Detection of Hemophilia Carriers During Pregnancy

By Leon W. Hoyer, Carl A. Carta, and Maurice J. Mahoney

The accuracy of hemophilia A carrier detection during pregnancy has been determined using combined measurement of VIII:CAg and VIIIR:Ag. These immunoassays detect determinants that are sufficiently stable in plasma that the assays could be done on frozen samples that had been obtained when women were seen for antenatal diagnosis studies (carrier women) or for routine prenatal care (controls). A linear discriminant was calculated that best separated the data for 32 normal women and 25 obligate carriers of the hemophilia gene. Twenty-three of 25 carriers (92%) and all 32 control women were correctly identified in this analysis. The overall classification accuracy (55/57, 96%) is comparable to that obtained by VIII:C and VIIIR:Ag measurements using freshly drawn blood samples in nonpregnant individuals. This study demonstrates that hemophilia A carriers can be detected during pregnancy with sufficient accuracy that the information may be used for genetic counseling.

The combined measurement of factor VIII coagulant activity (VIII:C) and factor-VIII-related antigen (VIIIR:Ag) has markedly improved the accuracy of carrier detection in classic hemophilia. The close relationship of VIII:C and VIIIR:Ag levels in normal plasma, reflecting the association of the separate proteins in a noncovalent complex, permits a much more satisfactory assessment than does measurement of VIII:C alone. The hemophilia carrier has, on the average, a plasma VIII:C level half that of normal women, since each somatic cell expresses either the normal or the hemophilic X chromosome. In contrast, the autosomal gene responsible for factor-VIII-related protein (von Willebrand’s factor) is normal in these women, and this is verified by the normal VIIIR:Ag levels. The combined measurement of the two proteins thereby improves carrier detection, as VIIIR:Ag values provide an “internal control” for VIII:C variation due to transient or sustained physiologic changes.

An important issue in carrier detection is its accuracy during pregnancy, since many women are first seen for genetic counseling when already pregnant. It is unlikely that a large number of pregnant carrier women will be seen in any one hemophilia center over a reasonable period of time, however, and this question has not previously been examined. While Bennett and Ratnoff found that data for two pregnant hemophilia carriers suggest that accurate identification is feasible, they could not establish the precision of genotype assignment during pregnancy.

The development of immunoassays for VIII:CAg and VIIIR:Ag, has been shown to improve slightly the precision of carrier detection in nonpregnant hemophilia carrier women. A systematic evaluation of carrier detection in pregnancy is, therefore, feasible, and our study of hemophilia antenatal diagnosis has provided a sufficient number of samples from obligate carriers of the hemophilia gene. We report here that carrier determination by combined measurement of VIII:CAg and VIIIR:Ag on frozen samples from women in the second trimester of pregnancy provides a sufficiently accurate evaluation of carrier status to be useful in genetic counseling.

Materials and Methods

Carrier and Control Groups

Blood samples from carrier women were obtained at the time of fetoscopy for hemophilia antenatal diagnosis at Yale-New Haven Hospital (19 patients), the University of California, San Francisco (4 patients), Royal Victoria Hospital, Montreal (1 patient), and the University of Lund, Sweden (1 patient). Obligate carriers were daughters of patients with severe classic hemophilia, women who had two sons with hemophilia, and women with a family history of hemophilia and one affected son. Potential carriers did not meet these criteria, but had a family history that indicated that they might be hemophilia carriers (prior probability of 0.25 or 0.5). In every case, the women were in their 18th-21st week of an otherwise uncomplicated pregnancy. A plasma sample from an affected relative was available in over 90% of the cases, and in each instance, it was consistent with severe CRM-negative classic hemophilia (VIIICAg < 0.05 U/ml with a normal VIIIR:Ag level).

The plasma samples from control women were obtained at Yale-New Haven Hospital during a routine visit that was part of their prenatal care. They had no personal or family history of any bleeding disorder, and they were in their 15th-21st week of pregnancy.

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Blood Collection and Analysis

Venous blood samples were anticoagulated with one-ninth volume of sodium citrate and the plasma was separated by centrifugation. Frozen plasma was held at -70°C in polystyrene tubes until it was analyzed 1-15 days later.

VIII:C:Ag was measured by an immunoradiometric assay using 125I-labeled Fab' prepared from the plasma of a nonhemophilic individual who developed an anti-VIII:C.11 VIIIIR:Ag was measured by an immunoradiometric assay using 125I-labeled IgG from a rabbit immunized with human factor VIII.11 The two assays are specific for the two components of the factor VIII complex and neither is affected by the level of the other protein. At least two dilutions of plasma were tested in each assay, and the standard was a plasma pool prepared as previously described and held at -70°C.11 Assay values are expressed by their relationship to this (1 U/ml) standard.

