THE THROMBOPLASTIN GENERATION TEST

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Physiological coagulation is dependent on the formation of a powerful thromboplastin within the blood. It is well recognized that coagulation defects, such as that in haemophilia, are due to a failure of the normal thromboplastin mechanism, but lack of knowledge has hitherto prevented any precise or detailed study of these abnormalities. Biggs, Douglas, and Macfarlane (1953a) have shown that three components, normal plasma treated with $\text{Al}(\text{OH})_3$, platelets, and normal serum, react to form thromboplastin. Further analysis has shown that two essential substances, anti-haemophilic globulin and factor V, occur in the $\text{Al}(\text{OH})_3$ -treated plasma, while serum contains two factors, factor VII and the Christmas factor, both of which are required for thromboplastin formation (Biggs, Douglas, and Macfarlane, 1953b). These five components—platelets, anti-haemophilic globulin, the Christmas factor, and factors V and VII—react together in the presence of CaCl_2 to form a labile thromboplastin as powerful as any so far described. A lack of any one of the five produces a coagulation defect associated with abnormal thromboplastin formation.

Factors V and VII are usually thought of as "accelerators" of prothrombin conversion because they are necessary for the rapid conversion of prothrombin to thrombin in the presence of brain thromboplastin. This conception is valid because brain extract does not contain a complete thromboplastin; it replaces part of the normal intrinsic thromboplastin system, but is incomplete because it lacks factors V and VII (Biggs, Douglas, and Macfarlane, 1953b). When brain extracts are added to plasma they first react with factors V and VII to form a complete thromboplastin which subsequently reacts with prothrombin. Thus in the absence of factor V or factor VII the conversion of prothrombin to thrombin with brain thromboplastin is delayed, and deficiency of either of these factors can conveniently be demonstrated by the classical one-stage "prothrombin" test.

Factors necessary for blood thromboplastin formation which do not lengthen the one-stage "prothrombin" time have hitherto been studied by such empirical procedures as the whole-blood clotting time, the calcium clotting time, and the prothrombin-consumption test. These techniques can be used to show that some abnormality in thromboplastin formation exists, but they are neither specific nor very sensitive. The whole-blood clotting time, for example, is often a good test for haemophilia (antihaemophilic globulin deficiency), but it is not a sensitive measure of the haemophilic defect. Some haemophilic patients, who suffer severe haemorrhagic episodes, have a normal whole-blood clotting time (Merskey, 1950, 1951), and after transfusion with normal blood the clotting time of haemophilic patients may be restored to normal while the defect must remain uncorrected because the haemorrhage continues.

In spite of the number of factors involved and the apparent complications of the reactions which produce thromboplastin, the thromboplastin generation test (Biggs, Douglas, and Macfarlane, 1953a) is simple to carry out and to interpret. Using this test together with the one-stage "prothrombin" time, it is possible readily to distinguish between deficiencies in the various factors which react to form thromboplastin. It is the purpose of this communication to describe the application of the thromboplastin generation test to the study of various coagulation abnormalities. Factor V was present in normal amounts in the blood of all of the patients studied. Factor V deficiency is not considered in this investigation.

Technique

Collection of Blood.—Venous blood is collected from a normal subject and the patient under investigation. Part of each sample is citrated by adding 1 part of 3.8% sodium citrate to 9 parts of blood. The plasma is separated after centrifuging at 2,000 r.p.m. for 15 minutes. Five millilitres of blood will suffice for the thromboplastin-generation test.

A further 3 ml. of the whole venous blood is placed in a tube with three glass beads. The tube is stoppered and inverted repeatedly until clotting occurs. This process encourages the conversion of prothrombin to thrombin during coagulation. When coagulation is complete the blood is allowed to stand at 37° C. for two hours or more for the complete neutralization of thrombin, maximum utilization of prothrombin, and disappearance of active thromboplastin. The serum is then separated after centrifuging and is diluted 1 in 10 with 0.85% saline for use in the thromboplastin-generation test. The "serum" factors are relatively stable on storage, and serum specimens may be used for some days after collection. Normal serum contains both factor VII and the Christmas factor.

Aluminium Hydroxide Ca.—This is prepared by the method of Bertho and Grassmann (1938) which is described by Biggs and Macfarlane (1953). It is referred to in this communication as "alumina."

Alumina Plasma.—To 1 ml. of citrate plasma is added 0.1 ml. of aluminium hydroxide suspension. The mixture is incubated at 37° C. for three minutes and the alumina is separated by centrifuging. The supernatant plasma is tested by the one-stage "prothrombin" test. By this test the clotting time should lie between one and four minutes. The clotting time should not exceed four minutes. This treated plasma contains the antihæmophilic globulin and factor V, but lacks significant amounts of prothrombin and the "serum" factors necessary for thromboplastin formation. For use in the thromboplastin generation test the treated plasma is diluted 1 in 5 with 0.85% saline. (It will be referred to hereafter as "alumina plasma.")

