Factor VIII-Related Antigen in the Detection of the Haemophilia Carrier State

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SUMMARY

The levels of factor VIII-related antigen (ERA) in normal White females, in White and Black obligatory haemophilia carriers and in possible haemophilia carriers were compared with the functional factor VIII activity. The results obtained compared favourably with those previously reported, in that the obligatory carrier group demonstrated excess antigen compared with functional factor VIII activity. The F VIII/ERA ratio obtained for each obligatory carrier was compared statistically with the ratio of the normal group. A ratio of 0.65 was the level below which haemophilia carrier status could be predicted within the 95% confidence limits.


The application of immunological techniques to the study of the various functional deficiencies of factor VIII (F VIII) has led research workers towards a fundamental review of the biochemical structure, function and genetic control of the F VIII molecule. The basic observation around which intensive research has revolved has been the finding of a protein molecular component which reacts to form precipitate with specific antisera raised in rabbits to crude and purified F VIII. This protein component has been observed to be present in normal plasma and haemophilic plasma, but was either reduced or absent in plasma from patients with Von Willebrand’s disease. Furthermore, these antisera react with normal F VIII and inhibit its procoagulant activity.

While many basic questions as to the structure and function of the F VIII molecule still remain unanswered, it has become apparent that this observation can be applied to solve certain problems, viz. the diagnosis of Von Willebrand’s disease and the haemophilia carrier state. It is generally known that the mean plasma level of F VIII in carriers is lower than that of a matched group of normal females. However, until recently the carrier state could only be detected with certainty in approximately 25% of haemophilia carriers, because of the wide range of F VIII levels observed in carriers and normals, which resulted in considerable overlap. In normal women, there is a close correlation between the levels of procoagulant F VIII and the immunologically reacting molecule (VIII-related antigen or ERA). This is not the case in the majority of carrier females, where it has been observed that there is excessive ERA protein in relationship to the clot-promoting activity.

Zimmerman et al. were the first to apply this technique to the detection of haemophilia carriers, and showed that over 90% of obligatory carriers could be positively identified. These studies have been confirmed and extended with some workers expressing their results as a ratio of procoagulant activity to ERA. With this ratio, the cut-off point between the normal and the carrier state was found to be around 0.7.

This article reports the diagnostic criteria for determining the haemophilic carrier status that have been established in our laboratory by the comparison of normal and obligatory carrier groups.

MATERIAL AND METHODS

Plasma from 15 haematologically normal White adult females was obtained, as well as plasma from 11 White and 2 Black obligatory haemophilia carriers. An obligatory carrier is defined as the daughter of a haemophiliac, the mother of two haemophilic sons, or the mother of one haemophilic son, but with other members of the family affected. Eighteen White, 1 Black and 4 Coloured possible carriers were also studied. The category of possible carrier includes the mother of one haemophilic son but with no family history of haemophilia, the daughter of a carrier and the cousin or any other female relative of a haemophiliac.

Blood was collected via plastic syringes into plastic tubes containing 3.8% trisodium citrate in a ratio of 9:1 (v/v).

A two-stage F VIII assay of procoagulant activity was carried out according to the procedure described by Biggs. One unit of activity is equivalent to the amount of F VIII present in 1 ml pooled normal plasma.

The ERA was assayed with a commercial anti-F VIII antigen (Behringwerke). The procedure used was a modification of that described by Zimmerman et al.

To 15 ml of boiled agarose solution cooled to 56°C, pH 8.6 (0.05M barbitral buffer), was added 0.015 ml of the anti-F VIII antiserum. The agarose was then poured onto a glass plate measuring 90 x 110 mm. The agarose was allowed to set before 5 μl of the test or control plasma was applied to the prepared sample wells of 2.5 mm diameter. Electrophoresis was carried out either over 3 hours (7 - 10 v/cm gel) or overnight (2 - 3 v/cm gel) at pH 8.6 and ionic strength 0.02 (0.05M barbitral buffer).
The gels were then flattened, washed initially in 0.9% saline and then in distilled water, and finally dried in a warm oven. They were stained with Coomassie brilliant blue R 0.8 g/100 ml in an ethanol : acetic acid : water mixture (4:2.5:4). The stained gels were de-stained by washing 3 times in an ethanol : acetic acid : water mixture (4.5:1:4.5).

One unit of ERA was equivalent to the amount of antigen present in 1 ml normal pooled plasma.

Each gel was standardised by running four dilutions of standard plasma obtained from a pool of the 15 normal adult females. The length of the rocket-shaped precipitates was measured and a standard curve was plotted on log-log paper. Each test sample was run in two dilutions and the concentrations of test ERA were obtained by reading off as a percentage from the standard curve, the final concentration being obtained by multiplying by the dilution factor. The gels were discarded if the precipitates did not run parallel to the sides of the glass plate and also if the standard curve failed to give a straight line. Test samples were re-run if the two dilutions failed to correlate closely. Many of the possible carriers were re-tested at least once; others were retested whenever the results were equivocal.

