Problems in the detection of carriers of haemophilia A

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SYNOPSIS Factor VIII activity and factor VIII related—or Willebrand—antigen were studied in 49 known carriers of haemophilia A and 31 normal women, and the data were analysed by four statistical approaches. Sixteen per cent of normals and 18% of carriers were misclassified, overlapping with the other group.

Although the percentage of carriers detected is higher when taking into account the results of both biological and immunological factor VIII, it is lower than others recently reported, and the discrepancies between the results obtained are discussed.

Haemophilia A is X-chromosome linked and is characterized by decreased plasma factor VIII (anti-haemophilic factor A) levels as measured by specific clotting assays. The detection of carriers of haemophilia A remains a difficult and still controversial problem. Previous attempts at defining them have depended upon the measurement of factor VIII clotting activity (Soulier and Josso, 1966). It has been shown that, as a group, carriers have a lower mean factor VIII activity than normal women; however, only 40-50% of carriers have low factor VIII values; there exists a large overlap between normals and carriers which may be related to the technical inadequacies of factor VIII determination and random inactivation of one of the two X chromosomes (Lyon, 1961; Russell, 1964). Furthermore, factor VIII varies widely in normal women (Ingram, 1965) as well as in carriers. As a result, this method alone is unsatisfactory since it allowed the detection of only 30-40% of carriers.

Recent studies (Zimmerman *et al*, 1971a; Stites *et al*, 1971; Meyer *et al*, 1972; Hoyer, 1972) have demonstrated the presence of normal or increased amounts of an inactive protein, antigenically similar to normal factor VIII, in the plasma of all haemo-

philia A patients tested. This protein is called 'factor VIII-related antigen' or 'Willebrand antigen' since it is present in normal individuals and haemophilia A patients and absent in patients with von Willebrand's disease. Using a quantitative immunological assay, Zimmerman *et al* (1971b) have shown that the ratio between factor VIII activity and Willebrand antigen was significantly lower in the cryoprecipitate of known carriers than of normal women. This ratio allowed Zimmerman *et al* (1971b) to identify with 99% certainty 92% of obligatory carriers. These data have been confirmed and extended to a larger series of heterozygotes by Bennett and Ratnoff (1973).

Factor VIII activity and Willebrand antigen were studied in 49 known carriers of haemophilia A and 31 normal women. Although in agreement with previous reports when the mean results of the two populations are considered, many problems concerning the discrimination of carriers from normal women remain. Therefore our data have been submitted to four different statistical approaches. An evaluation of their validity both in defining each population and in detecting the carrier state on an individual basis was attempted.

Materials and Methods

The group of carriers included 49 obligatory carriers (daughters of haemophiliacs, women with more than one affected son or with one affected son and a family history of haemophilia). None of the carriers was

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known to be pregnant. The control group included 31 non-pregnant females, laboratory and hospital personnel with no familial or personal bleeding tendency.

FACTOR VIII ASSAY

Blood was anticoagulated with 3.8% trisodium citrate (1 volume:9 volumes blood); the plasma was separated by centrifugation at 3000 g for 20 minutes at +4°C. The assay of factor VIII activity was carried out on fresh plasma within 3 hours after sampling with a modification of the partial thromboplastin time using kaolin and haemophilia A plasma (factor VIII <1%) as substrate. Normal range: 50-200%. The rest of the plasma was stored at -85°C and used for Willebrand antigen assay. All determinations were done in blind and duplicate. Standard reference plasma was a pool of plasmas from 20 normal women (age 20-55 years) kept at -85°C for no longer than two months. The same pool served as reference for the measurement of both factor VIII activity and Willebrand antigen.

WILLEBRAND ANTIGEN ASSAY

An antiserum to normal purified factor VIII was raised in rabbits and absorbed as previously described (Meyer *et al*, 1972). Such an antiserum reveals the existence of only one line of precipitation, present in normal and haemophilia A plasma, and lacking in von Willebrand's disease plasma. Assay of Willebrand antigen was carried out on fresh or frozen (-85°C) plasma by a modification of the Laurell (1966) quantitative immunoelectrophoretic technique using the previously described antiserum. Instead of measuring the antigen on cryoprecipitate (Zimmerman *et al*, 1971b), our modification was to use plain plasma at different dilutions (1/2, 1/4, 1/8, and 1/16) deposited in a single well (diameter 3 mm) (Meyer *et al*, 1974). All determinations were done in blind and triplicate.