Platelets and Substrate.—For the thromboplastin generation test 20 ml. of whole normal blood is collected into two silicone-treated 10 ml. graduated centrifuge tubes each containing 1 ml. of 3.8% sodium citrate. The blood is centrifuged at 1,500 r.p.m. for 10 minutes and the platelet-containing plasma is separated and put into silicone-treated tubes and centrifuged again for 15 minutes at 3,000 r.p.m., or at 15,000 r.p.m. for five minutes. The platelets are deposited and the clear supernatant plasma is separated and reserved as substrate for the test. The platelets are washed twice with saline, the platelet mass being fragmented by a wooden applicator stick and re-deposited on each occasion by centrifuging. After the second washing the platelets are resuspended in a volume of 0.85% saline equal to one-third of the volume of original plasma from which they were derived. This suspension of the platelets is facilitated by fragmentation of the platelet mass with a wooden applicator stick. The suspension is used undiluted for the test and may be used on the day after preparation.

Thromboplastin Generation Test.—Immediately before the test is started 0.1 ml. of substrate plasma is pipetted into each of six small tubes of uniform

diameter which are placed in a water-bath at 37° C. In a further tube in the water-bath at 37° C. is placed 0.3 ml. of alumina plasma diluted 1 in 5, 0.3 ml. of platelet suspension, and 0.3 ml. of serum diluted 1 in 10. To this is added 0.3 ml. of M-40 CaCl_2 and a stop-watch started. At intervals of one minute 0.1 ml. of the mixture is withdrawn into a graduated Pasteur pipette and, using the other hand, 0.1 ml. of M-40 CaCl_2 is withdrawn into a second pipette. The contents of the two pipettes are then discharged simultaneously into one of the tubes containing 0.1 ml. of substrate. The clotting times of the substrate samples are recorded. It is usually not necessary to continue the test for more than six minutes. The clotting times of the substrate give a measure of thromboplastin concentration in the incubation mixture and may be expressed in terms of thromboplastin concentration using a thromboplastin-dilution curve.

Thromboplastin Dilution Curve.—A potent preparation of blood thromboplastin, as made in the thromboplastin generation test described above, will usually cause clotting of normal plasma in eight to ten seconds. When made deterioration of the thromboplastin can be delayed by placing the tube in melting ice. The preparation can then be diluted 1 in 2, 1 in 4, 1 in 8, 1 in 16, and 1 in 32 with 0.85% saline. The dilutions are also kept at the temperature of melting ice and tested with normal plasma substrate as described above. A curve relating clotting time and thromboplastin concentration can then be drawn (Fig. 1).

Experimental Results

Normal Variation.—When platelets, alumina plasma, and serum prepared from a normal person are incubated with CaCl_2 a very powerful thromboplastin is formed. In Fig. 2 is shown the average result of carrying out this test on 40 different sets

FIG. 1.—Thromboplastin dilution curve: 0.1 ml. of various dilutions of blood thromboplastin and 0.1 ml. of M-40 CaCl_2 were added to 0.1 ml. of normal plasma and the clotting times were recorded. The curve shows the relation between clotting time and thromboplastin concentration where a clotting time of 10 seconds is taken to represent 100% thromboplastin.

FIG. 2.—The normal range of the thromboplastin generation test. The central curve represents the average of 40 different tests. The extreme limits indicate the range of variation obtained when all of the thromboplastin components are varied simultaneously. The shaded area shows the range of variation obtained in 38 of 40 observations in which the alumina plasma was obtained from different normal subjects, but serum and platelets were from one subject.

of reagents. It will be seen that a level of 100% of thromboplastin, indicating a plasma clotting time of 10 seconds, is achieved on an average after an incubation time of five minutes. This very rapid clotting time cannot be due to the transfer of thrombin from the incubation mixture because no significant amount of thrombin is transferred. In this test three components are used—platelets, alumina plasma, and normal serum; each of these may affect the amount of thromboplastin formed or the speed of its formation. There is therefore a wide range of variability between tests made on different days in which all three reagents are different (Fig. 2). It might be thought that this large variation greatly reduced the usefulness of the method, but in practice the test is not used to measure differences between two sets of the three components. In any particular case interest is centred on one component; on antihæmophilic globulin in hæmophilia, on a serum factor in Christmas disease, or on platelets in thromboasthenia. We have found that for this limited objective the normal range is less.

