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Research Article

A LABORATORY STUDY OF THE CARRIER STATE IN CLASSIC HEMOPHILIA[1]

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For many years it has been recognized that the hemorrhagic disorder now known as hemophilia is a hereditary disease of males transmitted to them only through females. From both the sociologic and medical points of view it would be important to detect those females who are conductors of the genetic defect responsible for this disease. On genetic grounds three groups of individuals can be assumed to be carriers, firstly, all daughters of a hemophiliac, secondly, the mother of two or more hemophiliacs, and thirdly, the mother of a single hemophiliac when she has other hemophilic relatives. The problem which cannot be answered on the basis of genetic hypothesis is which other female relatives of a hemophiliac are conductors. The published reports of studies of this problem are numerous and contradictory. It is the consensus that female carriers are usually asymptomatic, but there is considerable difference of opinion as to whether their blood has abnormalities detectable in the laboratory. It has only been within the last few years that the syndrome of hemophilia has been differentiated from Christmas disease (deficiency of plasma thromboplastin component). Possibly, the conflicting opinions concerning the detection of carriers have been due in part to the confusion of hemophilia with Christmas disease or with other hemorrhagic disorders. The present report concerns attempts to detect the carrier state in classic hemophilia by laboratory techniques. Among 19 presumptive and 8 possible carriers, an abnormally low concentration of antihemophilic factor was detectable in the plasma in only one instance.

MATERIALS

Blood was drawn from the antecubital veins of carriers, suspected carriers, patients with classic hemophilia, and normal adult humans, using No. 18 gauge needles coated with tris (2 hydroxyethyl) dodecyl-ammonium chloride (Monocote, Armour) and glass syringes coated with silicone (G.E. Dri-Film).

Oxalated and *citrated plasma* were prepared by mixing nine parts of venous blood with one part of 0.1 M sodium oxalate or 3.8 per cent (0.13 M) trisodium citrate dihydrate, and removing the cells by centrifugation at 2500 rpm for 15 minutes. This plasma was kept in ice water and used within a few hours, or stored at −25° C for as long as 3 months until used.

Barium sulfate-adsorbed plasma was prepared by adding one-tenth to one-fifth volume of powdered barium sulfate (Baker) to oxalated plasma which was either fresh or had been stored for as long as 1 month at −25° C. The mixture was stirred thoroughly, incubated at 37° C for 10 minutes, and then centrifuged. The supernatant barium sulfate-adsorbed plasma was kept in ice water and used within a few hours, or stored at −25° C for as long as 3 months. There was slight to moderate loss of anti-hemophilic activity in such plasma stored at −25° C for 3 months. However, in individual experiments, oxalated plasmas of similar ages were used.

Aluminum hydroxide-adsorbed plasma ("alumina-treated plasma") was prepared in the same way by adding one-tenth volume of aluminum hydroxide gel (Amphojel® Without Flavor, Wyeth) to citrated plasma which was either fresh or had been stored for as long as 3 months at −25° C, incubating the mixture at 37° C for 3 minutes, and separating the adsorbed plasma by centrifugation. The "alumina-treated plasma" was kept in ice water and used within a few hours, or stored at −25° C for as long as 8 months after the blood was drawn. In individual experiments, citrated plasmas of similar age were used although there was no detectable loss of anti-hemophilic activity in such plasma stored at −25° C for as long as 8 months. These plasmas, adsorbed with barium sulfate or aluminum hydroxide were deficient in prothrombin, proconvertin, and Christmas factor (plasma thromboplastin component).

Platelets were prepared by mixing 9 parts of blood and 1 part of 3.8 per cent trisodium citrate dihydrate in a silicone-coated lusteroid tube and separating the great bulk of the red cells and leukocytes by centrifugation at 1,000 rpm for 20 minutes at room temperature. The supernatant plasma was removed with a silicone-coated glass dropper, transferred to another silicone-coated lusteroid tube, and centrifuged at 3,000 rpm for 20 minutes. The *platelet-poor citrated plasma* was decanted and stored at −25° C. The sedimented platelets were sus-

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pended with 0.15 M sodium chloride solution, recentrifuged, and the process of washing then repeated. Finally the platelets were suspended in a volume of 0.15 M sodium chloride one-third to one-fifth of the volume of the plasma from which they were separated. The platelet suspension was stored in ice water and used within a few hours. Alternatively, the pellet of sedimented platelets was frozen without resuspension in sodium chloride solution, and stored at −25° C for as long as 5 days before use; it was then suspended in 0.15 M sodium chloride solution.

