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An Immunoradiometric Assay for Procoagulant Factor VIII Antigen: Results in Haemophilia, von Willebrand's Disease and Fetal Plasma and Serum

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Summary. An immunoradiometric assay (IRMA) has been developed based on the inhibitor which arose in a polytransfused severe haemophiliac. The two-site IRMA measures antigens closely associated with the procoagulant parts of the factor VIII complex, which are termed FVIIIC antigens or FVIIICAG. FVIIICAG was present in normal plasma and also, at a slightly lower concentration, in normal serum. In 37 patients with haemophilia A, 36 had FVIIICAG levels of less than 10% of the normal plasma pool. In patients with von Willebrand's disease the levels of FVIIIC and FVIIICAG were in good agreement, both before and after treatment with cryoprecipitate or DDAVP. FVIIICAG was relatively stable in plasma at 37°C and could also be detected in cord and fetal serum. The assay is of potential value for detecting reduced levels of factor VIII, for carrier detection and for the prenatal diagnosis of haemophilia.

Factor VIII is thought to circulate in plasma as a complex with three measurable entities. Procoagulant factor VIII (FVIIIC) is measured in clotting tests. Factor VIII related antigen (FVIIIRAG) represents the antigenic determinants detected by heterologous antisera raised to purified factor VIII and is measured by Laurell assay (Zimmerman *et al*, 1971b), radioimmunoassay (Paulssen *et al*, 1975), or by immunoradiometric assay (Counts, 1975; Ruggeri *et al*, 1976; Peake & Bloom, 1977). The third activity, ristocetin cofactor activity (FVIIIR:RistoCof), is measured by the ability of factor VIII, in the presence of the antibiotic ristocetin, to cause normal, washed or fixed platelets to aggregate (Howard & Firkin, 1971; Weiss *et al*, 1973; Macfarlane *et al*, 1975). The exact molecular relationship between these activities has been the cause of much discussion and is still not resolved (Bloom & Peake, 1977).

Studies on the relationship of FVIIIC to the rest of the complex have always been hampered by its lability. Inhibitor neutralization techniques have been used to study the presence (or absence) of non-coagulation active FVIIIC material both in haemophilic plasma (Denson *et al*, 1969; Briggs, 1974) and in tissue homogenates (Bloom & Giddings, 1972). However, this type of assay is difficult to perform with accuracy and is susceptible to the presence of other coagulant activities (e.g. tissue factor).

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This paper reports the development of a coated-tube two-site immunoradiometric assay (IRMA) based on the antibodies or inhibitor which arose in a polytransfused severe haemophiliac. The assay, which measures antigenic determinants closely related to the FVIIIC part of the factor VIII complex, has been used to study the nature and level of these determinants in haemophilia and von Willebrand's disease and also in certain fetal samples. The antigens detected are termed factor VIII clotting antigen or FVIIICAG, and the assay appears useful in the diagnosis of haemophilia in plasma, serum and perhaps fetal serum. Preliminary data with the assay have already been reported (Peake & Bloom, 1978).

MATERIALS AND METHODS

Source and Preparation of Anti-FVIIIC IgG Fraction

P.M. is a severe haemophiliac with an inhibitor to FVIIIC. During treatment with factor VIII concentrate for a serious bleed he developed an inhibitor which rose to a titre of >30 000 units/ml (Biggs & Bidwell, 1959). An IgG fraction was prepared as follows from a sample of the patient's plasma with a titre of 2000 u/ml obtained 20 d after the maximum titre had been reached. A plasma globulin fraction was prepared by precipitation with 50% ammonium sulphate, and after dialysis against 0·04 M phosphate buffer pH 8·0, the fraction was chromatographed on DEAE Cellulose (DE 52 Whatman) equilibrated in the same buffer. The eluent was collected and concentrated to the original plasma volume and was shown to have a titre of 1900 u/ml and a protein concentration of 14·2 mg/ml (by the method of Lowry *et al*, 1951). This IgG fraction was used both for the initial coating of the tubes in the assay system and as the fraction used for iodination (see below).

Preparation of Immunoadsorbent (IMAD)

The IMAD used in the iodination procedure was prepared from a factor VIII concentrate prepared by gel chromatography on Sepharose 6B (Pharmacia) of commercial factor VIII concentrate. The void volume fractions were concentrated to give a solution containing >50 u/ml FVIIIRAG. 1 ml of this solution was diluted 1:1 in 0·2 M borate buffer pH 8·6 and incubated with 2 ml of diazotized M-aminobenzyl oxymethyl cellulose (Miles-Yeda Ltd) as described by Counts (1975). The resulting factor VIII IMAD was washed and used in the iodination procedure described below.

