Diagnosis of haemophilia: use of an artificial factor-VIII-deficient human plasma system

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SYNOPSIS An artificial clotting system deficient in factor VIII has been made from normal human plasma. Factors XII and XI are supplied as 'activation product'. An eluate from $\text{Al}(\text{OH})_3$, which has been incubated with normal plasma, supplies factors X and IX in their 'plasma' (unactivated) form with II. Factor V is provided as the supernatant after the $\text{Al}(\text{OH})_3$ -treated plasma has been precipitated at one-third saturation with $(\text{NH}_4)_2\text{SO}_4$. Fibrinogen is freed of factor VIII by freezing and thawing a lyophilized preparation and then added. Of these, activation product and the fibrinogen may be prepared in advance and stored frozen, and the eluate and supernatant may be made on the day of testing. A phospholipid source and CaCl_2 -solution are also required. In use, a patient's and a control plasma are first diluted in a mixture of the eluate, supernatant, and fibrinogen solution, and clotting times are recorded after completing the system by adding the phospholipid, activation product, and calcium chloride. The clotting times from the mixtures containing the patient's and the control plasmas may then be compared.

The investigation of bleeding disorders is likely to be undertaken in an increasing number of laboratories which do not have access to natural reference plasmas. A further attempt has therefore been made to develop an artificial factor-VIII-deficient system, prepared from normal human plasma, for the investigation and management of haemophilia by simple procedures such as the partial thromboplastin time test.

Various artificial haemophilic 'substrates' previously proposed have included serum. Serum may validly be included in a two-stage test for haemophilia, as the thromboplastin generation test demonstrates; but in a one-stage system, such as the partial thromboplastin time test, the addition of serum will markedly shorten the clotting time of haemophilic plasma. Serum should not therefore be included in an artificial 'haemophilic' system for one-stage tests. The explanation for this shortening may be that serum contains factor X in the activated form (Xa); since the activation of X directly follows the activation of VIII in the clotting sequence (Biggs, Macfarlane, Denson, and Ash, 1965), the provision of Xa can presumably bypass a deficiency of VIII.

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THE TEST SYSTEM

The artificial VIII-deficient system is derived from normal plasma as follows:—

<i>Factors Supplied</i>	<i>Source</i>
XI, XII	'Activation product' (made in bulk)
V	Supernatant from $\frac{1}{3}$ -saturation with $(\text{NH}_4)_2\text{SO}_4$ after $\text{Al}(\text{OH})_3$ -adsorption
II, IX, X I	Eluate from $\text{Al}(\text{OH})_3$ used above VIII-poor purified fibrinogen (available lyophilized)

Factor X is thus provided in the unactivated plasma form. The methods for the preparation of these reagents have been arranged so that the labile reagent (the V-source) and the II-IX-X eluate derived from the same starting material may be prepared and the test carried out on the same day.

PREPARATION OF REAGENTS

CALCIUM CHLORIDE 0.025-M solution

AMINOTRIS(HYDROXYMETHYL)METHANE BUFFER (*Tris* buffer;

Gomori, 1964), 0.05M. This was prepared by dissolving 24.3 g. *tris* base in 1 l. distilled water. Of this solution 250 ml. was acidified with 42 ml. of M-HCl and made up to about 990 ml. with distilled water. The pH was adjusted to 7.2 at 37°C. (equivalent to 7.4 at 22°C.) and the volume made up to 1 litre.

PHOSPHATE BUFFER FOR ELUTION pH 8.0 (Biggs and Macfarlane, 1962) Dissolve (a) 11.88 g. $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, and (b) 9.1 g. KH_2PO_4 , each in 1,000 ml. distilled water; take 9.7 vol. of solution (a) with 0.3 vol. of solution (b).

INOSITHIN The granular soya bean extract Inosithin (Associated Concentrates, Inc., N.J.; V.A. Howe & Co. Ltd., London, importers), containing phospholipid, was used as a platelet substitute. A glairy solution was prepared by dissolving 0.2 g. in about 2.0 ml. ether, and agitating this in 5.0 ml. *tris* buffer (*i.e.*, 4.0 g./100 ml.) in a small ball-mill until the ether had evaporated. This stock solution was stored at -20°C. and diluted for use about 1 in 20 or more (1.2 mg./ml.). That concentration was used which gave the shortest clotting time. (The material may also be prepared by shaking a quantity of the granules directly in *tris* buffer until a stable opalescence is obtained, and determining empirically the optimum dilution for use.)

