ANTI-HEMOPHILIC GLOBULIN LEVELS IN CARRIERS OF HEMOPHILIA A *

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Hemophilia A (anti-hemophilic globulin deficiency) is transmitted as an incompletely recessive trait on the X chromosome. Therefore, the daughter of a hemophiliac must be a carrier. In contrast, the daughter of a carrier, e.g., the sister of a hemophiliac, is a "potential" carrier who has an equal chance of being either a carrier or normal. She doesn't know which she is until she bears a hemophilic son; it requires this event to identify her as a known or "obligatory" carrier.

The dilemma of the potential carrier has prompted many investigators to look for some clotting abnormality which might identify a carrier. Nilsson, Blombäck, Thilen and von Francken (1) have summarized the early studies with nonspecific techniques.

The first quantitative anti-hemophilic globulin (AHG) measurements in carriers were published in 1953 by Graham, McLendon and Brinkhous (2). They found low AHG levels in six of ten carriers of mild hemophilia and thought this was a peculiarity of the allelic mutant of the hemophilic gene responsible for mild hemophilia. In apparent confirmation, Gardikas, Katsiroumbas and Kottas (3) reported normal AHG levels in ten obligatory carriers of severe hemophilia. However, several subsequent groups of workers (4–6) demonstrated diminished levels of AHG in some carriers of both mild and severe hemophilia; and, recently, Nilsson and co-workers (1) reported that the vast majority of carriers have low levels of AHG.

The present paper reports on quantitative measurements of AHG made with the Pool-Robinson AHG assay technique (7) in a large number of obligatory carriers and normal women.

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METHODS

Test plasma. Blood was taken from nonfasting subjects between approximately 9 and 10 a.m. using Monocote-E (Armour and Co.) coated needles and silicone (G.E. Dri-film SC-87) coated syringes. Nine parts of blood were added to one part of 0.1 M sodium citrate anticoagulant in polyethylene tubes and centrifuged in the cold for 10 minutes at 10,000 rpm. The resultant platelet-poor plasma was stored frozen at −20° C in 0.7 ml filled, corked glass vials until tested in the assay approximately 3 hours later.

Standard reference plasma. Blood was taken from the same male subject at 2-week intervals. The plasma was prepared and stored as described above except that the plasma was centrifuged a second time to insure the removal of the platelets. Although fresh reference plasma was used every 2 weeks, the AHG content of the stored plasma did not fall over a 6 week period of storage.

The Pool-Robinson assay technique. Our reagents and technique for the Pool-Robinson assay have been described in detail elsewhere (8). The test plasma was assayed at dilutions of 1, 0.5 and 0.25 per cent and the clotting times converted to per cent AHG from the standard reference plasma curve for that day. The mean value for the three dilutions was taken as the final value. All assays were performed with a blind technique.

Thirty-five obligatory carriers and 30 normal women were studied. The obligatory carriers were *1*) daughters of hemophiliacs, *2*) mothers of two or more hemophilic sons, or *3*) mothers of one hemophilic son who had at least one other close male relative with hemophilia. The normal women were laboratory technicians and secretaries in the medical school without personal or family history of abnormal bleeding.

RESULTS AND DISCUSSION

A. Precision and accuracy. AHG was measured in four plasma samples taken from the subjects in one of two ways—either as duplicate samples (from the same venipuncture site but in separate syringes) on each of two successive days or as single samples on four successive weeks. These data are contained in Tables I and II; the weekly samples have been arranged by week of the men-

