A THROMBIN GENERATION TEST
THE APPLICATION IN HAEMOPHILIA AND THROMBOCYTOPENIA

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Much recent research on blood clotting has dealt with prothrombin and factors which influence its activation. In the development of such investigations experimental procedures have become increasingly complex and artificial. They usually involve the use of anticoagulants, often multi-stage fractionation by precipitation, adsorption or filtration, and, almost invariably, the addition of tissue extracts to promote thrombin generation. These techniques have yielded information of great importance, but they must have a limited application, since they are far removed from the natural process of clotting.

Normal blood taken by a clean venepuncture into a glass vessel clots firmly in a few minutes without any stimulus other than surface contact. It must therefore have some intrinsic source of thromboplastic activity, in the sense that something is formed which converts prothrombin to thrombin. It is clear that a study of this intrinsic thromboplastin is essential to further understanding of normal clotting and its various defects, because the ability of blood to clot without artificial assistance probably determines its haemostatic efficiency. Fractionation techniques can give no information on the earliest stages of natural clotting, which must, initially, be studied in blood which has been altered as little as possible. Estimations of clotting time and of prothrombin consumption can be made on unaltered blood, but because both give only a single figure which is influenced by several different factors, they are of little help in analysing the causes of delayed or deficient coagulation. More information would be provided if a series of determinations of the thrombin content of whole blood were made at intervals during clotting. In 1901 Arthus carried out an experiment of this sort. He took serial samples of dog's blood which was defibrinated by beating, added sodium fluoride, and after centrifuging determined their ability to clot fluoride dog plasma. A simplified technique described in the following paper uses unaltered whole blood from which serial samples are simply transferred to fibrinogen, the clotting time of which indicates the thrombin concentration of the blood at the time of sampling. By plotting thrombin concentration against time, curves are obtained which show the speed of thrombin generation and destruction and thus may indicate the rate of production and the activity of blood thromboplastin.

The generation of thrombin in whole blood can only reflect thromboplastic activity if irrelevant variable factors are controlled as much as possible. Variations in prothrombin, antithrombin, and calcium concentration are likely to have a considerable effect on thrombin generation, but they were avoided by the use of normal blood, or blood from cases of haemophilia or thrombocytopenia in which these factors are almost certainly normal.

Materials
Fibrinogen Solution.—Blood-bank plasma was adsorbed with Al(OH)₃ to remove prothrombin. From this prothrombin-free plasma the fibrinogen was separated by two successive precipitations with m-2 phosphate buffer at pH 6.6, and dialysed against citrate saline. Details of the method, originated by Jaques (1943), are given by Biggs and Macfarlane (1953). Alternatively, fibrinogen prepared by Dr. Kekwick of the Lister Institute, London, proved very satisfactory. The fibrinogen solutions used had a concentration of 100 mg. per 100 ml. of 0.85% saline.

Thrombin Dilution Curve.—A curve relating fibrinogen clotting times to concentration of thrombin was prepared for each batch of fibrinogen. The standard thrombin solution was a commercial preparation ("thrombin topical," Parke Davis) containing approximately 1,000 units per ml. The precise unitage was not determined, since for the purpose of comparison from one experiment to another it is not important. The standard thrombin was diluted to give 10 different concentrations ranging from 0.5 unit to 20 units per ml. Tubes containing 0.4 ml. of fibrinogen solution
were placed in the water-bath at 37° C., and 0.1 ml. of a thrombin dilution added to each, the clotting times being recorded. When plotted against clotting time the reciprocal of the concentration of thrombin should give a straight line passing near the origin. In practice, a simple curve of clotting times plotted against concentration is used for determining the thrombin content of an unknown solution in units per ml. With the fibrinogen solutions used 1 "unit" of thrombin per ml. of thrombin-fibrinogen mixture gave clotting times between 18 and 20 seconds at 37° C.

Silicone.—The silicone preparation used in some experiments was "drifilm."

Brain Thromboplastin.—This was prepared from human brain tissue by saline extraction of acetone-dried material.

Method
Blood was obtained by venepuncture, taking care to avoid contamination by tissue fluid, and 2 ml. placed, without delay, in a glass centrifuge tube containing 0.2 ml. of saline, shaken gently to mix, and the tube set up in the water-bath at 37° C. The purpose of the saline is to control the volume of reagents added in later experiments. One minute from the time of setting up this tube a sample of 0.1 ml. is removed from it, and added to the first of a row of $\frac{1}{2} \times 2\frac{1}{2}$-in. tubes also at 37° C., each containing 0.4 ml. of fibrinogen solution. Thereafter at one-minute intervals samples of 0.1 ml. of blood are removed in the same way and added to a tube of fibrinogen. The clotting times of these blood-fibrinogen mixtures are recorded. As sampling progresses, the blood clots in the centrifuge tube. The fibrin, as it forms, is pressed to one side of the tube, and removed by means of a wooden swab-stick, to which it adheres conveniently, so that fluid samples can continue to be taken without interruption. The concentration of thrombin in the whole blood at each time of sampling is then obtained from the thrombin-dilution curve. By plotting thrombin concentration against time, a curve of thrombin generation and destruction in the whole blood is obtained.