Citrated plasma was obtained by centrifuging a mixture of 9 volumes of normal human blood and 1 volume of 3.2% trisodium citrate solution, without glass contact. Non-contacted blood-bank plasma was sometimes used for the preparation of 'activation product'.

Activation product Following the method of Nossel (1964), normal human citrated plasma which had been taken into plastic equipment was incubated for 10 min. at 37°C. with Celite 512 (Johns-Manville Co. Ltd., Lompoc, Calif., and London S.E.1) 2 g. per 100 ml. plasma, with repeated mixing. The celite was spun down and, after discarding the supernatant, was washed thrice by resuspension in chilled saline and recentrifugation, and then eluted by resuspension in one-quarter the original plasma volume of 7% NaCl solution in 0.0125-M *tris* buffer for 10 min. at 37°C. with repeated mixing. The eluted celite was spun down and discarded and the supernatant dialysed overnight against about ten times its volume of 0.005-M *tris* buffer containing that quantity of NaCl required to bring the overall concentration to 0.85% after equilibration with the NaCl in the bag. After dialysis, Trasylol, 5 u./ml., was added to the reagent to inhibit fibrinolytic activity which has been variably found to contaminate different batches and which may affect the rate of thromboplastin formation (Amris, 1966).

Activation product may be prepared in bulk, using the largest convenient quantity of starting plasma, for which fresh plasma, frozen plasma, and supernatant plasma from which factor-VIII-rich cryoprecipitate has been prepared (Pool, Hershgold, and Pappenhagen, 1964), have all proved suitable.

The reagent loses activity on contact with glass; it is conveniently stored in polystyrene tubes and handled with a plastic or siliconed glass pipette. It is stable at -20°C. and should be kept chilled at the bench.

Satisfactory results were not obtained when dilutions

of test plasmas in the 'diluent mixtures' (see below) were preincubated with kaolin to activate factors XI and XII (Margolis 1957, 1958); activation product must therefore be used.

FACTOR-V-RICH SUPERNATANT (SUPERNATANT) Fresh plasma, 6-8 ml., was absorbed for 15 min. at 37°C. with one-tenth volume of alumina ($\text{Al}(\text{OH})_3$) suspension (aluminium hydroxide moist gel, B.D.H., diluted about 1 in 20 in *tris* buffer, so that the prothrombin time of the adsorbed plasma was 1½-4 min.). After centrifugation, the $\text{Al}(\text{OH})_3$ was retained for elution (see below). The supernatant was precipitated at room temperature with half its volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution, to remove factor VIII. The precipitate was removed by centrifugation and the supernatant dialysed overnight at 4°C. against about 100 times the volume of the starting plasma of a mixture of 9 parts of 0.85% NaCl and 1 part of *tris* buffer. It was found that this reagent could be used after only four hours' dialysis; so that it may be prepared on the day on which it is required.

ELUATE CONTAINING FACTORS II-IX-X (ELUATE) The $\text{Al}(\text{OH})_3$ separated above was twice washed by resuspension in 10 ml. chilled distilled water and recentrifugation. It was then eluted by resuspension and incubation at 37°C. for five min. in one-tenth the volume of the starting plasma of the phosphate buffer. After centrifugation the supernatant was diluted for use 1 in 8 in *tris* buffer. Dialysis is not required. This reagent may be stored at -20°C. but it is usually convenient to make it up in parallel with the supernatant for each test.

FIBRINOGEN Freeze-dried human fibrinogen was obtained from Dr. W. d'A. Maycock, The Blood Products Laboratory, Lister Institute, Elstree, Herts. It was reconstituted in distilled water to a concentration of 1.5 g./100ml. It was incubated at 37°C. until dissolved (about ½ hr.) and then frozen. On thawing, 48 hr. or more later, the factor-VIII content was found to be sufficiently reduced. Freeze-dried fibrinogen poor in factor VIII has also been prepared by Ortho Pharmaceutical Ltd. (Ream, Deykin, Gurewich, and Wessler, 1965).