The *buffer* used consisted of 7.30 gm. of sodium chloride, 2.76 grams of barbital and 2.06 grams of sodium barbital, diluted to a volume of 1 liter with distilled water, and was thus composed of 0.025 M barbital and 0.125 M sodium chloride at pH 7.5. Centrifugation was done at room temperature in an International SB Size 1 centrifuge. Unless otherwise stated, Pyrex tubes (internal diameter 8 mm.) were used.

METHODS

The *clotting time of whole blood* was measured by a modification of the method of Lee and White (1), using dry Pyrex or silicone-coated tubes (internal diameter, 11 mm.) (2). During the period of this study, the clotting time at 25° C in Pyrex tubes averaged 27.6 minutes (range, 19 to 38; standard deviation, 4.8 minutes) and in silicone-coated tubes averaged 131 minutes (range, 75 to 185; standard deviation, 26.5 minutes), in 36 normal individuals. The silicone clotting times are slightly longer, on the average, than our overall experience with this test, and seem to reflect an undefined change in our technique. Measurement of the clotting time at 25° C and in tubes wider than those originally used by Lee and White (1) prolongs the clotting time compared with conventional methods and exaggerates the difference between normal and abnormal bloods. These modifications increase the ease of detecting minor abnormalities in the clotting mechanism.

The *serum prothrombic activity* was measured in triplicate by a technique slightly changed from one previously reported (3). A volume of 2.25 ml. of blood was allowed to clot at 37° C in Pyrex tubes. One hour after the blood was drawn, a volume of 0.25 ml. of 0.1 M sodium oxalate solution was added, and the contents mixed thoroughly with a wooden applicator stick. After incubation at 37° C for 30 minutes to destroy any remaining free thrombin, the mixture was centrifuged and the serum removed. Using Alexander's modification (4) of the method of Owren and Aas (5), the prothrombic activity of the patient's serum was then compared with that of oxalated plasma which had been incubated for the same period of time. In 17 normal individuals, the serum prothrombic activity ranged from less than 10 per cent to less than 25 per cent of plasma.

The *anti-hemophilic activity* of barium sulfate-adsorbed oxalated plasma and of aluminum hydroxide-adsorbed citrated plasma was estimated by determining the plasma's capacity to correct the abnormality of plasma which was known to be deficient in anti-hemophilic factor, *i.e.*, hemophilic plasma. The plasma to be tested was diluted to the desired concentration with buffer. In duplicate, 0.1 ml. of hemophilic plasma, 0.1 ml. of the diluted test plasma, and 0.1 ml. of 0.025 M calcium chloride solution were mixed in Pyrex tubes immersed in ice water. The clotting time was then determined in a water bath at 25° C by tilting the tubes at approximately 30-second intervals. The clotting time was the period which elapsed from the time that the tubes were transferred to the 25° C bath until the appearance of a clot. The longer of the duplicate clotting times is recorded. When the clotting time was 12 minutes or less, the plasma in duplicate tubes usually clotted within one-half minute of each other. When the clotting time was 20 minutes or less, the plasma in duplicate tubes almost always clotted within one minute of each other.

The thromboplastin generation test was performed according to the method of Biggs and Douglas (6). Platelet solutions were used at either 3, 4, or 5 times the original plasma concentration. In one experiment the aluminum hydroxide-adsorbed citrated plasma was diluted with 39 volumes of 0.15 M sodium chloride solution instead of with 4 volumes, as originally recommended.

To assay for the presence of a *circulating anticoagulant*, 9 parts of the citrated plasma to be tested were incubated in Pyrex tubes with one part of normal citrated plasma at 37° C for 3 hours. At the same time, portions of the normal plasma and the plasma to be tested were incubated separately for 3 hours at 37° C and at the end of this time were mixed in the same proportions. Each mixture was then diluted with 19 volumes of buffer. A volume of 0.1 ml. of each dilution was mixed with 0.1 portions of hemophilic plasma and 0.1 ml. of 0.025 M solution of calcium chloride, and the recalcified clotting time measured at 25° C in Pyrex tubes. This test detects circulating anticoagulants not demonstrable by more usual techniques. A circulating anticoagulant has been demonstrated in 5 of 22 hemophiliacs studied in this laboratory.

