Detection of Carriers of Classic Hemophilia Using an Immunologic Assay for Antihemophilic Factor (Factor VIII)

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ABSTRACT The relation between functional antihemophilic factor (AHF) activity and AHF-like antigen was studied in the plasma of 25 known carriers of hemophilia. In 23 cases, this relationship was significantly different from that in normal women, at the 99% limit of confidence. In contrast, among families in which only one case of hemophilia had occurred, only five of nine mothers could be identified as carriers. This observation suggests that in some instances the hemophilia arose from a newly mutant gene. The data are consistent with the hypothesis that the proportion of antigen to AHF activity in carriers is determined by random activation or inactivation of the X chromosome.

INTRODUCTION

In classic hemophilia, a functional deficiency of antihemophilic factor (AHF, factor VIII) is inherited as an X-linked trait. Previous attempts to define the carrier state in this disease have depended upon measurement of AHF procoagulant activity (1–11). As a group, carriers of hemophilia have a lower mean AHF activity than normal women, while nearly half of individual carriers have titers of AHF that are distinctly low. In many carriers, however, AHF activity is within the normal range.

Recently, we developed an immunoassay for AHF, with which we demonstrated that plasma in classic hemophilia contains at least normal amounts of material antigenically related to AHF but in a form functionally defective in clotting systems (12). Using this assay, we have reexamined the problem of identifying carriers of hemophilia. Compared to normal individuals, carriers have less functional AHF activity than AHF-like antigen. This relative deficiency of functional activity compared to antigen has allowed us to identify 92% of carriers correctly at a level of certainty that would misclassify less than 1% of normal individuals as carriers. Our findings also support the view that some sporadic cases of hemophilia may arise from spontaneous mutation. Our data are consistent with the hypothesis of random activation or inactivation of the X chromosome in hemophilic carriers and predict the occasional carrier who is symptomatic.

METHODS

The techniques used for the preparation of *human plasma, crude ethanol concentrates of AHF,* and *absorbed antibody* against purified AHF, and for the *assay of functional AHF activity* and *AHF-like antigenic material* are described in the accompanying paper (12). The absorbed antibody, prepared in rabbits, behaved as if it were specific for antigens related to antihemophilic factor.

A "standard plasma" was prepared from pooled citrated plasma prepared from the blood of 25 normal white adult male subjects between the ages of 21 and 40 yr, as described in the accompanying paper (12). The pooled plasma, before freezing for storage, was said arbitrarily to contain 1.0 U of AHF activity per ml (13). The amount of antigenic material present in ethanol concentrates of frozen pooled plasma was arbitrarily defined as 1.0 antigen U/ml. *This arbitrary unitage is independent of that used to quantify functional AHF. There is no reason to assume that the unitages of functional and antigenic AHF are identical.* In each determination of antigenic activity, serial twofold dilutions of the ethanol concentrate of the standard plasma were tested along with suitable dilutions of similar concentrates of the plasma to be tested.

The term "carrier" will be used to designate the daughter of an individual with classic hemophilia, the mother of more than one hemophiliac, or the mother of a single hemophiliac who has other relatives with this disease. 25 such carriers, members of 17 families, were studied. The AHF activity in the affected males in these families ranged from less than 0.01 to 0.34 U/ml; the majority had titers less than 0.05

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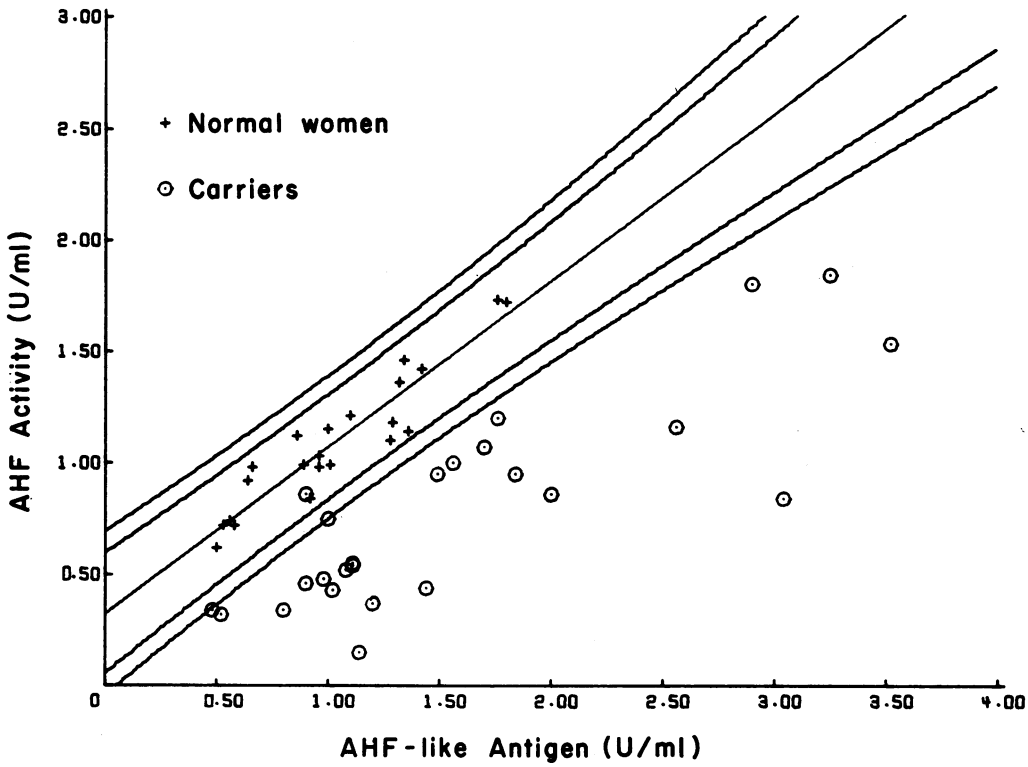