STATISTICAL ANALYSIS

The contingency table was calculated according to

Fisher (1936). The distribution of the mean ratio of factor VIII activity to Willebrand antigen and of the weighted mean of difference between the two entities were performed as described by Snedecor and Cochran (1967) using the rule of optimized decision of Bayes, described by Peterson (1965). Tolerance ellipses were calculated according to Seal (1968). The discriminant analysis was performed using a computer for treatment of the data. The distance (λ^2) of each individual point was calculated to the centrum of both ellipses, and each normal or carrier tested was affected to the group corresponding to the smallest distance, as measured with the equation of the ellipses. The confidence probability for each individual point to belong to one or the other group was estimated with the chi square table (Cramer, 1967) (freedom degree: 2) since λ^2 had a distribution corresponding to it.

Results

POPULATION ANALYSIS

Results of factor VIII activity, Willebrand antigen, specific ratio (factor VIII activity to Willebrand antigen), and difference between Willebrand antigen and factor VIII activity are listed in table I. As a mean, factor VIII activity and the specific ratio are lower in carriers than in normals. The difference between the two entities is significantly higher in carriers. Contrarily, Willebrand antigen is similar in both groups. In carriers of severe haemophilia A (table I), the mean of both factor VIII activity and Willebrand antigen appears lower than in carriers of moderate haemophilia A, or of haemophilia A with antibody to factor VIII, but these results may be of no significance since the number of carriers in the last two groups is small.

INDIVIDUAL ANALYSIS

The data obtained in normal women and carriers have been submitted to four different statistical approaches.

	Factor VIII Activity %	Willebrand Antigen %	Ratio	Difference	
Normals	93.81 ± 0.25 ¹	96.62 ± 0.25	0.98 ± 0.17	2.90 ± 2.40	(NS 5.10 ⁻³)
Carriers of haemophilia	61.09 ± 3.26	96.70 ± 3.66	0.64 ± 0.29	33.64 ± 7.65	(S < 1.10 ⁻⁴)
Severe (35)	59.35 ± 3.89	92.5 ± 4.07	0.68 ± 0.04	23.39 ± 4.77	
Moderate (7)	65.85 ± 8.43	100.00 ± 11.78	0.67 ± 0.11	42.26 ± 14.11	
With antibody (7)	66.78 ± 5.42	109.67 ± 9.42	0.64 ± 0.08	49.17 ± 15.60	

Table I Results of factor VIII activity, Willebrand antigen, specific ratio (factor VIII activity to Willebrand antigen), and difference between Willebrand antigen and factor VIII activity in 31 normals and 49 carriers, according to the type of haemophilia A (35 carriers of severe haemophilia A; 7 carriers of moderate haemophilia A; 7 carriers of haemophilia A with antibody to factor VIII)

¹Mean ± SEM

1. Fisher's Contingence Table

When taking into account the results of both populations together, the weighted mean of factor VIII activity and Willebrand antigen is respectively 74.9% and 96.6%. Using Fisher's contingency table (table II), the percentage of misclassification is 19.8% among normals (6 out of 31 = cases 2, 5, 6, 13, and 21) and 26.5% among carriers (13 out of 49 = cases 5, 6, 7, 12, 13, 19, 24, 28, 33, 38, 43, 46, and 49).

2. Distribution of the Ratio of Factor VIII Activity to Willebrand Antigen

Figure 1 shows the distribution of this specific ratio, with the determination of the optimized limit of

A			B
	8 N 3 C	17 N 10 C	
74.9			
	6 N 25 C	0 N 11 C	
C			D
	96.6		
	Willebrand antigen		

Table II Fisher's contingency table. Weighted mean of factor VIII activity (74.9%) and Willebrand antigen (96.6%). Percentage of misclassification : 19.8% for normals (N) and 26.5% for carriers (C)

The weighted mean of factor VIII activity is

$$\bar{y} = \frac{(n_N \bar{y}_N) + (n_C \bar{y}_C)}{(n_N + n_C)} = 74.90$$

n = number of cases

The weighted mean of Willebrand antigen is

$$\bar{x} = \frac{(n_N \bar{x}_N) + (n_C \bar{x}_C)}{(n_N + n_C)} = 96.67$$

Normals and carriers are affected to one of the four squares according to the results of both factor VIII activity (y) and Willebrand antigen (x)

A 8 N = cases 22-25, 26, 29, and 30
3 C = cases 28, 33, and 46

B 17 N = cases 1, 3, 4, 7, 8, 10, 11, 12, 14-20, 28, and 31
10 C = cases 5-7, 12, 13, 19, 24, 38, 43, and 49