Statistical Methods

VIII:C:Ag and VIIIIR:Ag values were analyzed after logarithmic transformation to normalize the distributions.11 Linear discriminant analysis and probabilistic classification was carried out by the methods described by Elston, Graham, and coworkers.6,8,11 The analysis did not examine a potential age effect in view of the narrow range of ages in the two study populations.

RESULTS

The VIII:C:Ag and VIIIIR:Ag values for 32 pregnant normal women and 25 pregnant hemophilia carriers are given in Fig. 1. A summary of the data and analysis is presented in Table 1. It is apparent that the combined measurement of VIII:C:Ag and VIIIR:Ag provides a good separation and that this is considerably better than the differentiation provided by measurements of VIII:C:Ag alone.

As expected, the VIII:C:Ag and VIIIR:Ag levels were increased in the pregnant normal women (1.17 and 1.23 U/ml) when compared to values (0.97 and 1.09 U/ml) obtained previously for similar groups of nonpregnant women.4,11 Pregnant carrier women had lower VIII:C:Ag levels (0.49 U/ml), however, even though they also had an increase in VIIIR:Ag to 1.42 U/ml. This antigen value is also greater than that observed in a comparable group of nonpregnant carrier women (1.21 U/ml).4

When the data for the two groups were analyzed, a linear discriminant function assigned each individual to one or the other group according to the immunassays: the control group had positive values for $D = 7.92 \log \text{VIII:C:Ag} - 7.66 \log \text{VIIIR:Ag} + 1.133,$ and the carrier group had negative values. Two of the 25 hemophilia carriers and none of the 32 control women were misclassified by this analysis based on VIII:C:Ag and VIIIR:Ag assays. This allocation (55 of 57 individuals assigned to the correct group) is better than that which most laboratories obtain using VIII:C and VIIIR:Ag assays for nonpregnant individuals.2,6

The analysis can be extended to calculate a probability that any potential carrier woman is, in fact, a hemophilia carrier. The VIII:C:Ag and VIIIR:Ag values and the prior probability for 31 potential carriers were examined according to the method described by Elston and coworkers,11 and the results indicate that useful information can be obtained for genetic counseling.

The combined probability for 11 women indicated that they had greater than a 90% likelihood of being hemophilia carriers, and the data for 13 women indicated that the likelihood of being a hemophilia carrier was less than 10%. Only 7 of the 31 women had combined probabilities between 0.10 and 0.90. Thus, 77% of potential carriers could be counseled with data,

### Table 1. Factor VIII Measurements in the Second Trimester of Pregnancy

<table>
<thead>
<tr>
<th></th>
<th>Normal Women (n = 32)</th>
<th>Hemophilia Carriers (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIII:C:Ag (U/ml ± SD)*</td>
<td>1.17 ± 0.33</td>
<td>0.49 ± 0.25</td>
</tr>
<tr>
<td>VIIIR:Ag (U/ml ± SD)*</td>
<td>1.23 ± 0.35</td>
<td>1.42 ± 0.44</td>
</tr>
<tr>
<td>Number classified by discriminant analysis as</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>Hemophilia carriers</td>
<td>0</td>
<td>23</td>
</tr>
</tbody>
</table>

*Geometric means obtained from a logarithmic distribution of the assay data.
indicating better than a 90% chance of carrier or noncarrier status.

While only a single plasma sample was examined for each of the normal and carrier women, we obtained preftoscopy samples from four obligate carriers studied during two different pregnancies. There was remarkable consistency in the paired values that were obtained (Table 2).

**DISCUSSION**

Although many women in hemophilia families first seek genetic counseling when they are pregnant, there have been no previous studies of carrier detection during pregnancy. While factor VIII is increased in pregnancy, it has been suspected that the physiologic changes have comparable effects on the procoagulant protein and the factor-VIII-related protein (von Willebrand factor). Data obtained by Bennett and Ratnoff for 31 pregnant women supported this assumption, and most VIII:C and VIIIR:Ag values were within an extension of the range observed for normal nonpregnant women. Two hemophilia carriers had values that could be considered abnormal in comparison to these control values. In studies as yet reported only in an abstract, Mibashan and coworkers have also demonstrated that VIIIR:Ag is increased in the second trimester of pregnancy for both controls and carriers and that VIII:C and VIII:CAg were also raised, though less, in the controls. Their abstract did not indicate the precision of carrier detection during pregnancy.