TABLE I

AHG in duplicate samples on successive days

Subject	Log per cent AHG			
	Day 1		Day 2	
	Sample 1	Sample 2	Sample 1	Sample 2
Carriers				
D.A.	1.78	1.79	1.83	1.87
L.G.	1.78	1.72	1.72	1.78
K.T.B.	1.63	1.65	1.51	1.48
S.F.	1.60	1.59	1.54	1.60
M.H.	1.64	1.62	1.58	1.56
How.	1.88	1.89	1.91	1.94
E.H.	1.94	1.93	1.85	1.91
R.R.	1.76	1.78	1.86	1.89
K.D.	1.89	1.80	1.75	1.83
J.F.	1.83	1.82	1.72	1.75
J.S.	1.68	1.69	1.67	1.66
C.W.	1.77	1.79	1.79	1.74
M.C.	1.81	1.75	1.81	1.83
A.W.	1.32	1.46	1.34	1.36
M.S.	1.82	1.86	1.77	1.80
E.J.	1.95	1.93	1.80	1.82
Normals				
J.R.	1.82	1.86	1.74	1.82
R.G.	1.83	1.83	1.83	1.86
J.C.	1.86	1.92	2.04	2.01
S.S.	2.00	2.01	1.95	2.06
J.G.	1.76	1.76	1.68	1.68
J.P.	1.72	1.75	1.72	1.68
N.H.	2.06	2.06	2.05	1.99
V.H.	2.01	2.01	1.90	1.94
J.L.	2.00	1.99	2.02	1.98
C.F.	1.97	1.95	1.96	1.91
S.L.	1.99	1.99	1.96	1.93
J.M.	1.79	1.82	1.90	1.89
C.C.	1.96	1.95	1.90	1.90

strual cycle. Log per cent AHG is given and was used in all statistical calculations because log transformation (9) resulted in a more normal frequency distribution of the data.

The methods of variance analysis (9) were used to compare error within days, error between days, and error due to possible variation of AHG during different phases of the menstrual cycle. The variances are given in Table III. As expected, the variances between individuals far exceeded those within or between days for the same individual. The variances between days are significantly greater than those within days ($p < 0.001$). In what part this reflects real differences in an individual's AHG level from day to day and in what part it represents technical differences in the test from day to day is not known.

The variances between days and the variances between weeks are of the same order of magnitude. Weekly sampling throughout the menstrual cycle did not bring out differences greater than those encountered on successive days. Thus, AHG may be measured in cyclic women without concern for variation due to the menstrual cycle.

As the very small variance within days indicates, duplicate determinations on the same day do little to increase the accuracy of AHG estimation over a single determination on one day. Greater accuracy requires single determinations on a number of days.

The accuracy with which a single determination on one day estimates the true level found by making single determinations on an indefinite number of days is a matter of practical importance. Figure 1 shows the 90 per cent confidence limits at various AHG levels for a single determination on one day (outer lines) and for the mean of four single determinations on four days (inner lines). These confidence limits were calculated from our combined data on carriers and normals sampled on four successive weeks.

The meaning of these confidence limits may be made more explicit with an example. If a single determination of AHG is 60 per cent, we may have 90 per cent confidence that the true value that might be found from an indefinite number of

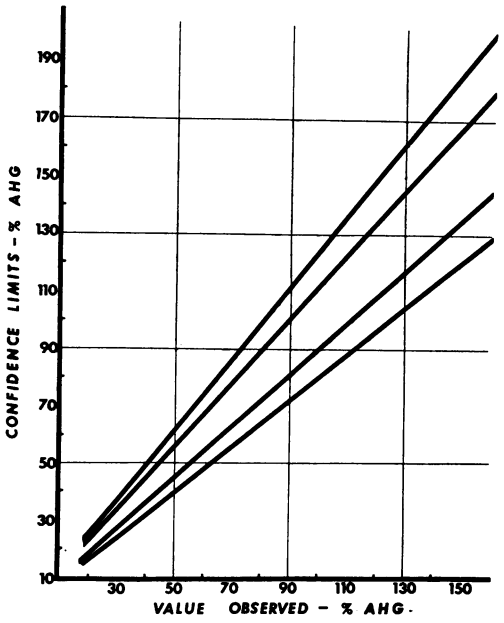

FIG. 1. NINETY PER CENT CONFIDENCE LIMITS OF A SINGLE DETERMINATION OF AHG (OUTER LINES) AND OF THE MEAN OF FOUR SINGLE DETERMINATIONS OF AHG (INNER LINES).