Iodination

20 μl of IgG solution (284 μg of protein) were iodinated by the chloramine-T method of Greenwood *et al* (1963). To the IgG solution in a glass tube was added 1 mCi of $Na^{125}I$ (Radiochemical Centre, Amersham). This was immediately followed by 10 μl of chloramine-T solution (0·018 M). After 30 s of rapid mixing, 100 μl of sodium metabisulphite solution (0·006 M) were added followed by 1 ml of potassium iodide solution (0·0012 M).

The reaction mixture was immediately chromatographed on Sephadex G-25 column (0·9 cm column containing 4 g of Sephadex G-25 (Pharmacia) swollen in 0·1 M phosphate buffered saline pH 7·2 (PBS) and equilibrated in PBS containing 1% bovine serum albumin (Miles). The radioactive protein peak routinely appeared in the 5–8 ml fractions and contained between 70% and 90% of the added radioactivity. More than 96% of this activity was precipitable with

10% trichloracetic acid. The labelled protein fractions were pooled and incubated with the factor VIII immunoadsorbent for 2 h at 37°C and 48 h at 4°C.

Elution of ^{125}I Anti FVIIIC Antibodies

After incubation the IMAD was spun down (3000 *g* for 10 min) and the supernatant was removed. The IMAD was now washed eight times by resuspension and centrifugation in 5 ml of 0·1 M barbital buffer pH 7·9 containing 0·15 M sodium chloride and 0·1% BSA (referred to as barbital buffer). The final wash was shown to contain negligible amounts of radioactivity. The IMAD was then resuspended in 5 ml of 10^{-4} M HCl and allowed to stand at room temperature for 5 min. The IMAD was then sedimented by centrifugation and the supernatant added to 5 ml of barbital buffer. This procedure was now repeated with 10^{-3}, 10^{-2} and 10^{-1} M HCl, and the supernatants were, in all cases, added to 5 ml of barbital buffer. Where necessary the pH of the eluates was adjusted to pH 7·9 with dilute NaOH. Maximum radioactivity ($\sim 1 \times 10^6$ cpm/ml) was obtained in the 10^{-2} M HCl eluate at a pH of 2·1. This fraction was stored at 4°C in the presence of 0·02% sodium azide and used in the immunoradiometric assay.

Immunoradiometric Assay

The assay system used was a coated tube method using 12 × 75 mm polystyrene tubes (W. Sarstedt, Leicester). Each tube was initially coated internally with 0·5 ml of a 1:3000 dilution of the haemophilic inhibitor IgG fraction in 0·1 M bicarbonate buffer pH 9·6, by incubation at 4°C

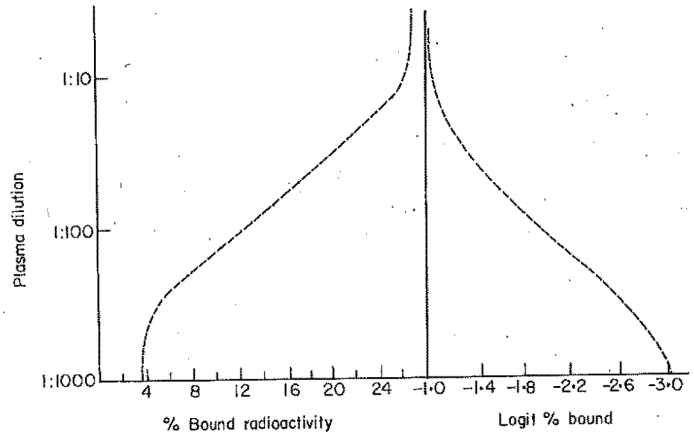

FIG 1. Immunoradiometric assay dose–response curves for a normal plasma pool.