RESULTS

The diagnosis of classic hemophilia was established in at least one male member of each family in the usual manner. In no instance was the plasma of one patient capable of correcting the defect in the plasma of another patient. Several of these hemophilic plasmas were tested for their ability to correct the defect in patients with Christmas disease (plasma thromboplastin component deficiency). In each instance there was full correction.

In all, twenty-seven carriers in eighteen families were studied (Table I). The carriers are numbered 1 through 27, and the families are numbered with Roman numerals (I through XVIII).

TABLE I

The clotting times and serum prothrombic activity of 26 carriers

Carriers*	Families	Normal range:	Glass clotting time (*min.*) 19–38	Silicone clotting time (*min.*) 75–185	Serum prothrombic activity (% *of plasma*) <25
1	I		26	125	<10, <10, <10
2	I		30	115	10, 10, 17
3	I		24	115	<10, <10, 15
4	I		25	125	<10, <10, 11
5	II		30	215	10, 13, 17
6	II		28, 33	>205, 175	11, 15, 20
7	III		28	155	17, 17, 22
8	II		24, 26	115, 115	10, 11, 13
9	II		27	135	<10, <10
10	IV		28, 23	135, 145	<10, <10, 12
11	IV		25	125	<10, <10, <10
12	IV		22	175	<10, <10, <10
13	V		27	155	10, 10, 12
14	V		32	115	<10, <10, <10
15	VI		29, 30	145, 205	11, 11, 12
16	VII				
17	VIII		25	155	12, 13, 14
18	IX		29	155	13, 13, 14
19	X		29	205	<10, <10, <10
20	XI		27	105	14, 19, 20
21	XII		21	75	10, 13, 14
22	XIII		30	155	<10, <10, <10
23	XIV		34	145	<10, 10, 11
24	XV		28	145	<10, <10, 12
25	XVI		22	115	<10, 10, 16
26	XVII		27	145	10, 10, 13
27	XVIII		24	125	11, 12, 15

* The same numbers are used to designate carriers in all subsequent tables.

Carriers 1 through 7 are daughters of hemophilic males. Carriers 8 through 19 are also genetically established carriers because of the presence of hemophilia in one or more sons and collateral lines. In contrast, carriers 20 through 27 have only one hemophilic son each; no additional familial bleeding history could be obtained. Thus carriers 1 through 19 are presumptive conductors, and carriers 20 through 27 are possible conductors of hemophilia. The term *carrier* will be used to denote both the presumptive and the possible carriers.

The clotting times in glass and in silicone-coated tubes of 26 carriers are shown in Table I. Clotting times of 4 carriers were determined on two separate occasions. In all cases the clotting times of blood in glass tubes were within normal limits. Slightly prolonged clotting in silicone-coated tubes occurred once each in carriers 5, 6, 15 and 19.

The residual serum prothrombic activity (Table I) was determined usually in triplicate, on one occasion in each of twenty-six carriers. All the determinations were within normal limits.

The thromboplastin generation test was performed in six experiments. Aluminum hydroxide-adsorbed citrated plasmas of 26 carriers and 28 normal controls were tested. In 17 instances the carriers were studied in at least 2 separate experiments. To increase the sensitivity of the test in detecting abnormal alumina plasma, the alumina-treated plasma was diluted 1 to 40 instead of 1 to 5 (Figure 1). Using the same normal platelets and normal serum throughout, the diluted plasmas of 13 carriers and 10 controls were tested. The ranges of the clotting times of platelet-poor citrated plasma are shown. There was no significant difference in the behavior of alumina-treated plasma of the carriers and of the controls.

In order to increase its sensitivity in detecting abnormal alumina plasma, the thromboplastin generation test was modified in another way in 3 experiments. Normal platelets and normal serum were used throughout, but the alumina plasma consisted of 4 parts of hemophilic alumina-treated plasma and 1 part of the test alumina-treated plasma; the mixture was diluted 1 in 5. In 1 of these experiments the alumina-treated plasma of