TABLE II

AHG in single samples on successive weeks

Subject	Log per cent AHG			
	Menstrual cycle			
	Days 1–7	Days 8–14	Days 15–21	Days 22–28
Carriers				
L.L.	1.42	1.52	1.58	1.46
S.S.	1.58	1.59	1.78	1.54
M.F.	1.72	1.72	1.65	1.63
B.L.	1.79	1.82	1.73	1.80
L.E.	1.74	1.73	1.81	1.76
L.H.	1.71	1.72	1.76	1.75
O.S.	1.97	2.06	2.06	2.04
E.S.	1.99	1.91	1.90	1.89
A.G.	1.74	1.81	1.80	1.81
T.P.	1.84	1.69	1.88	1.77
Normals				
S.A.	1.94	1.96	2.00	2.00
L.B.	2.00	2.06	2.06	2.06
R.P.	2.01	2.14	1.98	2.18
P.O.C.	1.83	1.99	1.90	1.93
R.L.	1.90	1.86	1.96	1.91
R.G.	2.04	2.16	2.06	2.04
P.S.	1.93	2.04	1.91	1.95
B.D.	2.05	2.09	2.09	2.12
T.W.	2.04	2.14	2.15	2.16
H.B.	1.86	1.92	1.92	1.97
F.S.	2.05	2.09	2.05	2.06
C.M.	2.07	2.03	2.12	2.01
M.J.	2.16	2.15	2.06	2.06
M.L.	1.99	1.80	1.83	1.89

tests over a four week period would have an average value that fell between 48 and 75 per cent AHG. If four single determinations give a mean value of 60 per cent AHG, the 90 per cent confidence limits narrow to between 54 and 67 per cent AHG.

B. Levels of AHG in carriers and in normal subjects. AHG levels for 35 carriers and 30

TABLE III

Summary of variance analysis of data in Tables I and II

Subject	Variation	Sum of squares	Degrees of freedom	Variance
	Duplicate samples on successive days			
Normals	Between individuals	0.5412	12	0.0451
	Within days	0.0199	26	0.0007
	Between days	0.0532	13	0.0041
	Total	0.6143	51	0.0117
Carriers	Between individuals	1.3534	15	0.0902
	Within days	0.0243	32	0.0007
	Between days	0.0787	16	0.0049
	Total	1.4564	63	0.0231
	Single samples on four successive weeks			
Normals	Between individuals	0.3727	13	0.0287
	Between weeks	0.1281	42	0.0030
	Total	0.5008	55	0.0091
Carriers	Between individuals	0.7977	9	0.0887
	Between weeks	0.1023	30	0.0034
	Total	0.9000	39	0.0231

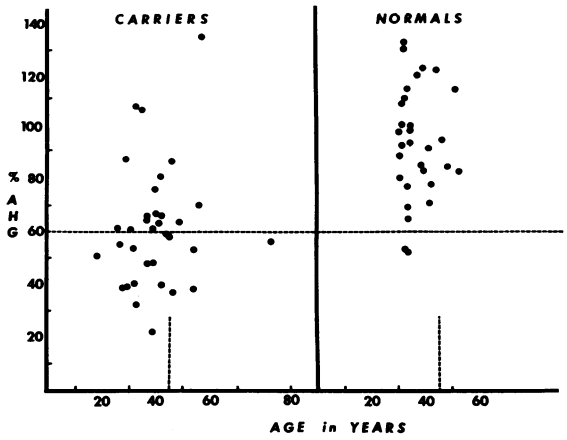

FIG. 2. AHG LEVELS IN CARRIERS AND IN NORMAL SUBJECTS PLOTTED ACCORDING TO AGE IN YEARS. The vertical dotted lines are at age 45 years.

normal women are plotted by age in Figure 2. Except for three subjects from whom only three samples could be obtained, each dot represents the mean of four determinations made from samples taken by either method described above.