for 18 h. The tubes were then washed twice with barbital buffer and twice with distilled water. Dilution of normal plasma or test plasma or serum was prepared in barbital buffer containing 4% BSA and 0·2 ml was added to each tube (using duplicates). The assay tubes were then incubated at 37°C for 24 h. After a single wash with barbital buffer 0·2 ml of ^{125}I anti FVIIIC antibodies (containing ~4000 cpm) were added to each tube which were then incubated for 24 h at 37°C and 24 h at 4°C. After finally washing twice with distilled water each tube was counted for 2 × 5 min on a Micromedic MS 588 γ counter. The bound radioactivity in each

tube was expressed as a percentage of the total activity added, and a plot of this percent bound against the log of the plasma dilution resulted in a typical dose–response curve with the linear portion of the curve between dilutions of 1:10 to 1:320. A plot of logit % bound (ln %B/(100−%B)) against the log of the plasma dilution gave a straighter line with a sensitivity limit of 1:1000 dilution of normal plasma pool (i.e. 0·1% FVIIICAG). Typical dose–response curves for the normal plasma pool are shown in Fig 1. Test samples were assayed at at least three duplicate dilutions.

Collection of Samples

Blood was taken by venepuncture and immediately divided into two. One half was anticoagulated with one part of trisodium citrate (38 g/l w/v) to nine parts of blood and centrifuged at 3000 ***g*** for 15 min to obtain platelet poor plasma (PPP). This was assayed for FVIIIC activity as soon as possible. All other assays were generally performed on plasma (or serum) stored at −20°C. The second aliquot of blood was allowed to clot at 37°C for 2 h and then placed at 4°C for 18 h. The resulting serum was aspirated and stored at −20°C. All results were expressed as a percentage of the levels of activity present in a lyophilized pool of normal plasma containing plasma aliquots from 30 normal individuals and stored at −80°C. This pool was also used as the control for FVIIIRAG assays. Serum results were corrected for the absence of the dilution effect of anticoagulant in plasma.

Procoagulant factor VIII (FVIIIC) was assayed by a two-stage method similar to that described by Pool & Robinson (1959) and standardized against the International factor VIIIC standard (National Institute for Biological Standards and Control, Holly Hill, London).

Factor VIII related antigen (FVIIIRAG) was assayed by the immunoelectrophoretic method of Laurell as applied to normal plasma (Bloom *et al*, 1973) or by immunoradiometric assay (Peake & Bloom, 1977). Both techniques utilized rabbit anti-human FVIIIRAG, prepared as previously described (Bloom *et al*, 1973). Results were expressed as a percentage of that present in the pool of normal plasma.

Factor VIII related ristocetin cofactor activity (FVIIIR:RistoCof) was measured using the fixed platelet method of Macfarlane *et al* (1975) using an aggregometer (H. Upchurch, Leicester). The results were expressed as a percent of the normal plasma pool.

Factor VIII inhibitor neutralization activity (FVIII:INA) was assessed by the method of Giddings *et al* (1977), using the same haemophilic inhibitor plasma as used in the IRMA.

RESULTS

Normal Plasma and Serum

FVIIIC and FVIIICAG levels were measured in plasma samples from 26 normal individuals. The results are shown in Fig 2, and show a correlation coefficient (r) of 0·74. The mean FVIIIC level (95·4%) was not significantly different from the mean FVIIICAG level (87·8%). Serum was obtained from 14 of these normal people and FVIIICAG was assayed. The IRMA dose–response curves for serum were parallel to those obtained with plasma and a comparison of the plasma and serum results in these normals is shown in Fig 3. An r value of 0·89 was obtained, but the mean FVIIICAG level in serum was significantly lower than that in plasma (mean plasma level 85·4%, mean serum level 69·6%, $P<0·001$).

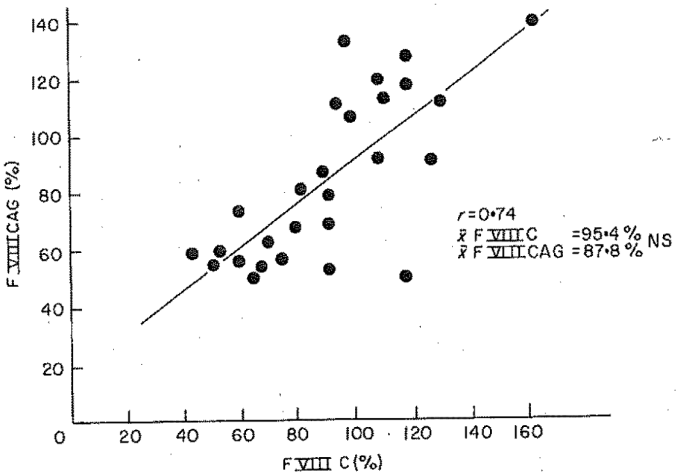

FIG 2. A plot of FVIIIC against FVIIICAG in 26 normal plasmas.