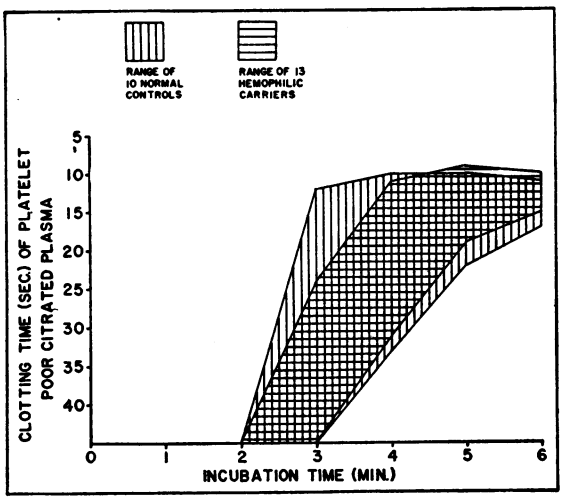

FIG. 1. THE THROMBOPLASTIN GENERATION TEST IN HEMOPHILIC CARRIERS

Equal volumes of 1) aluminum hydroxide citrated plasma, diluted 1/40, 2) normal pooled serum, diluted 1/10, 3) normal platelets concentrated 5 times, and 4) 0.025 M calcium chloride solution were incubated at 37° C in Pyrex tubes (internal diameter 11 mm.). At intervals of one minute beginning with the second minute 0.1 ml. aliquots of this "incubating mixture" and 0.1 ml. of 0.025 M calcium chloride solution were added to 0.1 ml. of platelet-poor citrated plasma, and the clotting time determined.

carrier 6 produced the longest clotting times of platelet-poor plasma. Otherwise there was no apparent difference between the carrier and the control groups.

In another experiment "thromboplastin generation" was measured in the manner just described, but hemophilic platelets were used instead of normal platelets. There was no appreciable difference in the clotting times of platelet-poor plasma upon the addition of an incubation mixture containing normal aluminum hydroxide-adsorbed plasma or aluminum hydroxide-adsorbed plasma prepared from carriers.

In another experiment the test was performed in the manner described, using normal platelets and normal serum. The activity of the alumina plasma of the 4 carriers of family II (including carrier 6) was compared with that of the normal controls. There was no appreciable difference in the clotting time of platelet-poor plasma upon the addition of incubating mixture containing normal alumina-treated plasma or alumina-treated plasma prepared from carriers.

Anti-hemophilic activity may be assayed more specifically by testing the ability of diluted barium sulfate-adsorbed oxalated plasma ("test plasma") to correct the prolonged recalcified clotting time of hemophilic plasma. By comparing the clotting times recorded for various dilutions of the test plasmas, one can compare their anti-hemophilic activities. Nineteen separate experiments were performed with the plasmas of 21 carriers and of 26 normal controls. A typical experiment is shown in Table IIA. The anti-hemophilic activity of the plasmas of carrier 4 and of carrier 20 was at least equal to that of the 2 normal controls. It is of interest that the plasma of the first normal control has twice the anti-hemophilic activity of the plasma of the second control. Occasionally by this technique as great as a three-fold difference in anti-hemophilic activity may be demonstrated among normal plasmas. Therefore, when the anti-hemophilic activity of a carrier's plasma was not as great as that of any one of 3 or 4 control plasmas, the carrier's plasma was compared with a pool of 4 to 6 normal plasmas. If the plasma of the carrier had at least half the anti-hemophilic activity of the normal pooled plasma, the carrier's plasma was not considered to be significantly deficient in anti-hemophilic activity. Of the 21 carriers tested, only the barium sulfate-adsorbed plasma of carrier 6 was grossly deficient in anti-hemophilic activity (Table IIB), having only one-fourth the corrective effect of normal control plasma or of pooled normal plasma.

TABLE II

Assay for anti-hemophilic activity in barium sulfate-adsorbed oxalated plasma *

		Initial dilution of test plasma			
A.	Test plasma	1/5	1/10	1/20	1/∞†
	Carrier No. 4	8	9	10	17
	Carrier No. 20	8	10	11	
	Normal control	8	9	10	
	Normal control	9	10	11	
B.	Carrier No. 6	12	13	14	18
	Normal control	10	10	11	
	Normal control	9	11	11	
	Normal control	10	10	12	

* *Clotting time* (min.) at 25° C in Pyrex tubes containing 0.1 ml. each of 1) hemophilic citrated plasma, 2) barium sulfate-adsorbed oxalated plasma of person to be tested, diluted with buffer as indicated, and 3) 0.025 M solution of calcium chloride.

† Buffer.