C 6 N = cases 2-5, 6, 9, 13, and 21

25 C = cases 2-4, 9-11, 16-18, 20-22, 25, 26, 29, 30, 32, 34-36, 40-42, 44, and 45

D 11 C = cases 1, 8, 14, 15, 23, 27, 31, 37, 39, 47, and 48

Fig 1 Distribution of the ratio of factor VIII activity to Willebrand antigen in normals (31) and carriers (49): T = discriminant optimized limit to the decision rule of Bayes

$$T = h_N \frac{R_N + hc}{h_N + hc} R_C = 0.8456$$

$$h \text{ weighted coefficient} = \frac{1}{\sqrt{2s^2}}$$

s^2 = estimated variance

N = normals

C = carriers

$\bar{R}$ = mean ratio of factor VIII activity to Willebrand antigen.

The small numbers represent the carriers and normals:

Misclassified normals: 2, 5, 10, 16, 20, 29, 31

„ carriers: 5, 7, 9, 12, 13, 15, 24, 26, 28, 32, 33, 40, 42, 46

Bayes (T); 22.5% of the normals (7 out of 31) and 28.5% of the carriers (14 out of 49) are misclassified.

3. Distribution of the Weighted Mean of Difference between Willebrand Antigen and Factor VIII Activity

The same mode of representation was used, except

Fig 2 Dispersion diagram of Willebrand antigen and factor VIII activity in normals and carriers, with 50% (inner) and 95% tolerance ellipses.

$$\lambda^2 = \frac{1}{1 - \rho^2} \left[\left(\frac{x - \bar{x}}{sx} \right)^2 - 2\rho \left(\frac{(x - \bar{x})(y - \bar{y})}{sx sy} \right) + \left(\frac{(y - \bar{y})}{sy} \right)^2 \right]$$

λ^2 = statistical distance (see Methods)

ρ = correlation coefficient

x = factor VIII activity

y = Willebrand antigen

s = standard deviation

The equation shows that each value of λ^2 corresponds to an ellipse. For increasing values of λ^2 , these ellipses contain an increasing percentage of the samples studied.

that for this analysis all the results of both assays for the two groups were taken into account, and not only their mean as previously; 29% of normals and 22.4% of carriers are misclassified.

4. Discriminant Analysis

Figure 2 shows the results of factor VIII activity and Willebrand antigen, transformed in log values, together with the 50% and 95% tolerance ellipses for both groups. These ellipses are different since the correlation coefficient (ρ) is distinct in the two groups. The long axis of the ellipses corresponds to the variability of Willebrand antigen and is similar in both groups; the other axis is quite different since the variability of factor VIII activity is small for the control group and large for the carrier group. The distinction between the two groups studied is not clear since the distance between the centrum of the two ellipses is small.

A discriminant analysis using a computer was then applied to the results. The distance (λ^2) of each individual point was calculated to the centrum of both ellipses, and examples of results are given in table III. For case 1 among the normals, the distance towards the centrum of the normal ellipse (0.129) was smaller than that towards the centrum of the carrier ellipse (1.760). She had a 94% confidence probability of belonging to the normal group and 42% of belonging to the carrier group. Therefore she was classified as normal. For case 33 among the carriers, the distance was smaller towards the centrum of the normal ellipse. She was classified as a normal, with 97% confidence probability. According to this analysis, 6 out of 31 normals (19.3% = cases 2, 5, 9, 16, 20, and 31) and 9 out of 49 carriers (18.3% = cases, 5, 7, 12, 13, 15, 24, 28, 33, and 46) are misclassified.

The misclassified normals and carriers according

		Factor VIII Activity %	Willebrand Antigen %	λ^2 Normal Group ¹	λ^2 Carrier Group ¹	% Confidence Probability		Classification
						Normal	Carrier	
Normals	1	97	105	0.129	1.760	94	42	N
	2	60	71	1.873	0.510	47	78	C
	5	60	88	4.258	0.046	12	97	C
Carriers	7	121.5	136	1.982	4.383	42	12	N
	25	56.5	90	5.965	0.038	6	97	C
	33	86.5	91	0.079	1.213	97	55	N

Table III Examples of computerized results of a discriminant analysis in normals (N) and carriers (C)

¹See fig 2

Fig 3 Misclassified normals and carriers (designated by their order number) according to 1, 2, 3, or 4 of the statistical approaches used. For example, 2 normals (cases 2 and 5) and 5 carriers (cases 7, 13, 28, 33 and 46) are misclassified by all (4) statistical approaches.

to the four statistical approaches are listed in fig 3; 16% of normals and 18% of carriers are misclassified by at least three statistical methods.