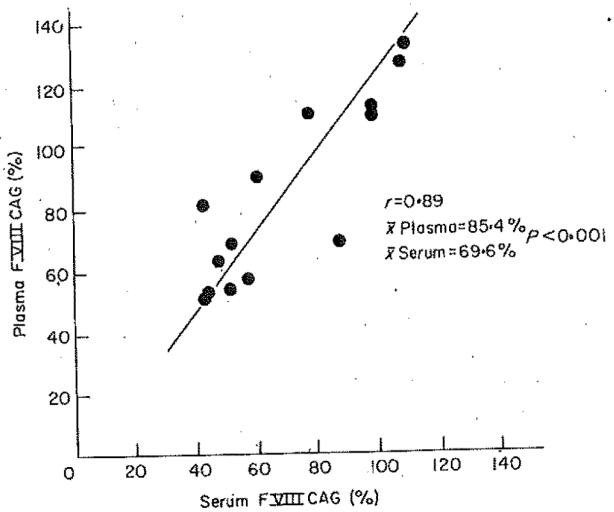

FIG 3. Plasma and serum levels of FVIIICAG from 14 normal donors.

Haemophilic Plasma and Serum

Plasma and serum samples were obtained from 37 patients with mild or severe haemophilia A. The results obtained for FVIIIC, FVIIICAG, FVIIIRAG and FVIII INA in plasma, and FVIIICAG in serum are shown in Table I. FVIIIC levels ranged from 30% to 0% and FVIIIRAG levels were normal or raised. However, in these only one had a FVIIICAG level of greater than 10% (No. 22, FVIIICAG 41%). As Fig 4 shows, there is an apparent complete lack of correlation between plasma levels of FVIIIC and FVIIICAG, except that, of course, they are

both markedly lower than normal. Serum FVIIICAG levels agreed well with the plasma levels, as did serum FVIIIRAG with plasma FVIIIRAG (results not shown). The results may be summarized as follows. Of the 37 haemophiliacs studied 16 had plasma FVIIIC levels of 0% and of these 12 had FVIIICAG levels of <0·1%. In all 24 patients had FVIIICAG levels of <0·1%. Only nine had measurable levels of both FVIIIC and FVIIICAG and, as seen in Fig 4, there was no obvious correlation between these results.

TABLE I. Results in haemophilic plasma and sera*

Patient No.	*% FVIIIC*	*% FVIIICAG plasma*	*% FVIIICAG serum*	*% FVIIIRAG plasma*	*% FVIII:INA plasma*
1	0	<0·1	<0·1	200	NT
2	0	<0·1	<0·1	128	NT
3	0	<0·1	<0·1	86	NT
4	0	<0·1	<0·1	234	NT
5	0	<0·1	<0·1	217	<10
6	0	<0·1	<0·1	259	NT
7	0	<0·1	<0·1	121	<10
8	0	<0·1	<0·1	235	NT
9	0	<0·1	<0·1	156	NT
10	0	<0·1	<0·1	275	NT
11	0	<0·1	<0·1	256	NT
12	0	<0·1	<0·1	234	NT
13	0	2	2	191	NT
14	0	6	5	97	NT
15	0	6	7	128	<10
16	0	2	4	65	NT
17	1	1	1	215	NT
18	1	5	9	88	NT
19	2	4	7	164	NT
20	3	<0·1	<0·1	50	NT
21	3	<0·1	<0·1	198	NT
22	3	41	41	158	65
23	3	<0·1	<0·1	52	NT
24	5	<0·1	<0·1	198	NT
25	6	<0·1	<0·1	143	<10
26	6	7	9	259	25
27	8	<0·1	<0·1	152	<10
28	20	7	9	120	NT
29	22	6	9	62	20
30	12	<0·1	<0·1	145	NT
31	13	<0·1	<0·1	109	NT
32	27	<0·1	<0·1	115	NT
33	10	<0·1	<0·1	86	<10
34	17	2	3	66	<10
35	30	1	1	184	NT
36	23	<0·1	<0·1	75	NT
37	20	<0·1	<0·1	109	NT

NT=not tested.

* All data as % of normal plasma pool.