TABLE III

Assay for anti-hemophilic activity in aluminum hydroxide-adsorbed citrated plasma *

	Test plasma	Initial dilution of test plasma 1/5	1/10	1/20	1/∞†
A.	Carrier No. 27	8	9	10	18
	Normal control	8	9	11	
	Normal control	8	10	11	
	Hemophiliac	18	19	18	
B.	Carrier No. 6	10	12	12	18
	Normal control	8	9	10	
	Normal control	8	9	10	

* *Clotting time* (min.) at 25° C in Pyrex tubes containing 0.1 ml. of 1) hemophilic citrated plasma, 2) aluminum hydroxide-adsorbed citrated plasma of person to be tested, diluted with buffer as indicated, and 3) 0.025 M calcium chloride solution.

† Buffer.

Similarly, the ability of aluminum hydroxide-adsorbed citrated plasma of 21 carriers (including the six whose barium sulfate-adsorbed plasmas were not tested) and of 19 normal individuals to correct the prolonged recalcified clotting time of hemophilic plasma was compared in 16 separate experiments. A typical experiment (Table IIIA) shows that the anti-hemophilic activities of the alumina-treated plasma of carrier 27 and of the two normal controls are roughly similar, but that the alumina-treated plasma of a classic hemophiliac is completely deficient in anti-hemophilic activity. Of the 21 carriers tested by this technique, only the alumina-treated plasma of carrier 6 was grossly deficient in anti-hemophilic activity (Table IIIB), having only one-fourth the corrective effect of normal control plasma or pooled normal plasma.

It has been suggested that the inability of the plasma of a carrier to correct the hemophilic defect may be due to the presence in that plasma of a circulating anticoagulant which actively destroys the anti-hemophilic substance (7). The plasma of carrier 6 did not inactivate the anti-hemophilic factor of normal plasma even after prolonged incubation, and presumably did not contain a detectable circulating anticoagulant.

DISCUSSION

Characteristically, hemophilia is a disorder limited to males and inherited in a manner suggesting that the defect is transmitted by a sex-linked recessive gene. On theoretic grounds, one would expect that females would inherit the disease only if they were offspring of the union of a hemophilic man and a heterozygous conductor woman. Rare instances of this disorder in females have indeed been reported both in humans (8–10) and in dogs (11, 12), a species in which a disease strikingly similar to human hemophilia may occur.

A mass of conflicting information has been reported concerning the hemostatic mechanisms in the female conductors of hemophilia who are presumably heterozygous with respect to the defective gene. Many authors have described minor hemorrhagic phenomena in these heterozygous conductors. However, such women are of course likely to be acutely aware of bleeding phenomena, and may exaggerate otherwise unremarkable symptoms. It is not as easy to dismiss the many reports of abnormalities detectable in the laboratory. Among the coagulative defects which have been described in hemophilic carriers are prolonged whole blood clotting times (13–21), abnormal "utilization" of prothrombin during clotting (22–28), diminished activation of proconvertin and formation of proaccelerin during clotting (29), diminished "tolerance" of whole blood to heparin (30) and abnormal thromboplastin generation tests (30). It has also been reported that the plasma of carriers will not correct the defect in hemophilic plasma as readily as will normal plasma (30, 31).

An equally impressive array of data have been published supporting the view that the blood of hemophilic carriers does not contain detectable hemostatic abnormalities. Thus it has been stated that the blood of carriers has a normal clotting time in glass (26, 30, 32–35) or in silicone-coated (31) tubes, and a normal "tolerance" to heparin (26), and significantly shortens the clotting time of hemophilic blood (33, 35). The "consumption" of prothrombin during clotting (30, 31, 33, 35, 36) and thromboplastin generation tests (3, 37) are said to be normal.

There are at least two possible explanations for these discordant results. In the first place, the various authors may not have been studying the same disease in their various patients. It has only recently been appreciated that the symptom complex originally termed hemophilia might result from a variety of abnormalities in the plasma. Thus, several groups of investigators described

a disorder strikingly similar to hemophilia but due instead to a deficiency of another factor, variously called plasma thromboplastin component (38) or Christmas factor (39). Although the inheritance of Christmas disease superficially resembles that of hemophilia, the female carriers of the former may have hemorrhagic symptoms (26, 40) and evidences of a partial deficiency of Christmas factor in their plasma (26, 37, 39, 40). More recently, Rosenthal, Dreskin, and Rosenthal (41) observed a hemorrhagic disorder resembling mild hemophilia but occurring in both sexes, and attributed to a deficiency of yet another factor in plasma, which they called plasma thromboplastin antecedent. It is possible that certain of the reports in which the identification of the carrier state was described may have concerned patients with Christmas disease or plasma thromboplastin antecedent deficiency. However, it must be remembered that most patients with hemophilia-like disease do have classic hemophilia. Our own experience, which now includes 28 families with classic hemophilia but only 6 families with Christmas disease, is typical of data which have been reported by others. This would suggest that misdiagnosis plays at most a small part in the contradictory results which have been reported.