USE OF THE REAGENTS

Working in polystyrene tubes, equal volumes of supernatant and fibrinogen were mixed with 2 volumes of eluate. This formed the *diluent mixture*, and dilutions of the unknown (patient's) and control plasmas were made in it. For testing, 0.1 ml. quantities of Inosithin were pipetted into a sufficient number of polystyrene clotting tubes at 37°C.; 0.1 ml. of a dilution was added to a tube, followed successively by 0.1 ml. activation product and 0.1 ml. CaCl_2 solution, and the clotting time recorded. By this procedure, the ability of the unknown plasma to correct the VIII-deficient system is compared with that of the control. From the comparison, the factor VIII content of the unknown may be inferred; and by arranging the tests formally as an assay, a potency ratio may be calculated for the unknown with respect to the control.

Experiments carried out to test the validity of such comparisons are described in the following section.

TESTS ON THE VALIDITY OF THE SYSTEM

SENSITIVITY OF THE SYSTEM To assess how completely the system had been made deficient in factor VIII, the least concentrations of normal plasma perceptibly shortening the clotting time of the system and of natural haemophilic plasma were determined in parallel as follows.

Serial 5-fold dilutions of a normal plasma in the diluent mixture were successively tested until dilutions were reached which gave clotting times clearly within the range of 'blank' readings on the diluent alone. Sufficient quantities of each dilution had been made so that a second clotting time could be measured on each, working in the reverse order. Similar readings were then obtained on dilutions of the same normal plasma in natural haemophilic plasma. The results are plotted in Fig. 1, which shows that the artificial system and the natural haemophilic plasma yielded similar dose-response curves, diverging only at concentrations below about 1 in 500. The residual factor-VIII activity of the diluent mixture was thus about 0.2% of that of normal plasma.

COMPARATIVE FACTOR VIII ASSAYS IN THE PROPOSED SYSTEM AND IN NATURAL HAEMOPHILIC PLASMA The most stringent *in vitro* test of validity would appear to be to compare assay results from parallel tests in the artificial system and in natural haemophilic plasma. Plasma samples were therefore obtained from normal persons with various factor VIII levels and from haemophiliacs with varying but low factor VIII levels following treatment, and assayed in batches of two to four plasmas. In each assay, the

FIG. 1. Serial dilutions of normal plasma in haemophilic plasma ●—●, and in the artificial system ×—×.

FIG. 2. Comparative factor VIII assays using haemophilic plasma and the artificial system for diluting the plasmas tested. The correlation coefficient is 0.89 on 30 pairs of readings.

plasma of one or other of us was always used as standard, and the potency ratios of the other samples calculated against it. Each plasma to be tested was diluted 1/6, 1/36, and 1/216 in the diluent mixture and also in natural haemophilic plasma. The two series of dilutions were tested in parallel, in the same way, by completing the clotting system with Inosithin, activation product, and CaCl_2 solution. The potency ratios were calculated as described by Ingram (1965). The results are shown in Fig. 2, which demonstrates a sufficient correspondence between the two methods for ordinary clinical purposes. The correlation coefficient (r) for these data (30 pairs) was 0.89.

DISCUSSION

There will always be some uncertainty in using artificial substitutes for natural plasmas with known deficiencies of clotting factors. However close a correlation is obtained between the results from parallel tests, the possibility can never be excluded that under some special circumstances a discrepancy would appear. Nevertheless artificial substitutes have a place if they permit work to be done which would otherwise not have been possible. It is hoped that the system suggested above will be useful to this extent, when natural haemophilic plasma is not available.

The present technique, and the method suggested by Knights and Ingram (1967) based on similar

reagents, together make it possible to use the partial thromboplastin time test to diagnose haemophilia and Christmas disease without access to plasmas naturally deficient in factors VIII or IX, and to control treatment in haemophilia. The versatility of the partial thromboplastin time thus becomes comparable to that of the thromboplastin generation test for these purposes.

It is suggested that in the diagnosis of haemophilia, test and control plasmas are diluted 1 in 20 in the diluent mixture, as suggested by Matchett and Ingram (1965) when using natural haemophilic plasma.

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