Results
Normal Whole Blood.—The thrombin generation test was carried out on six men and six women of the laboratory staff. The curves all fell within the shaded area of Fig. 1, the maximum thrombin concentration in each case being not less than 7 units per ml. of blood. The average curve, derived from the 12 results, is given as a thick black line within the shaded area. In each subject the first appearance of thrombin in the blood was nearly coincident with the first appearance of fibrin.

From a study of these normal curves certain characteristics are apparent. There is a well-defined latent period of from two to four minutes during which no thrombin is detectable. This is followed by a sudden, rapidly rising thrombin concentration reaching 7 to 15 units per ml. At a point about six minutes from the start of the experiment the thrombin concentration begins to decline, the rate of fall being initially as rapid as the rate of increase, but becoming progressively less as the base line is approached.

The generation of thrombin was then studied in whole blood to which had been added enough brain thromboplastin contained in 0.2 ml. of saline to produce coagulation in from 15 to 30 seconds. The results obtained are illustrated in Fig. 2, which shows a typical curve obtained from a mixture of normal blood and brain extract compared with the average curve of untreated blood. It is clear that the latent period is abolished by the addition of brain thromboplastin, which causes immediate thrombin production, but the rate of thrombin production judged from the slope of the two curves is little greater than the maximum rate of thrombin production in the untreated blood. Thus untreated blood behaves as if an optimum amount of thromboplastin had been suddenly added to it some minutes after its collection. This naturally suggests that an intrinsic thromboplastin is gener-
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Fig. 2.—Curve A represents thrombin generation in blood to which brain thromboplastin has been added. Curve B is the average curve of untreated normal blood, and the shaded area the limits of normal.

It will be seen from these results that above a certain minimum a reduction in the number of platelets causes a corresponding reduction in the rate of thrombin generation and in the amount of thrombin formed, but has relatively little effect on the time at which thrombin generation begins. Thus a reduction of the platelet content of the plasma from 100,000 per c.mm. to 2,000 per c.mm. causes a lengthening of the latent period of only one minute though the amount of thrombin formed is greatly reduced. Below a level of about 1,000 per c.mm., there is a significant lengthening of the latent period.

Thrombin Generation in Clinical Thrombocytopenia.—The thrombin generation test was carried out on blood from seven cases of thrombocytopenic purpura in which the platelet count was 25,000 per c.mm. or less. The results are given in Fig. 4. In general, they confirm those obtained by experimental reduction of the platelets. In one case the thrombin concentration reached the lower limit of normal, though there was some delay in its production. Apart from this case, the pattern of a reduced thrombin production without much lengthening of the latent period was evident. The thrombocytopenia in these patients was idiopathic in four cases, following rubella in two, and due to aplasia of the bone marrow in one case. In cases where active platelet destruction may be proceeding, the possibility of functionally active platelet fragments being present in the plasma but not counted as platelets cannot be eliminated.
Thrombin Generation in Haemophilic Blood.—The thrombin generation test was carried out on the untreated blood of seven haemophilic patients in whom the clotting time was more than 30 minutes. In none of these tests could any measurable amount of thrombin be demonstrated, even though samples continued to be taken long after clotting had occurred.

The test was then performed on seven haemophilics in whom the clotting time was usually short, often within normal limits. These are illustrated in Figs. 5 and 6, the three curves in the latter figure being from haemophilic triplet brothers aged 22, in whom the Lee and White clotting time was usually between 10 and 12 minutes.

These results show a definite abnormality of the thrombin generation curve, despite the almost normal clotting times. The amount of thrombin produced may be normal, but the latent period is prolonged by several minutes.

When increasing proportions of normal plasma are mixed with haemophilic blood there are corresponding improvements in thrombin generation. Fig. 7 shows the results of a typical experiment in which the blood of a patient with a long clotting time (over 30 minutes) was used. Without added plasma there is no detectable thrombin generation. With 0.2% of normal plasma thrombin generation begins at 11 minutes and reaches 3 units; with 1% it begins at six minutes and reaches 6 units, and with 10% plasma it begins at five minutes and reaches 5.5 units. The amount of thrombin actually
produced by these proportions of normal plasma would be too small to affect directly the thrombin titration. The effect observed must be on the thrombin generation of the haemophilic blood. Similar results were obtained with additions to haemophilic blood of normal fibrinogen prepared by ammonium sulphate precipitation, or by the ether precipitation method of Dr. Kekwick. It is known that antihaemophilic globulin is present in such fractions. A number of observations were also made on the effect of blood and plasma transfusions and the administration of fibrinogen fractions to haemophilic patients in vivo. In these cases it was observed that, though the clotting time could be restored to normal by such therapy, the thrombin generation test, though improved, still gave abnormal results.

