THE KAOLIN CLOTTING TIME

A RAPID ONE-STAGE METHOD FOR DIAGNOSIS OF COAGULATION DEFECTS

BY

J. MARGOLIS*

From the Pathology Department, Radcliffe Infirmary, Oxford

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A major variable in blood coagulation tests is the nature and the amount of surface with which the blood comes in contact. When tests on re-calcified plasma are used the methods of preparation and storage of the plasma have also to be considered. The prolonged contact of plasma with a large surface, e.g., with glass powder or asbestos, is accompanied by the loss of many coagulation factors, including prothrombin and fibrinogen (Tocantins, 1945). Even a more limited exposure to glass, such as is likely to be encountered in normal practice, may be followed by considerable prolongation of the plasma clotting time (Margolis, 1957). The loss of clotting ability under these conditions is primarily due to the disappearance of the plasma thromboplastin antecedent (Rosenthal, 1955) which is the component most easily destroyed by contact with glass (Margolis, 1958).

An additional difficulty when dealing with plasma is that, as the number of platelets decreases with increasing centrifugation, the clotting time becomes more and more sensitive to variations in the platelet count and the results become more unpredictable.

Previous investigations (Margolis, 1957) indicated that the clotting time of re-calcified plasma can yield both sensitive and reproducible results when both the contact surface and the platelet content are controlled. The purpose of the present paper is to demonstrate the usefulness of this procedure for the diagnosis of plasma clotting defects.

Methods

The patient's plasma is re-calcified after the addition of different preparations derived from normal blood. Instead of relying on the walls of glass tubes as the activating surface, this is supplied much more effectively by the addition of a suspension of kaolin. By lowering the platelet content the system is made more sensitive, and the differences between normal

and abnormal are magnified. The amount of platelet material is controlled by adding to rapidly-spun plasma a small standard amount of lysed platelets sufficient to mask the uncontrollable platelet activity left behind after centrifugation of the plasma samples.

All samples of blood and its derivatives are collected and handled in siliconed glassware unless otherwise stated.

Reagents.—The following are required :

(1) *Citrated Plasma*.—Venous blood is mixed with 20% trisodium citrate (0.2 ml. to 10 ml. of blood) and centrifuged at about 1,000 r.p.m. for 10 min. to obtain platelet-rich plasma. Platelet-poor plasma is obtained by further centrifuging at least at 3,000 r.p.m. for 30 min. and carefully removing the upper two-thirds of the plasma. The platelet count should not exceed 15,000/c.mm. It is important to prevent the formation of dried blood films on the walls of the centrifuge tubes, as this leads to the release of disrupted cellular material which is not easily sedimentable. A convenient method is carefully to transfer the citrated blood into a clean centrifuge tube, minimizing contact of blood with the tube above the level of the meniscus.

(2) *Aged Serum*.—Normal blood is allowed to clot in a glass centrifuge tube, incubated for several hours at 37° C., and then left for at least 18 hours at room temperature. The serum is decanted and centrifuged at 3,000 r.p.m. or more rapidly ; it is then transferred into siliconed tubes and may be stored at -20° C.

(3) *Al(OH)₃-Adsorbed Plasma* ("Alumina Plasma").—This reagent is prepared according to the methods of Biggs and Macfarlane (1957).

(4) *Heated Plasma*.—Normal plasma is heated at 56 to 57° C. for 20 min., and centrifuged to remove the denatured fibrinogen precipitate. A similar preparation heated at 60° C. is also useful.

(5) *Kaolin Suspension*.—Light kaolin, 200 mg., is suspended in 5 ml. of physiological saline.

(6) *Lysed Platelets*.—Platelet-rich plasma is centrifuged at 3,000 r.p.m. or more rapidly. The sediment is washed twice with saline and suspended in a volume of distilled water equal to that of the original plasma. The lysis is completed by freezing and thawing after which any remaining clumps are allowed to settle for a few minutes, and the super-

*Present address : Pharmacology Department, Middlesex Hospital Medical School, London, W.1.

nantant is used. The platelet extract is then diluted with approximately 20 vol. of saline, preferably buffered with glyoxaline at pH 7.3 (Biggs and Macfarlane, 1957). The diluted extract is mixed with an equal volume of M/20 CaCl_2 immediately before use. This mixture provides platelet material equivalent to 5,000–20,000 platelets/c.mm., and the final concentration of the extract should be adjusted to give a clotting time of approximately 90 sec. with normal plasma. (After the first centrifuging a layer of platelets adheres to the sides of the tube and should be scraped off under saline with a wooden applicator. The platelet deposit is dispersed by vigorously rubbing in a drop of saline after the second and third centrifugations.) If it is desired to increase further the sensitivity of the system a higher dilution of the platelet extract is used, but this requires additional precautions (see below).