In tests on 40 samples in which the platelets and serum factor were constant, but the alumina plasma was prepared from different normal samples, the observations exceeded the range shown by shading in Fig. 2 on only two occasions. Similar results were obtained in 15 samples in which the alumina plasma and platelet preparations were constant but the source of serum was varied.

The observed range in two experiments is shown in Fig. 3. In each of these experiments five different samples of alumina plasma were tested with the same preparation of serum and platelets. It will be seen that although the results in two experiments are different the range of observations in one experiment is not wide. To assess, for example, whether or not a preparation of anti-hæmophilic globulin is abnormal the alumina plasma is prepared simultaneously from normal and from the patient's plasma and tested with the same preparations of platelets and serum. When expressed graphically the difference between normal and abnormal should be wider than the total normal range shown in the shaded area of Fig. 2. If the normal, for example, corresponds to the upper limit, curve C in Fig. 3, then the

FIG. 3.—The curves show the range of variation in thromboplastin formation in two experiments. In each five samples of normal alumina plasma were tested with the same preparations of platelets and serum.

abnormal should lie rather below curve D before a deficiency in antihæmophilic globulin could be deduced. No exact quantitative measure of deficiency in any factor can be made by this method, but a distinction between normal and abnormal is possible. A rough quantitative measure can often be achieved by comparing the relative effects on thromboplastin formation of dilutions of normal and abnormal samples. For example, if a serum factor is thought to be deficient an approximate measure of the extent of the deficiency can be obtained by carrying out the thromboplastin generation test on a 1 in 5 or 1 in 10 dilution of the patient's serum and comparing the resulting curve with a series of curves prepared using dilutions of normal serum. If the 1 in 10 dilution of the patient's serum gives a curve similar to that of the 1 in 100 dilution of normal serum, the patient's serum may be said to have 10% of

the normal thromboplastin-forming capacity. The method is not exact because the second serum factor will be diluted in the normal serum to a greater extent than in the patient's serum. In most of the conditions studied the difference between normal and abnormal was very large, and for diagnostic purposes finer criteria of distinction were unnecessary.

Antihæmophilic Globulin Deficiency (Hæmophilia).—When hæmophilic plasma is treated with $\text{Al}(\text{OH})_3$ and supernatant alumina plasma used to replace normal alumina plasma in the thromboplastin generation test thromboplastin formation is greatly reduced. In Fig. 4 are shown the results of carrying out this test with plasma samples from eight hæmophiliacs. In each of these experiments the alumina plasmas from the normal and the

FIG. 4.—Thromboplastin formation in eight cases of hæmophilia. The shaded area shows the total range of thromboplastin formation using normal alumina plasma samples tested in parallel with the samples from hæmophilic subjects. The curves show the results of replacing the normal alumina plasma by alumina plasma from hæmophilic blood.

hæmophilic subjects were compared in their ability to form blood thromboplastin using preparations of platelets and serum from normal subjects. In these experiments no fine distinction between normal and abnormal was required and the total observed range of normal variation is shown.

The test is more sensitive than other laboratory tests for hæmophilia. Three patients whose whole-blood clotting times and prothrombin-consumption indices were normal showed grossly reduced ability to form blood thromboplastin.

The sensitivity of the thromboplastin generation test to the hæmophilic defect is well illustrated by experiments on mixtures of normal and hæmo-

FIG. 5.—The curves show the progress of thromboplastin formation using normal and hæmophilic alumina plasma (upper and lower curves) and using mixtures of the two. The figures are the average results of four similar experiments.

philic plasma. When one part of normal is mixed with nine parts of hæmophilic plasma the clotting time and the consumption of prothrombin during clotting are usually normal in the mixtures. On the other hand, when mixtures of normal and hæmophilic plasma treated with $\text{Al}(\text{OH})_3$ are tested by the thromboplastin generation test the results are not normal (Fig. 5). Even 50% of $\text{Al}(\text{OH})_3$ -treated normal plasma will not eliminate the hæmophilic defect.

When a hæmophilic patient is transfused hæmorrhage may continue despite the normal results of whole-blood clotting time and prothrombin consumption tests. On one occasion it was necessary to remove two teeth from a child aged $6\frac{1}{2}$, weighing 40 lb. He was given one pint of fresh plasma and two-thirds of a pint of fresh whole blood before the extractions. His whole-blood clotting time and prothrombin consumption index were normal, but he bled rather more than normal and his thromboplastin generation remained abnormal (Fig. 6). The thromboplastin generation test may prove to be the most reliable guide to the efficiency of treatment in hæmophilia.