Discussion

In normal blood placed in contact with glass the sudden generation of thrombin after an initial latent period of several minutes suggests that thromboplastin is suddenly produced as the result of some time-consuming reaction. The idea that the first stage of coagulation is the activation of inactive "prothrombokinase" was put forward many years ago by Collingwood and MacMahon (1912) and again by Milstone (1949). It may be argued that the latent period might be required, not for the activation of a thromboplastin precursor but for the formation of "active" prothrombin, or an essential accelerator of prothrombin conversion, or for the removal of an inhibitor. But when active thromboplastin (brain extract) is added to blood immediately on collection thrombin generation occurs without a latent period, suggesting that no appreciable time-consuming reaction interposes between the appearance of thromboplastin and the conversion of prothrombin. Moreover, thrombin generation in untreated blood, though it occurs later, is almost as rapid as it is in blood treated with brain extract. Thus, intrinsic thromboplastin, once formed, is apparently as powerful as that produced by tissue extracts, and the relatively long clotting time of untreated blood reflects the delay in its production, not a low potency. Tissue extracts shorten the clotting time merely by abolishing this delay.

The effect of a reduction of the platelets is to reduce proportionally the rate of thrombin generation and the amount of thrombin produced. Since, in these observations, there was no reduction of prothrombin, such an effect probably indicates a reduction in the amount of thromboplastin available. It seems, therefore, that the platelets are concerned quantitatively with the amount of intrinsic thromboplastin produced in the blood. Within wide limits, however, they do not affect the duration of the latent period, indicating that they are not concerned quantitatively with the speed of the reaction which leads up to the production of thromboplastin. These results explain why, in cases of thrombocytopenia, the clotting time is normal, though prothrombin consumption is reduced. The normal clotting time reflects the normal latent period; the reduced prothrombin consumption indicates the deficiency of thromboplastin.

The action of antihaemophilic globulin on thrombin generation is quite different from that of platelets. The most striking effect of a reduction in this factor is a lengthening of the latent period, which may reach the point at which no detectable thrombin is produced at all. Antihaemophilic globulin concentration seems to control the time at which intrinsic thromboplastin is formed. The clotting time is therefore usually prolonged in haemophilia, but this is not invariably so. In severe cases no thrombin can be detected in the thrombin generation test, even though clotting may
occur during the sampling process. Fibrin formation in such cases must be produced by a slow formation of thrombin which never reaches detectable levels. In less severe cases the clotting time may be within normal limits, though the measurable generation of thrombin is delayed by several minutes. In these cases there may be a slow and undetected generation of thrombin causing partial clotting within the normal time, followed by a delayed outburst of thrombin production. It follows that the thrombin generation test is a more sensitive and reliable indicator of the haemophilic defect than is the simple clotting of the blood.

In practice, the test should have useful applications. It is simple to carry out, needs no special apparatus, and, apart from fibrinogen, no special reagents. Since the blood being tested is unaltered by manipulation, or the addition of anticoagulants, calcium, or thromboplastin, misleading artefacts are avoided. The thrombin generation test will reveal abnormalities of intrinsic thromboplastin production, and will distinguish clearly between a deficiency of platelet factor and a deficiency of antihaemophilic globulin. Its sensitivity may be of assistance in the diagnosis of haemophilia, and particularly in assessing the potency of antihaemophilic preparations in vitro and in vivo. Its application to the study of other factors in the formation of thromboplastin will be considered in a later communication.

Summary and Conclusions

A simple technique for following thrombin concentration during natural blood coagulation is described.

The sudden generation of thrombin in normal blood after a latent period of some minutes suggests that thromboplastin is also generated suddenly as the result of a slow reaction initiated by surface contact.

Brain extract causes immediate thrombin generation, but, though it occurs earlier, its rate is little faster than it is in untreated blood. Intrinsic thromboplastin is therefore of the same order of activity as brain thromboplastin.

The long clotting time of untreated blood is due to the latent period preceding thrombin production, not to a low potency of blood thromboplastin. Tissue extracts accelerate clotting by abolishing the latent period.

Within limits the rate of thrombin production, and the amount of thrombin produced, is proportional to the number of platelets, but the latent period remains unaffected. Thus the platelets appear to be concerned with the amount of thromboplastin formed, but not with the time required for its appearance. The blood clotting time is therefore normal in thrombocytopenia, but prothrombin consumption is reduced.

The duration of the latent period is affected by the concentration of antihaemophilic globulin. In severe cases of haemophilia no observable thrombin generation occurs in the blood at any time; in less severe cases, or with added antihaemophilic globulin, it may be normal in rate but delayed in time. Thus antihaemophilic globulin appears to be concerned with the rate of the reaction which leads up to the formation of thromboplastin.

The thrombin generation test is sensitive to any deficiency in blood thromboplastin production and distinguishes between at least two different causes for such a deficiency. It should therefore be useful in diagnosis and in assessing the effect of treatment in haemophilia and other conditions due to deficient thromboplastin production.

References