(7) *Alternative to Platelet Extract.*—As an alternative to the platelet extract, chloroform brain extract (Bell and Alton, 1954) may be used. The dilution must be empirically adjusted to give a normal clotting time of about 90 sec. (usually 1/1,000–1/10,000 of the stock suspension). This eliminates the somewhat time-consuming preparation of lysed platelets.

Procedure.—This is in two steps.

(a) *Screening Test.*—Into two glass clotting tubes ($\frac{3}{8}$ in. $\times$ $2\frac{1}{2}$ in. is a convenient size) are added in the following order:

Saline	...	...	...	0.05 ml.
Test plasma (patient's or normal)	...	...	...	0.1 ml.
Kaolin suspension	...	...	...	0.05 ml.

The tubes are incubated with occasional shaking for 2 min. at 37° C. and are then re-calcified with 0.1 ml. amounts of CaCl_2 -platelet reagent. The samples are shaken at intervals and inspected for clotting. The end-point is sharp and is preceded by the kaolin flocculating coarsely 2–5 sec. before the formation of solid clot. In addition it is advisable to test the system for "platelet tolerance" by including two further tubes in which a mixture of equal parts of the patient's and normal plasma are re-calcified as above. In one of these tubes the platelet extract is replaced by saline. The clotting time without the platelets must be at least 30 sec. longer than the one with the extract (Table III). A difference of less than 30 sec. indicates the need for centrifuging the plasma further. This is particularly important when high dilutions of platelet extract are used as shown in Table II (B).

(b) *Full Test.*—The reagents (1–4) to be added to the test plasma are first diluted with equal volumes of saline. A series of clotting tubes is set up and the procedure carried out as in (a), saline being replaced with one of these preparations (Tables I and IV). With the aid of several stop-watches all the tubes can be dealt with at the same time. Alternatively the series may be split into groups of two or three tubes, provided these are tested within reasonable time of each other.

Since kaolin provides an internal activating surface, the clotting time is quite independent of the size of the containers and the absolute volumes of the reagents. These can be proportionally increased, if desired, to facilitate a more accurate measurement of the components.

Results

Reproducibility.—Table I shows the results of repeated tests on plasma from a patient under phenindione treatment. The figures show a high degree of reproducibility of the tests carried out within short intervals of each other and that there is little change in the clotting times after keep-

TABLE I
KAOLIN CLOTTING TIME (SEC.) OF DINDEVAN PLASMA*

Preparations Added to Dindevan Plasma				
Dindevan Plasma	Normal Plasma	Alumina Plasma	Aged Serum	Heated Plasma (56° C.)
130 (92)†	101	153	102	159
128 (93)	102	148	99	155
129 (95)	98	150	97	153
128 (91)	100	149	101	155

* Samples of the patient's plasma, mixed with various preparations (25% v/v test plasma) and kaolin suspension, were re-calcified with CaCl_2 -platelet reagent (see methods). The test was repeated four times within one hour to demonstrate the reproducibility of the results.

† Prothrombin index* of 44.

‡ The figures in brackets refer to normal plasma tested in parallel.

ing all the reagents for one hour at room temperature. Leaving small volumes of diluted blood derivatives on the bench for periods longer than two hours should be avoided as they tend to lose their corrective activity.

Sensitivity.—Table II (A) shows the clotting times of mixtures of normal plasma and plasma from a moderately affected haemophilic patient. Assuming the concentration of antihæmophilic globulin (A.H.G.) in the patient's plasma to be near zero, and that of normal to be near 100%, the mixture containing 25% A.H.G. can be confidently diagnosed as deficient. Clinically this

TABLE II
KAOLIN CLOTTING TIME (SEC.) OF MIXTURES OF NORMAL AND HAEMOPHILIC PLASMA

Patient	Concentration of Platelet Extract	Concentration of Normal Plasma in Mixture (%)				
		100	50	25	12½	0
A	1	93	102	136	152	570
		95	100	132	155	550
B	1	95	95	110	125	205
		92	98	113	122	198
	1:10	135	154	171	183	360
		138	155	177	185	345

A = plasma from a high-grade haemophilic.

B = plasma from a moderately severe haemophilic.

In each test all five mixtures were re-calcified in parallel and a duplicate series was tested within 10 min. of the first one.

concentration of A.H.G. would represent a very mild bleeding tendency (Biggs and Macfarlane, 1957).

As was pointed out, the difference between normal and abnormal can be magnified by further reducing the number of platelets added to the system. The effect of this treatment is shown in Table II (B). The haemophilic plasma used in this experiment was obtained from a patient less severely affected than the patient in II (A). The test was performed with the usual strength and also with a 1 in 10 dilution of the same platelet extract. It is clear that the results are more informative when the diluted extract is used. To ensure that the plasma mixtures were sufficiently free of platelets the clotting times with and without added platelets were compared as described above. As shown in Table III, the clotting time of the saline control was well in excess of that with the added extract and the conditions of the test were therefore satisfied.