AHG levels in normal women varied between 52 and 133 per cent; only 2 of the 30 normal women had AHG levels below 60 per cent of our reference plasma. The carrier range was much wider, between 22 and 135 per cent. Although approximately one-half of the carriers had AHG levels below 60 per cent, many others had levels well within the normal range. AHG did not increase with age in the carrier population; we could not confirm Nilsson and associates' finding (1) of higher AHG levels in carriers over the age of 45 years.

In Figure 3 the values for each group, expressed as log per cent AHG, have been plotted on probability paper against accumulated frequency expressed in per cent. The reasonable linearity of both plots is consistent with sampling from homogenous populations. The wide range of AHG values indicates that many factors in addition to the single gene controlling hemophilia must influence the AHG level in both carriers and normals. Our data do not identify such factors but do exclude age and menstrual cycle.

It can be seen from Figure 3 that 20 carriers per 1,000 may be expected to have AHG levels below about 25 to 30 per cent of normal. This is low enough to predispose to abnormal bleeding. Studies of heterozygous carriers with clinically significant bleeding episodes have been published by Douglas and Cook (10), and by McGovern and Steinberg (11).

The carrier in our series with 22 per cent AHG had bled on the tenth day after tonsillectomy, for three days after one tooth extraction, and for seven days after another. She had developed massive ecchymoses following a minor injury to her leg. Her well documented family history of hemophilia, which followed a sex-linked recessive pattern over several generations, coupled with her normal bleeding time leaves little doubt that she represents another example of significant bleeding in a heterozygous carrier and not of vascular hemophilia.

This carrier comes from a kindred with mild hemophilia (AHG levels of 15 to 20 per cent in affected males). It is of interest that the carriers described by Douglas and Cook (10) came from a family with mild hemophilia. This may also be true of the patient described by McGovern and Steinberg (11). Therefore, although it is well established that low AHG levels may be found in carriers of either mild or severe hemophilia, the question arises as to whether or not there is a rare form of carrier population with *excessively* low AHG levels and clinically significant bleeding episodes in kindreds with mild hemophilia. If our carrier were removed from our series on these

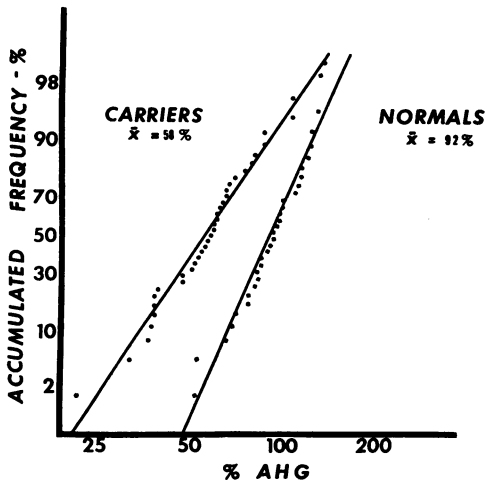

FIG. 3. ACCUMULATED FREQUENCY IN PER CENT PLOTTED ON PROBABILITY PAPER (9) AGAINST OBSERVED VALUE OF AHG IN PER CENT (LOGARITHMIC SCALE). The carriers are identified by circles, the normal subjects by squares.

grounds, then the mean shown in Figure 3 would remain essentially unchanged, but the variances of the carrier and normal groups would become more nearly equal. This would alter slightly the data shown in Figures 4 and 5 (see below). However, in view of the scantiness at present of the evidence for a separate carrier population, we have considered her as an ordinary carrier in our statistical calculations.

Seven of our carriers had AHG levels between 30 and 40 per cent. None had bled abnormally despite the fact that all had had teeth extracted and many had been through major surgery. This is indirect evidence that AHG levels above 30 per cent are usually adequate for hemostasis.

