefficient of variation by ELISA was 10.0%. Of the 14 individuals tested, the titer of AHF-related antigen was below a measurable range in 8

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using the modified Laurell technique (Table 2). In those individuals in whom values could be obtained, the coefficient of variation was 16.1%, a value greater than that obtained from any other group of individuals studied by the Laurell method.

**Detection of the Carrier State of Classic Hemophilia**

Nonparametric discriminant analysis using procoagulant AHF titers and concentration of AHF-like antigens, as determined by either the modified Laurell technique or the ELISA method, was applied to the laboratory detection of the carrier state. Thirty-seven obligate carriers were tested using the ELISA, and 110 obligate carriers were tested using the modified Laurell technique.

The geometric mean of functional AHF titers was 0.59 U/ml and the geometric mean of AHF-like antigens was 1.31 U/ml in the 37 obligate carriers tested with the ELISA method. Discriminant analysis of the individual values demonstrated that all of 37 carriers differed significantly from a group of 84 normal women at the 95% level of certainty. At the 99% level of certainty, 35 of 37 (95%) of the obligate carriers could be identified.

The geometric mean of functional AHF titer was 0.54 U/ml and the geometric mean of AHF-like antigen was 1.21 U/ml in the 110 obligate carriers tested with the Laurell technique. Similar discriminant analysis of the individual values yielded a detection rate of 104/110 (95%) at the 95% level of certainty. Of the obligate carriers, 95/110 (86%) could be detected at the 99% level of certainty. The control group for this analysis consisted of 236 normal women.

**Table 2. AHF Values in von Willebrand’s Disease**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Procoagulant AHF (U/ml)</th>
<th>ELISA Antigen (U/ml)</th>
<th>Laurell-Antigen (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. I.</td>
<td>0.27</td>
<td>0.13</td>
<td>&lt;0.15*</td>
</tr>
<tr>
<td>D. I.</td>
<td>0.26</td>
<td>0.18</td>
<td>&lt;0.15*</td>
</tr>
<tr>
<td>J. I.</td>
<td>0.45</td>
<td>0.13</td>
<td>&lt;0.15*</td>
</tr>
<tr>
<td>F. K.</td>
<td>0.35</td>
<td>0.03</td>
<td>&lt;0.15*</td>
</tr>
<tr>
<td>S. K.</td>
<td>0.45</td>
<td>0.06</td>
<td>&lt;0.15*</td>
</tr>
<tr>
<td>R. W.</td>
<td>0.18</td>
<td>0.10</td>
<td>&lt;0.15*</td>
</tr>
<tr>
<td>L. B.</td>
<td>0.08</td>
<td>0.07</td>
<td>&lt;0.15*</td>
</tr>
<tr>
<td>A. G.</td>
<td>0.40</td>
<td>0.24</td>
<td>&lt;0.15*</td>
</tr>
<tr>
<td>L. M.</td>
<td>0.45</td>
<td>0.26</td>
<td>0.67</td>
</tr>
<tr>
<td>D. M.</td>
<td>0.22</td>
<td>0.13</td>
<td>0.30</td>
</tr>
<tr>
<td>W. G.</td>
<td>0.39</td>
<td>0.28</td>
<td>0.61</td>
</tr>
<tr>
<td>D. S.</td>
<td>0.36</td>
<td>0.34</td>
<td>0.66</td>
</tr>
<tr>
<td>A. J.</td>
<td>0.89</td>
<td>0.53</td>
<td>0.78</td>
</tr>
<tr>
<td>J. T.</td>
<td>0.56</td>
<td>0.45</td>
<td>0.61</td>
</tr>
</tbody>
</table>

*In these individuals, AHF-like antigen was not measurable by the Laurell technique; the data are arbitrarily assigned values of <0.15 U/ml, the minimum observable value.

†M. I., D. I. and J. I. are related, as are F. K. and S. K., and L. M. and D. M.

Carrier detection was also performed in the subset of women whose AHF-like antigen was determined by both the ELISA and Laurell techniques. A trivariate linear discriminant function was constructed using logarithms of procoagulant AHF, ELISA AHF-like antigen, and Laurell AHF-like antigen from 34 obligate carriers and 44 normal women.23 The AHF-like antigens determined by the Laurell technique could be safely omitted without compromising discrimination (F-to-remove = 2.50, df = 1 and 74, p > 0.05), but those measured by the ELISA technique could not (F-to-remove = 5.84, df = 1 and 74, p < 0.05). In other words, the ELISA AHF-like antigen values are more clearly separated in the two groups, relative to within-group variability (Table 1). By nonparametric discriminant analysis, all 34 obligate carriers were correctly classified using either ELISA or Laurell; carriers were correctly classified using either ELISA or Laurell; among 11 of these who could not readily be classified by visual inspection, 9/11 (82%) were more strongly classified as carriers by the ELISA values than by the Laurell values.

**DISCUSSION**

Since its introduction by Engvall and Perlmann in 1971,21,22,26 enzyme-linked immunosorbent assay (ELISA) has attained widespread use. Modifications of the basic principles have now been applied to assays of such determinants as viral antibody titers,27 antibodies resulting from infection with malaria,28 insulin,29 thyrotropin,30 and rheumatoid factor31 titers, and the detection of hepatitis A virus antigen and antibody,32 hepatitis B surface antigen, and hepatitis “e” antigen.33 Recently, ELISA has been used by several authors for the determination of AHF-related antigen (factor VIII:Ag).9,13

Using heterologous antisera directed against AHF, Bartlett et al.9 developed a noncompetitive ELISA for AHF-related antigen. Eight obligatory carriers of classic hemophilia, 10 hemophiliacs, 30 individuals with von Willebrand’s disease, and 20 normal individuals were studied. AHF-related antigen was measured with a modified Laurell technique and with the ELISA. In general, the correlation between the results of the two techniques was good. In some determinations, however, the error approaches 100%, perhaps related to the difficulties encountered when measuring low AHF-related antigen levels in individuals with von Willebrand’s disease using the modified Laurell technique.34

Yorde et al.10 have developed a competitive enzyme-linked immunoassay for AHF-related antigen. In a study of 33 normal donors, the assay was found to be reproducible and sensitive to 0.05 U/ml.
DETECTION OF CLASSIC HEMOPHILIA CARRIERS

Utilizing this modified statistical technique of discriminant analysis, we have determined that all of the 37 obligate carriers could be identified at the 97% level of certainty. In contrast to that obtained in other series, the use of the ELISA technique yielded interpretable results in all groups except the obligate carriers. The results in all groups except the obligate carriers were highly correlated with those obtained by the modified Laurell technique. Examination of the values obtained in normal subjects demonstrated that these were not distributed in a normal bivariate fashion even when the data were transformed to logarithms. This anomaly was due to the inclusion of apparently normal individuals in whom inactivation of one X chromosome occurs in each female somatic cell at an early stage of embryonal development. This results in occasional obligate carriers at a level of certainty that misclassified 1% normal women as carriers. This detection rate is similar to that of the modified Laurell technique. Undoubtedly, 100% detection of classic hemophilia with that of the modified Laurell technique.

The correlation coefficient because of the known difficulties encountered when assaying AHF-related antigen levels as determined by the modified Laurell technique was 0.871. Comparison of both results in all groups except the obligate carriers revealed an excellent agreement. The detection rate of the carrier state for classic hemophilia using an immunologic assay for antihemophilic factor (factor VIII) produced the following results in the 123 subjects tested.

7. Stites DP, Hershgold EJ, Perlman JD, Fudenberg HH: Factor VIII:C.


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