FIGURE 1 The relationship of AHF activity to AHF-like antigen in carriers and normal women. The center line is the regression line for the data obtained from normal women, the outermost lines represent the 99% confidence belt, and the other two lines represent the 95% confidence belt. The regression equation for normal women was $y = 0.33 + 0.74x$, where y is AHF activity in units per milliliter, and x is the concentration of antigen in antigen units per milliliter. The regression equation for the carriers was $y = 0.08 + 0.45x$. The slopes of the two regression lines did not differ significantly from each other.

U/ml. The term "possible carrier" describes those women who had only one affected son and no other known affected relatives.

AHF activity and *concentration of AHF-like antigen* were determined in 22 normal female subjects between the ages of 21 and 54 yr. AHF activity was plotted against antigen concentration, and a regression line was calculated by the method of least squares. The 95 and 99% confidence belts for individual observations were calculated by a standard formula (14). The frequency distribution of AHF activity in normal subjects is slightly skewed, and skewness is reduced by the use of a logarithmic plot (5, 13). For this reason, geometric means for AHF activity and AHF-like antigen were calculated as the antilog of the mean of the logarithm of individual values.

RESULTS

The regression line relating AHF activity in plasma to AHF-like antigen in ethanol concentrates of plasma in 22 normal women was drawn along with the 95 and 99% belts for individual observations (Fig. 1). Among the 25 carriers tested, the relationship between AHF activity and AHF-like antigen differed from normal at the 99% limit of confidence in all but two cases. The two carriers not identified belonged to families in which a typical pattern of inheritance was present; the disorder was severe in one family and mild in the other.

AHF activity alone was not as satisfactory a way of discriminating the plasmas of carriers from those of normal women, as only 12 of the 25 fell below the 95% range of normals, and six fell below the 99% range. The geometric mean AHF activity of 22 plasmas, obtained from normal women, was 1.06 U/ml; the lower end of the 95% confidence range of normal plasma was 0.61 U/ml, and the lower end of the 99% confidence range was about 0.44 U/ml. The geometric mean AHF activity of the carriers was 0.67 U/ml.

The geometric mean AHF-like antigen concentration in normal women was 0.97 U/ml, while that of carriers was 1.37 U/ml (significantly different at the 0.001 level). On the average, the arithmetic ratio of AHF activity to antigen was 1.11 in normal women and 0.52 in carriers. The ratio of activity to antigen ranged from 0.84 to 1.49 in the normal subjects and from 0.13 to 1.04 in the carriers.

Nine suspected carriers were tested. In four of these individuals the relationship of antigen to activity was within the 99% confidence belt for normal women (Fig. 2). This proportion was significantly greater than for the carrier group ($P = 0.03$ by the Fisher exact test). None of the four women not identified as carriers by this method had abnormally low plasma AHF activity.

DISCUSSION

Many investigators have attempted to identify hemophilic carriers on the basis of their functional AHF titers (1–11). In all sufficiently large modern series, some carriers had abnormally low titers of AHF, but in most the majority fell within the normal range. Determination of functional AHF activity relative to AHF-like antigen has allowed us to identify 92% of a group of carriers as outside the 99% limits of normal. By contrast, using the AHF titers alone, only 48% were identified as being outside the range which included 95% of the normals, and only 24% outside the 99% range.