A second explanation for the variation in opinion concerning the detection of hemophilic carriers may reside in the technical methods used. For example, many of those who have claimed that the clotting time of the blood of hemophilic conductors is prolonged used the method of Bürker (42). In this test, the end-point is more subjective than in the Lee-White (1) technique. In the last analysis, the only tests which are specific for a deficiency in anti-hemophilic factor are those in which the capacity of the unknown plasma to correct the defect of known hemophilic plasma is measured. Such non-specific tests as the clotting time or the prothrombin consumption test may be abnormal in any given subject because of quite irrelevant factors. Indeed, the converse is often true, namely, that these tests may give normal results in classic hemophiliacs (43–50). However, any abnormality present in a hemophilic carrier might be manifested by a true decrease in the plasma's anti-hemophilic activity, were the defective gene "incompletely recessive."

In the present study, a significant reduction in anti-hemophilic activity compared with normal plasma was noted in the plasma of only one carrier, No. 6. She is the asymptomatic 9-year-old daughter of a true hemophiliac. This same young girl had a long silicone clotting time on one of two occasions. Her sister, No. 5, had a long silicone clotting time, but assays of her plasma did not clearly demonstrate a deficiency of anti-hemophilic factor. Two other proved carriers in this family had no demonstrable abnormalities, although they bruised easily and bled after dental extractions. The two known hemophiliacs in this family bled only occasionally, and although hemarthroses were observed in one, no crippling deformities ensued. However, the father of carriers No. 5 and No. 6 bled to death after exploratory laparotomy.

The data we have presented make it appear unlikely that with the laboratory techniques presently available a significant percentage of female carriers in families with classic hemophilia can be identified by a study of the concentration of anti-hemophilic factor in their blood. It is of interest that in the one family in which it was possible to demonstrate any changes, the hemophilia appeared to be relatively mild. This observation is of particular interest because of the study by Graham, McLendon, and Brinkhous (51) of a family of classic hemophiliacs in whom the disorder was mild. None of their patients had suffered a hemarthrosis. Graham, Collins, Godwin, and Brinkhous (52) and Langdell and Wagner (53) assayed the plasma of 10 (presumed or possible) carriers (in this family) by a method specific for anti-hemophilic factor, and demonstrated that 6 of the 10 had concentrations of 31 to 54 per cent of normal, values which they considered clearly pathologic.

Similarly, the Moena family, originally reported to have a unique disorder, was also noteworthy because of the association of mild hemophilia (54) and detectable changes in the blood of female carriers (23). These families, then, resemble that of carrier No. 6 in the present report.

Although the hemophilia was mild in those families in which carriers were detected, this may be fortuitous. Lewis (55) has observed a significantly low titer of anti-hemophilic activity in 7 of 40 presumed or possible carriers. In her series there did not appear to be any correlation between

the severity of the disease and the anti-hemophilic titer in the female carriers.

Because the clinical severity of hemophilia varies in different families, and because of the abnormalities found in the blood of carriers of mild hemophilia, it has been suggested that the genetic transmission of hemophilia is not simply dependent upon the presence or absence of a single recessive gene. Rather it has been suggested that there is a series of isoallelic genes (51), each manifested clinically by hemophilia of a given severity, and each associated with a corresponding level in the blood of anti-hemophilic factor. An alternate but basically similar explanation is that a single recessive gene is responsible for the disease, but that there are also modifying genes (37, 56) which alter its clinical expression. The data which have been presented do not lend themselves to the support of one or another of these hypotheses.

SUMMARY

The medical literature relating to the detection of hemophilic conductors by laboratory techniques is conflicting. Routine clotting studies and assays for anti-hemophilic factor were performed in 27 carriers of classic hemophilia. A definitely reduced concentration of anti-hemophilic factor was demonstrated in only one carrier, although minor abnormalities of the clotting mechanism were found in several others. In the families of hemophiliacs, it is possible to detect only a small number of carriers of the trait with currently available techniques.

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