TABLE III
KAOLIN CLOTTING TIMES WITH VARYING CONCENTRATIONS OF PLATELET EXTRACT

Concentration of platelet extract (%)	100*	33	11	3.7	0
Clotting time (sec.)	98	123	145	169	203

The plasma used was the 50% mixture of normal and haemophilic plasma shown in Table II (B).
* Standard diluted extract.

Differential Diagnosis of Coagulation Defect.—The type of clotting defect is identified by assessing the corrective effect of added reagents prepared from normal blood. Examples of tests on patients belonging to different groups are shown in Table IV. Haemophilia is corrected by alumina plasma and Christmas disease by serum. Neither is corrected by heated plasma (56° C.), which fact distinguishes these conditions from the plasma thromboplastin antecedent (P.T.A.) de-

TABLE IV
KAOLIN CLOTTING TIME IN SOME HAEMORRHAGIC CONDITIONS (SEC.)

Test Plasma	Preparations Added to Test Plasma					
	Patient's Plasma	Normal Plasma	Alumina Plasma	Aged Serum	Heated Plasma (56° C.)	Heated Plasma (60° C.)
Normal	—	90	85	70	92	—
Haemophilic (moderate)	360(90)*	120	132	238	240	—
Christmas disease (mild)	160(95)	122	169	100	168	—
P.T.A. deficiency (mild)	195(83)	114	118	76	116	200
Hageman trait	1,320(90)	99	97	90	105	350

* The figures in brackets refer to normal plasma tested in parallel.

ficiency and the Hageman trait (Ratnoff and Colopy, 1955). The distinction between the latter two is not quite as clear, as the Hageman trait is partly corrected but not the P.T.A. defect by plasma heated at 60° C., but this is hardly likely to present a practical difficulty as the Hageman trait is a very rare condition and it presents an entirely different clinical picture. (For further information see Biggs, Sharp, Margolis, Hardisty, Stewart, and Davidson, 1958 ; Margolis, 1958.)

The range of the added preparations can be modified to suit individual cases and extended by the inclusion of plasma from cases with known coagulation defects (direct matching).

Discussion

The theoretical considerations, with particular reference to the effect of contact, on which the methods are based, have been discussed elsewhere (Margolis, 1957). It has since been demonstrated that both the Hageman factor and P.T.A. are concerned in the activation of clotting by contact (Biggs *et al.*, 1958). It is therefore not surprising that in the absence of satisfactory control of contact conditions the results of clotting tests in cases of deficiency of these factors were often difficult to interpret. What may not, at first, appear obvious is why it should be necessary not only to control but also to reduce the platelet concentration in order to increase the sensitivity of the system to deficiency of A.H.G. and Christmas factor. One might expect that an arrangement where all the components except the one being assayed are kept within normal limits would be quite satisfactory, since the latter would become the limiting factor in the reaction. The clotting time is, however, a resultant of a complicated chain of successive overlapping reactions. The phase of coagulation in which A.H.G., Christmas factor, and platelets are directly involved probably represents only a fraction of the total clotting time. When the platelet concentration is greatly reduced and the clotting time thereby increased, it is presumably this phase of the reaction which is selectively lengthened. Under these conditions any deficiency of A.H.G. or Christmas factor which is reflected by a further prolongation of the relevant stage of the reaction will have a proportionally greater effect on the total clotting time. The present results are in agreement with the observations of Quick (1951) that the difference between the clotting time of normal and haemophilic plasma is magnified by using rapidly centrifuged specimens of blood. By extending this argument, a similar increase in the sensitivity of

the method could be expected if, instead of platelets, other components of blood thromboplastin were reduced. This is, in fact, borne out by a device often used in the diagnosis of mild coagulation defects, where the plasma of the suspected patient is diluted in the plasma of a severely affected case. This method has been used for the assay of A.H.G. by one-stage clotting procedures (Soulie and Larrieu, 1953; Langdell, Wagner, and Brinkhous, 1953), but suffers from the disadvantage of requiring a stock of suitable abnormal plasma as one of the reagents. Moreover, the results of these methods showed considerable variations between different experiments, presumably owing to the inadequate control of the contact surface.

The present method appears to be free from these drawbacks, the reagents being readily available and the results reproducible from day to day. The sensitivity of the system can be adjusted at will and is apparently limited only by how effectively platelets are removed from the plasma. In addition, the technique is simple and, being based on clearly defined principles, can be easily modified to suit individual requirements.

Summary

A simple, one-stage method for the detection and diagnosis of plasma coagulation defects is

described. The test is based on the clotting time of re-calcified plasma in which the conditions are controlled by the addition of kaolin and a diluted platelet or platelet-substitute reagent. The type of deficiency is determined by assessing the corrective effect on the clotting time of the abnormal plasma of various derivatives of normal plasma. The procedure appears to be adequate for detecting even mild degrees of deficiency of plasma clotting factors.

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