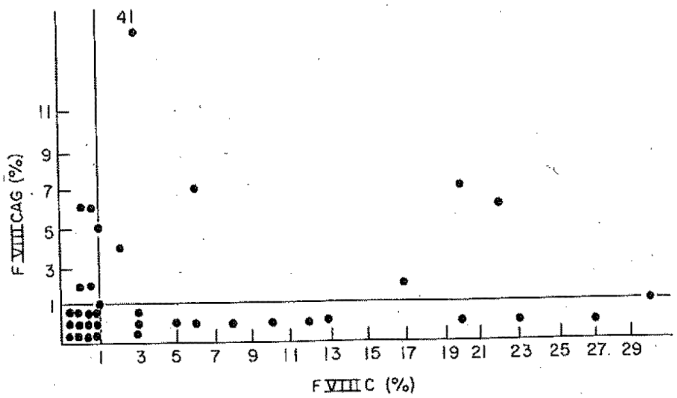

FIG 4. FVIIICAG and FVIIIC in plasma from 37 haemophilia A patients. Cut-off lines shown at the 1% level.

Von Willebrand's Disease (v Wd)

The results obtained in three types of von Willebrand's disease are shown in Table II. In three severe vWd patients, with very low levels of FVIIIC and no detectable FVIIIRAG by the sensitive IRMA, FVIIICAG levels of 3%, 4% and 4·5% were obtained suggesting the presence of low circulating levels of non-active procoagulant factor VIII. In five patients with intermediate vWd, levels of FVIIIC, FVIIICAG and FVIIIRAG were all reduced to approximately the same extent. In one of these, treatment with cryoprecipitate resulted in an increase in all three parameters as expected. However, there was still good agreement between FVIIIC and FVIIICAG. In a single patient with atypical vWd (FVIIIRAG with increased electrophoretic mobility) levels of FVIIIC and FVIIICAG were very similar.

TABLE II. Results in von Willebrand's disease plasma

	FVIIIC (%)‡	*FVIIICAG (%)‡*	*FVIIIRAG (%)‡*
Severe vWd			
1	0	4	<0·01 (by IRMA)
2	0–1	3	<0·01 (by IRMA)
3	0	4·5	<0·01 (by IRMA)
Intermediate vWd			
1	10	5	14
2	6	5	8
3	22	13	13
4	7	6	7
5	5	4	8
5 post cryoprecipitate*	39	32	110
Atypical vWd†			
1	20	23	46

* Following treatment with cryoprecipitate.

† FVIIIRAG has increased electrophoretic mobility on two-dimensional crossed immunoelectrophoresis.

‡ As % of normal plasma pool.

A sixth intermediate vWd patient, with initial levels of FVIIIC, FVIIICAG, FVIIIRAG and FVIIIR:RistoCof all below 10% but with a normal bleeding time, was treated with 1-deamino-8-D-arginine vasopressin (DDAVP) as suggested by Mannucci *et al* (1977) to control bleeding at hysterectomy. The patient was given DDAVP on three occasions during an 8 h period (0·3 μg/kg on each occasion) and the plasma levels of the four FVIII related activities were assayed during this period. The results are shown in Fig 5. The response to the first dose of DDAVP was considerably greater than subsequent doses, and in general all four activities increased to similar extents, except for FVIIICAG after the first injection, when the increase was considerably more than that of the other activities. The significance of this is at present uncertain.

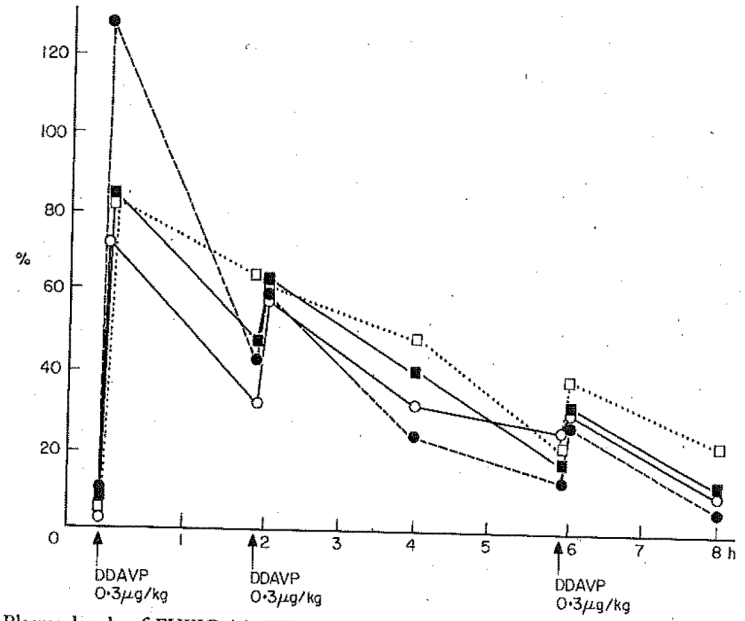

FIG 5. Plasma levels of FVIIIC (■), FVIIICAG (●), FVIIIRAG (□) and FVIII:RistoCof (○) in an intermediate vWd following treatment with l-deamino-D-arginine vasopressin (DDAVP 0·3 μg/kg).