Potential difficulties in carrier detection during pregnancy were suggested by the report of van Royan and tenCate, however, for they detected a significant increase in the VIIIR:Ag to VIII:C ratio in late pregnancy. While Denson also detected an increased ratio in late pregnancy, his studies and those of Thornton and Bonnar indicated that the ratio of the two components was minimally changed during the second trimester.

Our laboratories are now able to provide hemophilia antenatal diagnosis for women at risk of having a hemophilic son, and this service has made it possible to collect a large number of samples from pregnant hemophilia carriers. More than 50 women have been studied by fetoscopy during the past 3 yr, and a plasma sample has been analyzed for each patient. When the values for obligate hemophilia carriers are compared to those of normal women at the same stage of pregnancy, it is apparent that a satisfactory separation is obtained by the combined measurement of VIII:CAg and VIIIR:Ag (Fig. 1). Analysis by linear discriminant function correctly classified 55 of 57 women (96%) in this study. Similar precision was obtained when the data were examined by linear regression analysis, i.e., 24 of 25 carrier women and 1 of 32 noncarriers had values outside of the 95% confidence limits for control pregnancies. While both control and carrier women have increased levels of VIII:CAg and VIIIR:Ag, when compared to nonpregnant individuals, and the relationship of the two components is slightly altered, the separation is, if anything, more clear-cut than in previous similar studies of nonpregnant individuals.

While misclassification has been observed in every previous study of hemophilia carrier detection, we have carefully examined the two misclassifications in this series in order to understand their possible bases. As only single determinations were carried out during each pregnancy for both control and carrier groups, it is possible that assay error was responsible for the misclassifications. Although this cannot be determined with certainty, an estimate of the reproducibility of the measurements in two different pregnancies was obtained in four women (Table 2) and it suggests that this is an unlikely explanation. One of the misclassified carrier women, a daughter of a hemophiliac, had repeat determinations 7 mo after the pregnancy, and once again the values fell into the normal range (VIII:CAg = 1.04 and VIIIR:Ag = 1.13 U/ml). There are no other genetic studies available to verify paternity in this individual. The other misclassified carrier woman has had one hemophilic son and an affected fetus, so that the classification as hemophilia carrier is undoubtedly correct. The probability of the carrier state based on assays alone (VIII:CAg = 0.79 and VIIIR:Ag = 1.31 U/ml) was 0.36 in this case and we do not have subsequent assays for this patient. It is recognized, of course, that the random inactivation of one of the X chromosomes in each somatic cell is expected to cause some misclassifications when the assays measure X chromosome gene products.

Our analysis, and the data recently reported by Peake and coworkers, indicate that carrier detection in hemophilia can be done by immunologic assay of VIII:CAg and VIIIR:Ag. In fact, these measurements

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pregnancy</th>
<th>VIII:CAg (U/ml)</th>
<th>VIIIR:Ag (U/ml)</th>
</tr>
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<tbody>
<tr>
<td>CONN 8</td>
<td>1</td>
<td>66</td>
<td>161</td>
</tr>
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<td></td>
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appear to provide somewhat better discrimination between carrier and normal groups. As a consequence, carrier detection appears to be satisfactory for assays carried out on frozen plasma samples, since there is minimal change in VIII:CAg upon freezing and storage. The assay is sufficiently precise that good separation of the two groups has been obtained with a single plasma sample from each tested individual. While slightly greater accuracy might be possible with analysis of several samples, this was not feasible in the group of patients that we studied, and it does not appear to be necessary.

Thus, immunoassays for the two components of the factor VIII complex provide good discrimination between normal women and hemophilia carriers during the second trimester of pregnancy. As a result, genetic counseling is possible for women who have not had carrier studies prior to a pregnancy and it permits the analysis to be carried out at regional or national centers. The results obtained for most potential carriers permit more accurate genetic counseling than is possible by family history alone. Nevertheless, as we and others have recognized in previous studies of hemophilia carrier detection, there is overlap between the carrier and control populations and a few carrier women appear to have normal assay values. This limitation in the present method must be clearly recognized by all individuals who provide genetic counseling for women who are at risk of transmitting hemophilia.

ACKNOWLEDGMENT

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REFERENCES

13. Peake IR, Newcombe RG, Davies BL, Furlong RA, Ludlam CA, Bloom AL: Carrier detection in haemophilia A by immunological measurement of factor VIII related antigen (VIIIIRAg) and factor VIII clotting antigen (VIIICAg). Br J Haematol 48:651, 1981
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