Seven known female carriers of the hæmophilic trait were tested in the hope that this more sensitive test might reveal some difference from normal, but in no instance was the amount of antihæmophilic globulin significantly reduced as judged by the narrower criterion of significance given in Fig. 2.

In addition to its sensitivity to the hæmophilic defect the thromboplastin generation test gives a more specific diagnosis of hæmophilia than other tests. The test makes possible a distinction between hæmophilia and Christmas disease, which is not easy using other methods.

FIG. 6.—The lower curve represents the progress of thromboplastin formation using the alumina plasma collected immediately after the transfusion to a haemophilic child of about 600 ml. of fresh normal plasma. The upper curve represents thromboplastin formation using normal alumina plasma.

Deficiency of Serum Factors

Christmas Disease (Biggs et al., 1952).—Christmas disease is a recently recognized condition closely resembling haemophilia. It differs from haemophilia in that the antihæmophilic globulin is present in normal amounts as tested by the thromboplastin generation test and in that the plasma of patients with Christmas disease will correct the abnormality of hæmophilic plasma. Thromboplastin formation is deficient in Christmas disease due to a reduction in the blood of a factor which differs from the antihæmophilic globulin and is present in normal and hæmophilic serum. The distinction between Christmas disease and hæmophilia can readily be made using the thromboplastin generation test. When alumina plasma and platelets are made from normal or from the patient's plasma and the diluted serum from a patient with Christmas disease is added the amount of thromboplastin formed is reduced (Fig. 7). If hæmophilic or normal serum is used the generation of thromboplastin is normal. The patient's blood therefore contains normal amounts of antihæmophilic globulin, but lacks a factor present in normal and hæmophilic serum.

"Tromexan" Therapy.—The administration of "tromexan" reduces a substance in the blood called factor VII (Koller et al., 1951). Factor VII is necessary for the action of brain thromboplastin; it is obtained from the same fraction of the serum as is the Christmas factor. The reduction of factor VII in the blood of patients receiving tromexan is the main factor which controls the one-stage prothrombin time (Douglas, 1953). If the serum of patients treated with tromexan is tested with alumina plasma and platelets prepared

FIG. 7.—Thromboplastin formation in five cases of Christmas disease. The shaded area shows the total range of thromboplastin formation using normal alumina plasma and serum samples which were tested in parallel with the samples from the subjects with Christmas disease. The curves show the results of replacing the normal serum by the patients' serum.

from normal plasma it is found that the ability to form thromboplastin is reduced parallel to the reduction of factor VII (Fig. 8).

The thromboplastin generation test shows that in the blood of patients with Christmas disease and in the blood of patients receiving tromexan there is a deficiency of a factor necessary for thromboplastin formation. Christmas disease and the defect in tromexan therapy are obviously very different. In Christmas disease there is a normal one-stage "prothrombin time" and characteristically a long whole-blood clotting time; in tromexan therapy the one-stage "prothrombin" time is long and the whole-blood clotting time is usually normal. It is now clear that these two conditions

FIG. 8.—The upper curve shows the ability of the serum from patients treated with tromexan to form blood thromboplastin from normal platelets and alumina plasma. The lower curve shows the factor VII content of plasma samples collected at the same time measured by the one-stage technique (Douglas, 1953). The figures are expressed as percentages of normal and represent the average results obtained with six cases.

lack different factors, both of which are necessary for thromboplastin formation (Biggs, Douglas, and Macfarlane, 1953b; Douglas, 1953).

Functional Platelet Deficiency (Thromboasthenia)

The concept of functional platelet deficiency was put forward by Glanzmann in 1918. Originally, patients who would now be said to have thrombocytopenic purpura or von Willebrand's disease were included in the category "thromboasthenia." The name "thromboasthenia" should be reserved for the few patients described who have functionally and morphologically abnormal platelets. These cases are reviewed by Biggs and Macfarlane (1953).

We had the opportunity to examine a patient whose blood contained giant platelets resembling those described in cases of thromboasthenia; these platelets behaved abnormally in the thromboplastin generation test (Fig. 9). In this experiment alumina plasma and serum were prepared from normal blood and the normal and abnormal platelets were compared. In Fig. 9 the normal and abnormal platelet preparations contain approximately equal numbers of platelets. From experiments with dilutions of platelet suspensions it appeared that the normal platelets were about ten times as effective as those from the patient.

Thromboplastin Inhibitors

There are now a number of reports of patients who acquire a disease closely resembling haemophilia in adult life. We have found records of about 15 patients with this type of abnormality. These patients may have in their blood an anti-

FIG. 9.—The curves show the thromboplastin generated from normal alumina plasma and serum using normal platelets (upper curve) and the abnormal platelets from a patient with thromboasthenia (lower curve).