C. Recognition of the carrier by AHG assay. Since the daughter of a carrier has an equal chance of being either normal or a carrier, the accumulated frequencies shown in Figure 3 may be used to calculate her chances of being a *true carrier at or below* any observed AHG level. This is shown in Figure 4. Although each laboratory uses a different 100 per cent reference plasma, the value for the mean of a normal population will probably be found within the limits of 90 to 110 per cent of the particular reference plasma, respectively. Linear interpolation between these curves is probably adequate for practical purposes. Therefore, once the normal mean for a reference plasma has been established, Figure 4 may be used to estimate the probability that a *potential carrier* may be a *true carrier at or below* any observed per cent AHG of that reference plasma.

The mean of our carrier series was 58 per cent AHG. Figure 4 shows that 95 of 100 *potential* carriers with an AHG level *equal to or below* 58 per cent of our reference standard will be *true* carriers. Approximately three-fourths of our carriers had AHG levels below 70 per cent. From line A of Figure 4 we see that a *potential* carrier with an AHG level *at or below* 70 per cent of our reference standard has better than four chances out of five of being a *true* carrier. Thus, it would appear that AHG assay by the thromboplastin generation technique enables one to recognize with reasonable accuracy about 75 per cent of the *true* carriers in a *potential* carrier population.

In a similar fashion it is possible to estimate the probabilities that a *potential* carrier may be *normal* when her AHG level *equals or exceeds* any observed value. This is shown in Figure 5.

As seen from line A of Figure 5, a potential carrier with an AHG level *at or above* 80 per cent of our reference standard has four chances out of

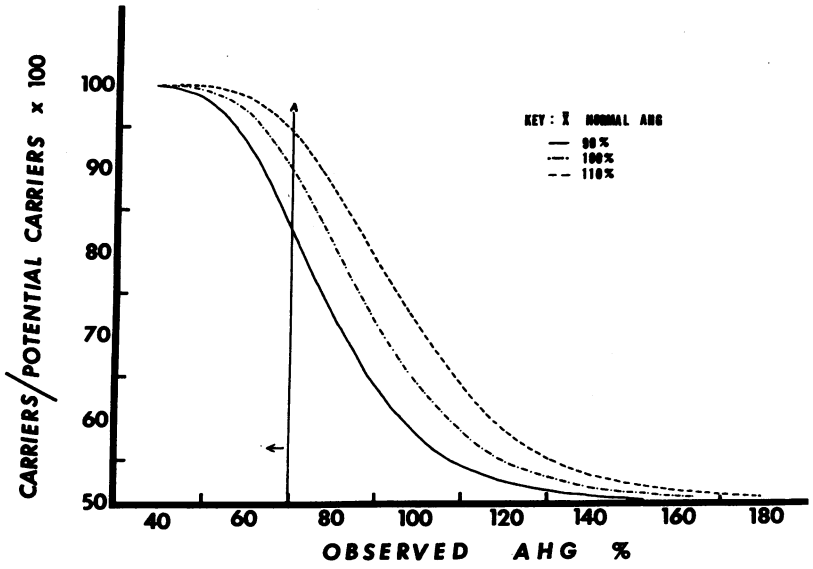

FIG. 4. THE PERCENTAGE OF CARRIERS IN A POTENTIAL CARRIER POPULATION *AT OR BELOW* ANY GIVEN OBSERVED LEVEL OF AHG. Values are plotted for reference plasma which gives a mean for a normal population of 90, 100 and 110 per cent, respectively.