FVIIICAG in Fetal Samples

As the results in serum have shown, FVIIICAG is readily measurable in normal serum (Fig 3). FVIIICAG was also shown to be present in cord serum and Fig 6 shows a comparison of FVIIICAG in plasma and serum from cords of six normal full-term infants. The results were within the normal range and there was no significant difference between them. Assays were also performed on 10 samples of amniotic fluid (FVIIICAG <0·1%) and seven samples of serum obtained directly from fetuses after termination of pregnancy (Table III). Of the latter samples five were from fetuses of normal mothers. In cases 6 and 7 the mother was an obligate carrier of haemophilia and the fetuses were initially shown to have male karyotype by amniocentesis. In both cases no FVIIICAG was detectable in the serum samples obtained, compared to 10–23% in the fetuses from normal mothers.

TABLE III. FVIIICAG in fetal samples

Sample		*FVIIICAG (%)**	*FVIIIRAG (%)**	*Maternal status*	*Fetal ABF (weeks)*	*Fetal sex M/F*
Amniotic fluid (10 samples)		<0·1	<0·1	Normal	—	—
Fetal serum	1	23	NT	Normal	18	M
	2	17	NT	Normal	19	F
	3	17	272	Normal	21	F
	4	10	53	Normal	21	M
	5	15	264	Normal	16	F
	6	<0·1	254	Haemophilia carrier	16	M
	7	<0·1	144	Haemophilia carrier	22	M

NT=not tested.

* As % of normal plasma pool.

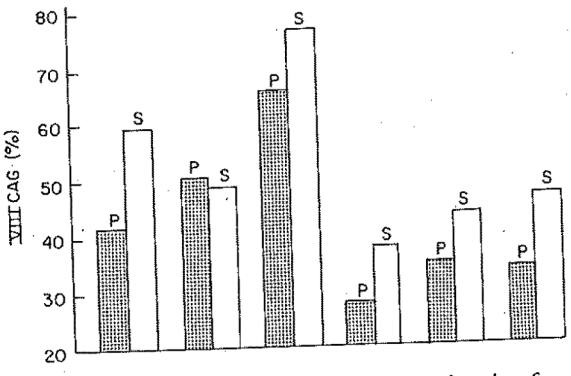

FIG 6. FVIIICAG levels in six cord plasma (P) and serum (S) samples taken from cords of normal full-term fetuses.

Stability of FVIIICAG at 37°C

Normal plasma was incubated at 37°C for up to 26 h. Samples were removed at various time intervals, frozen at −80°C and then assayed together for FVIIICAG, FVIIIRAG and FVIIIC. Fig 7 shows the results of a typical experiment. As expected, FVIIIC levels fell to 30% of the initial level within 24 h. However, the level of FVIIICAG remained constant. Levels of FVIIIRAG showed a slight initial rise.

DISCUSSION

An immunoradiometric assay has been developed based upon an inhibitor which arose in a polytransfused severe haemophiliac. The method used is a coated tube assay very similar to that

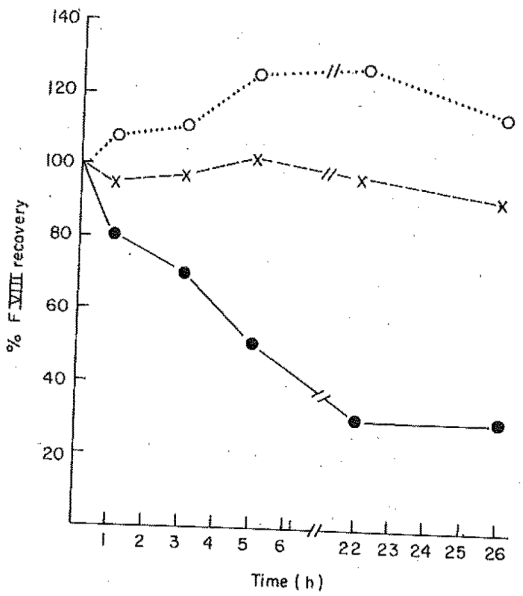

FIG 7. Levels of FVIIIC (●), FVIIICAG (×) and FVIIIRAG (○) in plasma incubated at 37°C. Percent recovery of each activity shown. Initial plasma levels were FVIIIC 125%, FVIIICAG 82%, FVIIIRAG 86%. After 26 h FVIIIC 37%, FVIIICAG 76%, FVIIIRAG 98%.