FIG. 10.—The curves show the thromboplastin generated from a mixture of normal alumina plasma, serum, and platelets, and from the same mixture to which was added various concentrations of alumina plasma containing an inhibitor.

coagulant which interferes with intrinsic thromboplastin formation. In addition, true haemophilic patients may develop a very similar anticoagulant, possibly as a result of immunization following transfusion. There are records of at least 12 such patients.

The thromboplastin generation test has proved very useful for defining the site of action of the anticoagulant. We have now examined the blood of four patients; in three the disease arose in adult life, with no previous history of a haemorrhagic diathesis; the other patient had haemophilia. In all cases it was found that the anticoagulant inhibited the formation of active thromboplastin but did not interfere with the ability of this thromboplastin to form thrombin from prothrombin. The result of an experiment on thromboplastin generation is shown in Fig. 10. In this experiment alumina plasma, platelets, and serum were prepared from normal blood. In three tests final dilutions of 1 in 100, 1 in 250, and 1 in 500 of the patient's alumina plasma were added to the mixtures forming thromboplastin. It will be seen that at a concentration of 1 in 250 the patient's plasma inhibited the formation of plasma thromboplastin. The patient's serum was similarly inhibitory.

In these three cases the inhibitor apparently interfered with the activity of both antihæmophilic globulin and the Christmas factor, because neither antihæmophilic globulin activity nor serum activity could be demonstrated in the patient's blood. In the fourth case, a woman aged 46, the serum activity was normal but the patient's plasma

TABLE I

RESULTS OF THE ONE-STAGE "PROTHROMBIN" TIME AND THE THROMBOPLASTIN GENERATION TEST ON PATIENTS WITH VARIOUS COAGULATION DEFECTS

Technique	Source of Thromboplastin Components			Type of Coagulation Defect					Patient with Thromboplastin Inhibitor
				Haemophilia	Christmas Disease	Factor V Deficiency	Factor VII Deficiency	Thrombosthenia	
One-stage "prothrombin" time	—			Normal	Normal	Prolonged	Prolonged	Normal	Normal
Thromboplastin generation test	Alumina plasma	Serum	Platelets						
	Patient	Normal	Normal	Abnormal	„	Abnormal	Normal	„	Abnormal
	Normal	Patient	„	Normal	Abnormal	Normal	Abnormal	„	May be abnormal
	„	Normal	Patient	„	Normal	„	Normal	Abnormal	Normal

lacked antihæmophilic globulin. In this case it appears that the inhibitor specifically neutralized antihæmophilic globulin. Factors V and VII were unaffected by the inhibitor in all these cases because the one-stage "prothrombin" time using brain thromboplastin was normal.

Discussion

The thromboplastin generation test is technically simple, the final test system being essentially a series of one-stage "prothrombin" times. The preparation of the platelet suspension is time-consuming, but not more so than the preliminary stages in, say, the red-cell fragility test. Since the thromboplastin generation test is not required very frequently in ordinary routine diagnosis this disadvantage is not important. Used in appropriate cases, the test has great advantages over any previously described technique. Using this test, it is possible to differentiate between the abnormalities in which there is a reduction in the formation of intrinsic thromboplastin. Moreover, the test may be a much more sensitive indicator of abnormality than any other test.

Using the thromboplastin generation test, it is possible to make a certain diagnosis of hæmophilia even in mildly affected individuals, and it may be the most reliable index of the effectiveness of treatment in hæmophilia. The thromboplastin generation test makes possible a definite differentiation between hæmophilia and Christmas disease, and this distinction is of practical importance because the treatment of the two conditions is different. In addition, the thromboplastin generation test can be used to detect abnormal platelet function and to define the site of action of anticoagulants which depress thromboplastin formation. For these reasons we believe that the thromboplastin generation test may prove an unusually valuable test of clotting function. The

results of carrying out the test together with the one-stage "prothrombin" time on the blood of patients with various clotting abnormalities are summarized in Table I.

Summary

The thromboplastin generation test is described in detail. This test is the most reliable method for the diagnosis of hæmophilia because, using this test, cases which are mildly affected can be diagnosed with certainty and because it permits a differentiation between hæmophilia and the closely related condition, Christmas disease. Being the most sensitive test for deficiency of antihæmophilic globulin, the thromboplastin generation test gives the best indication of the effectiveness of treatment in hæmophilia.

The thromboplastin generation test has also proved valuable in the study and diagnosis of patients with Christmas disease, with thrombosthenia, and with circulating anticoagulants which inhibit the normal intrinsic thromboplastin system.

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