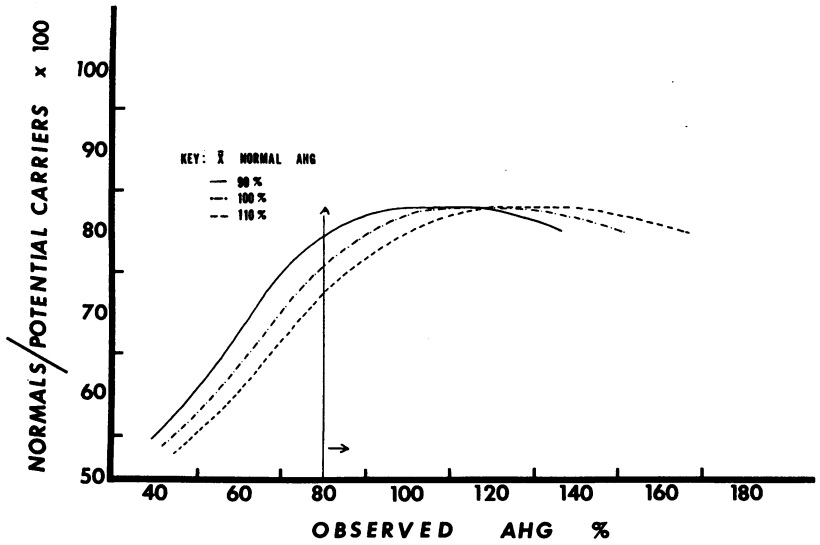

FIG. 5. THE PERCENTAGE OF NORMAL SUBJECTS IN A POTENTIAL CARRIER POPULATION *AT OR ABOVE* ANY GIVEN OBSERVED LEVEL OF AHG. Values are plotted for reference plasma which gives a mean for a normal population of 90, 100 and 110 per cent, respectively.

five of being *normal*. More than two-thirds of the normal members of a potential carrier population would fall into this group. The chances of being normal do not increase significantly *at or above* higher observed AHG values due to the difference in the slope of the two lines in Figure 3. Thus, a *potential* carrier with a high normal AHG level still retains one chance in five of being a *true* carrier.

Our data confirm and extend the observations made with thromboplastin generation techniques of AHG assay in groups of carriers by Pitney and Arnold (6) and by Nilsson and co-workers (1). The latter group also measured AHG in their carriers by a second technique, based upon the correction of the prolonged recalcification time of hemophilic plasma, which resulted in a clearer separation of carriers from normals.

Review of their data discloses *1*) that both techniques yielded the same mean and wide range of AHG levels in normal subjects, and *2*) that, although the majority of carriers had lower values with the recalcification technique, occasional carriers had lower values with the thromboplastin generation technique. These observations raise the possibility that the seemingly more distinct separation by the recalcification time technique may be due to the measurement of a second variable which helps to distinguish carriers from normals. Otherwise, one must postulate that thromboplastin generation techniques of AHG assay give falsely high values in carriers but not in normal subjects. A comparison of these two methods of AHG assay in a variety of conditions in which AHG levels vary appears necessary before this question can be resolved.

SUMMARY

1. Plasma AHG levels were measured in obligatory carriers of hemophilia A and in normal women by the Pool-Robinson assay technique. Four samples were taken by methods which permitted the evaluation of error between duplicates on the same day, between single determinations on successive days, and between single determinations on successive weeks. AHG levels did not vary with the phase of the menstrual cycle.

2. The normal women had a mean AHG value of 92 per cent of our reference plasma (range 52 to 133 per cent). The carriers had a mean AHG value of 58 per cent (range 22 to 135 per cent). Both sets of data appeared to be normally distributed when log per cent AHG was plotted on probability paper.

3. One carrier with 22 per cent AHG had experienced clinically significant bleeding. Seven

carriers with AHG levels between 30 and 40 per cent had not bled abnormally. The distribution of AHG values in our carrier series suggests that AHG levels low enough to predispose to bleeding, i.e., below 30 per cent should occur in about 20 carriers per 1,000.

4. Probability calculations indicate that this AHG assay will detect with reasonable accuracy (four chances out of five) about 75 per cent of the *true* carriers in a *potential* carrier population and about 66 per cent of the *normal members* in a *potential* carrier population. However, a *potential* carrier with even a very high AHG level still retains one chance in five of being a *true* carrier.

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