In any series of hemophiliacs, a sizable portion of patients do not have a family history of this disorder. In our series, sporadic cases were detected in 9 of 26 families studied. Of these nine, four mothers fell within the 99% confidence limits of normality, as judged by the ratio of AHF activity to antigen concentration; none of the four had abnormally low AHF activity in her plasma. In contrast, only two of the 25 known carriers fell within this range. The difference between these two groups is statistically significant. At least some of these mothers of patients lacking a family history of hemophila are probably not carriers, the aberration arising in the affected patient by mutation within the X chromosome of the ovum from which he developed.

Hemophilic males are known to have at least normal amounts of antigenic material related to AHF in concentrates of their plasma despite their functional AHF deficiency (13). Thus, they possess a functionally defective form of AHF, the production of which is governed in some way by a gene on the X chromosome. Carrier women had about one-half the ratio of AHF activity to antigen (0.52) compared to normal women (1.11), suggesting that they too synthesize some functionally inactive AHF. Similar findings were observed in two carriers in one hemophilic family reported in

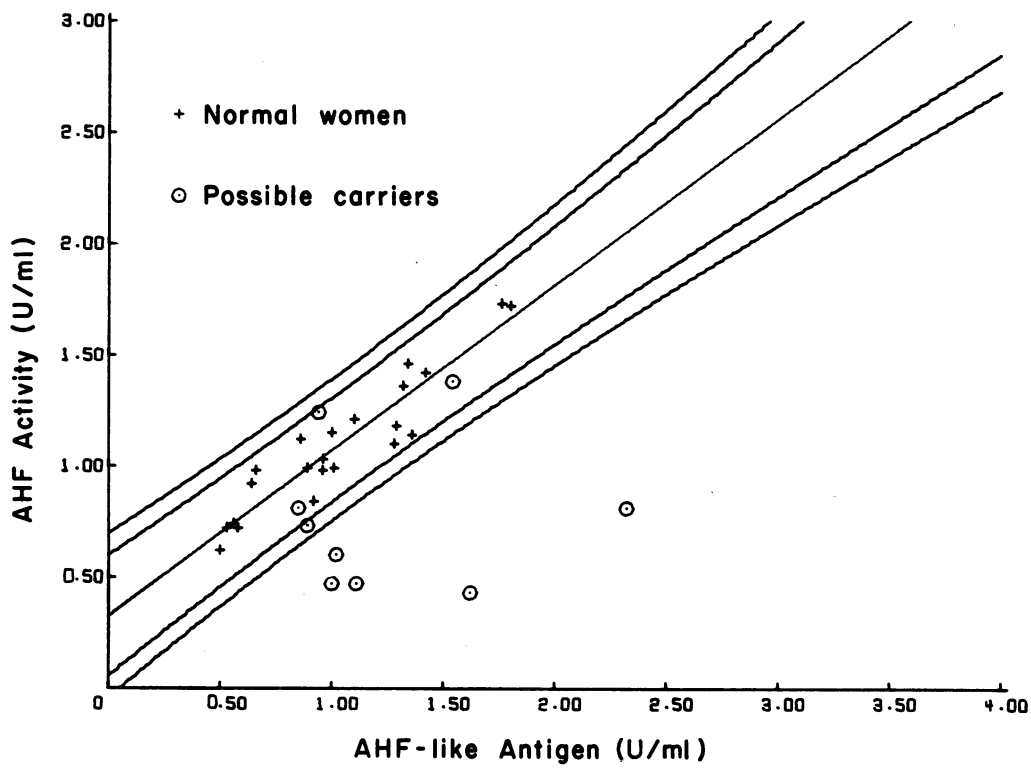

FIGURE 2 The relationship of AHF activity to AHF-like antigen in possible carriers and normal women. The center line is the regression line for the data obtained in normal women, the outermost lines represent the 99% confidence belt, and the other two lines represent the 95% confidence belt.

abstract form by Bennett (15). The ratio of activity to antigen in carriers varies greatly, from 0.13 to 1.04. These findings are consistent with the hypothesis of random activation (16) or inactivation (17) of the X chromosome. Random activation or inactivation of one of the two X chromosomes in the somatic cells of carriers of hemophilia should result in the production of varying proportions of active and inactive AHF in different individuals, but, on the average, approximately one-half should be active and one-half inactive, the result observed. In the extreme case, a carrier might synthesize little or no functional AHF and because of this be symptomatic (18). The study of AHF-like antigen in such instances would be of interest.

Notably, the geometric mean AHF-like antigen concentration was significantly higher in carriers than in normal women. The cause of this elevation was not evident; it was apparently unrelated to the administration of oral contraceptive agents. It is curious, however, that patients with hemophilia also had significantly more antigen than normal male subjects (12).

None of the carriers tested was known to be pregnant. The titer of functional AHF rises during pregnancy in normal women. Whether a disproportionate rise in antigenic AHF occurs during pregnancy is not known; were this the case, the technique described would not be of value for the detection of carriers during pregnancy.

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