RESULTS

Table I shows the mean levels and ranges of F VIII, ERA and the F VIII/ERA ratio established for the normal group and also for the obligatory carriers (White). Statistical analysis showed that the normal female group differed significantly from the obligatory carrier group in respect of their mean F VIII levels and F VIII/ERA ratios ($P > 0.001$). Nevertheless, the ERA levels did not differ significantly. When individual obligatory carrier F VIII/ERA ratio levels were compared with that of the normal group as a whole, it was found that the ratio of 0.65 was the cut-off point for statistical significance ($P > 0.5$); below this there would be a 95% chance of demonstrating haemophilia carrier status. These results are shown graphically in Fig. 1.

It may be seen that the results obtained for the White obligatory carriers apply equally well to those of the 2 Black subjects. While 8 of the obligatory carriers showed F VIII levels below 50%, all 13 women showed an abnormal F VIII/ERA ratio.

The results obtained by comparing the normal female levels with those of the obligatory carriers were then applied to a group of possible carriers (Fig. 2). The possible carriers can be divided into two groups: those falling below the 0.65 ratio level (11 individuals), and those lying above this level (12 individuals).

Table I. Means and Ranges of F VIII, ERA and the F VIII/ERA Ratio in 15 Normal White Females and in 11 Obligatory White Haemophilia Carriers

<table>
<thead>
<tr>
<th></th>
<th>F VIII (%)</th>
<th>ERA (%)</th>
<th>F VIII/ERA ratio</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>Normal White females</td>
<td>15</td>
<td>47 - 130</td>
<td>95</td>
</tr>
<tr>
<td>Obligatory carriers (White)</td>
<td>11</td>
<td>25 - 67</td>
<td>45</td>
</tr>
</tbody>
</table>
Only 1 individual classified as ‘normal’ had a F VIII functional level of less than 50%. The ratio in this case, however, was 0.89, well above the 0.65 cut-off point. Four of the individuals classified as ‘carriers’ demonstrated F VIII activity levels greater than 50%, but in only 1 case did the ratio approach that of the cut-off point. One possible carrier closely approached the 0.65 level. She demonstrated a F VIII activity of 68%. All 5 of the non-White possible carriers fell within the expected range of their respective groups. The detailed results of each of the Black and Coloured obligatory or possible carriers are shown in Table II.

<table>
<thead>
<tr>
<th>Black Obligatory Carriers</th>
<th>ERA</th>
<th>F VIII</th>
<th>F VIII/ERA Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>197</td>
<td>47</td>
<td>0.41</td>
</tr>
<tr>
<td>2</td>
<td>111</td>
<td>57</td>
<td>0.51</td>
</tr>
<tr>
<td>Possible Carriers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (sister)</td>
<td>199</td>
<td>176</td>
<td>0.88</td>
</tr>
<tr>
<td>Coloured Possible Carriers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (sister)</td>
<td>154</td>
<td>57</td>
<td>0.37</td>
</tr>
<tr>
<td>2 (aunt)</td>
<td>154</td>
<td>162</td>
<td>1.1</td>
</tr>
<tr>
<td>3 (cousin)</td>
<td>157</td>
<td>156</td>
<td>1.0</td>
</tr>
<tr>
<td>4 (sister)</td>
<td>133</td>
<td>182</td>
<td>1.3</td>
</tr>
</tbody>
</table>

### DISCUSSION

The normal ranges and means for F VIII, ERA and the F VIII/ERA ratio were established for our laboratory by testing 15 normal White women. A study of 13 obligatory carriers showed that the F VIII levels and F VIII/ERA ratios differed significantly from those of the normal group. The ERA levels, as expected, did not. These results correlate closely with those observed by Zimmerman et al. and Denson and Ingram. The differences established for each obligatory carrier were significant up to the 95% confidence limits, and were similar for White, Black and Coloured groups.

Examination of the F VIII/ERA ratio of each obligatory carrier and its statistical comparison with that of the normal group, showed that the ratio of 0.65 was the point beyond which statistical significance was obtained for this carrier group. Other workers have found a ratio of 0.7 to be the cut-off point between the normal and the carrier state. It is suggested, therefore, that the cut-off point should be assessed independently for each laboratory.

On application of these techniques to 23 possible carriers, two groups of approximately equal numbers were separated by the 0.65 ratio cut-off level. The total group was a heterogeneous one, being composed of mothers of one haemophilic son (with no family history), sisters of haemophilia carriers, and also cousins and other relatives of haemophilia sufferers. A few individuals showed a ratio close to the cut-off point. In each of these, as well as in the majority of possible carriers studied, the assays were repeated at a later date to confirm the initial assessment.

The identification of haemophilia carriers, using both the F VIII functional assay and the ERA levels to assess carrier status, is not a simple procedure. The techniques employed to measure these two parameters are subject to artefact and have inherent limitations. They must be carefully controlled and standardised. In addition, the levels of F VIII and ERA in any one individual on any particular day are subject to a variety of biological control factors and frequently vary from day to day.

In view of the limitations of the two techniques used to detect the haemophilia carrier state, and because of the influence the result would have upon subsequent genetic counselling, it is considered important to repeat the studies, to confirm or reject the initial assessment. Where the results do not correlate, a third assessment is warranted.

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### REFERENCES