Discussion

The measurement of factor VIII activity alone is a poor way of identifying carriers of haemophilia, except in 30-40% of them with factor VIII below 50% on three different occasions (Soulier and Josso, 1966). No judgment can be made concerning the carrier state in a woman whose level of factor VIII is above 50%. It has been stated that estimation of the ratio of factor VIII activity to Willebrand (or factor VIII-related) antigen is of valuable help in genetic counselling for haemophilia A (Zimmerman *et al*, 1971b; Bennett and Ratnoff, 1973). This specific ratio has been found to increase the score of carrier detection to 92-95%, both studies using the same source of antiserum, and the determination of factor VIII-related antigen being done first on cryoprecipitate (Zimmerman *et al*, 1971b), then on plasma (Bennett and Ratnoff, 1973). Similar data, but based on the study of only a small number of carriers, were reported by Bennett and Huehns (1970) using an inhibition neutralization technique. According to Bennett and Ratnoff (1973), 50% of daughters of known heterozygotes (with an even chance of being normal) were tested as carriers, and this detection was also possible in pregnant women,

although this fact is disputed by others (van Royen and Ten Cate, 1973).

In this study of a large series of obligatory carriers (49) and normal women (31), we found that the mean Willebrand antigen was not significantly different in carriers from that of normals, unlike Zimmerman *et al* (1971b) who found it higher. The data in the two populations were analysed by four statistical methods and the normals and carriers were considered to be misclassified when the results of at least three statistical approaches (including the discriminant analysis using a computer) were in agreement. Using such criteria, 16% of normals and 18% of carriers were found to overlap with the other group.

Although the percentage of carriers detected is higher when taking into account the results of both biological and immunological factor VIII, it is low when compared to the others previously reported (Zimmerman *et al*, 1971b; Bennett and Ratnoff, 1973). These discrepancies could theoretically be related to technical differences in the assay of both factor VIII activity and Willebrand antigen or to the use of antisera with slightly different specificity. Furthermore, our results were analysed by a different approach from that previously used since both parameters studied (factor VIII activity and Willebrand antigen) are variable. However, our present data are in agreement with those of Bouma *et al* (1975), Prentice and Forbes (1974), and Zimmerman (1974) who tested another and larger series of carriers. According to these authors, 20-30% of 'hidden' known carriers are not detected by the methods presently used.

The observation that the plasma of female carriers contains functionally active and antigenically detectable, but inactive, material was reported to be consistent with the Lyon (1961) and Russell (1964) hypothesis of random inactivation of a portion of both normal and mutant X chromosome. According to this hypothesis, the average concentration of factor VIII activity in carriers should be 50%. Results in individual carriers vary widely; in the extreme case, a carrier might synthesize little or no

functional factor VIII and because of this be symptomatic. Since it is X chromosome dependent, the Lyon hypothesis may explain results of factor VIII activity in carriers. However, factor VIII related (or Willebrand) antigen is autosomal dependent and thus the Lyon hypothesis should not be applied to it. It remains to be established whether further progress for the detection of the carrier state will be achieved by refinement of the determination of both factor VIII activity and Willebrand antigen, or whether in a proportion of carriers there may be inactivation of all the X chromosomes from the abnormal parent, resulting in such a carrier in normal amounts of factor VIII activity. These individuals thus would not be identified by the present methods.

It is not yet known what autosomal dependent entity(ies) is (are) measured with the heterologous antisera employed and what is the relationship between factor VIII activity (X chromosome dependent) and Willebrand antigen (autosomal dependent). Both entities vary in a parallel manner in a number of physiological or non-physiological conditions (Bennett and Ratnoff, 1972; Prentice *et al*, 1972). However, they may be separated in a number of situations: transfusion in von Willebrand's disease (Bennett *et al*, 1972), immunoadsorption (Zimmerman and Edgington, 1973) or dissociation with either sodium chloride 1 M (Weiss *et al*, 1972; Meyer *et al*, 1974) or calcium chloride 0.25 M (Owen and Wagner, 1972). Thus factor VIII activity and Willebrand antigen seem to be two biologically linked but distinct molecules. If this is so, more data concerning the synthesis and the metabolism of these entities are needed before elucidating with certainty the problem of the detection of carriers.

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