used for the assay of FVIIIRAG (Peake & Bloom, 1977) and the antigen(s) measured by this assay has been termed factor VIII clotting antigen as FVIIICAG. A comparison of FVIIIC and FVIIICAG in 26 normal plasmas showed reasonable agreement between these two activities ($r=0{\cdot}74$). The reason for the relatively low coefficient of correlation is unclear at present but may be a reflection of the difference in stabilities of the activities. As described in this paper, FVIIICAG is stable in plasma at 37°C for 24 h, while FVIIIC is, of course, labile and loses up to 70% of its activity. A second factor is, of course, that the two assays are totally different in nature and are subject to very different inherent errors. Even so, the agreement in this particular group of normals was better than that of FVIIIC against FVIIIRAG (unpublished observation).

FVIIICAG was also shown to be present in serum and the parallelism with the plasma dose–response curves suggests considerable similarity between plasma and serum FVIIICAG. Although there was a good correlation coefficient between the levels of FVIIICAG in normal plasma and sera ($r=0{\cdot}89$) there was a significant difference in the mean values (plasma 85·4%, serum 69·6%). The reason for this difference is probably connected with the clotting or fibrinolytic process which has occurred and which has resulted in either consumption of some FVIIICAG, or slight antigenic alteration of the molecule, possibly by proteolysis. The results obtained in this paper on normal plasma and serum appear to be very similar to those of Lazarchick & Hoyer (1977), as yet only published in abstract form. The results of their fluid phase assay also suggest better agreement between FVIIIC and FVIIICAG, than FVIIIC and FVIIIRAG, and also indicate the greater stability of FVIIICAG.

Of particular interest are the results obtained with the 37 haemophiliac patients. In all cases except one (No. 22, Table I) both FVIIIC and FVIIICAG were reduced, and FVIIICAG was

also reduced to a similar extent in serum. Thus in these 36 patients a diagnosis of haemophilia could be made based on the plasma or serum FVIIICAG results, combined with a normal or raised FVIIIRAG, normal bleeding time, etc. Patient 22, with 41% FVIIICAG and 3% FVIIIC, represents a CRM+ haemophiliac (Denson *et al*, 1969) as confirmed by the FVIII:INA result of 65%.

As may be readily seen from the haemophilic data there was no apparent relationship between levels of FVIIICAG (as measured by the IRMA) and FVIIIC. Thus with the IRMA the level of FVIIICAG does not indicate the severity of the haemophilia, except that the proportion of patients with FVIIICAG <0·1% in severe haemophiliacs (FVIIIC 0%, 12 out of 16) was greater than in those with measurable FVIIIC levels (10 out of 21). In several patients (32, 33, 36 and 37, Table I) a FVIIIC level of >10% was accompanied by no detectable FVIIICAG. The reason for this is likely to be a combination of the defect in the FVIIIC molecule and the specificity of the antiserum. One interesting possibility is that, since the assay requires that at least two antigenic sites are present on the FVIIIC molecule, the existence of a FVIIIC molecule with some FVIIIC activity, but with only one site available to the antibody(ies), would not be detected by the FVIIICAG assay as performed. Similarly, a FVIIIC molecule altered to have no FVIIIC activity, but still possessing at least two antibody binding sites, would be detected and measured by the assay (e.g. No. 14 and 15, Table I). Quite clearly further studies with different antibodies are necessary, but a complex heterogeneity in haemophilia is suggested. Although detailed family studies have not been performed, the presence of similar results in two pairs of brothers (32 and 33, 14 and 15, Table I) may indicate homogeneity within affected families.

In von Willebrand's disease the close agreement between FVIIIC and FVIIICAG in the intermediate and variant patients further enhances the relationship between these two measurements. The finding of low but measurable levels of FVIIICAG in three severe patients was unexpected, and suggests the presence of a small amount of non-coagulation active FVIIIC in the circulation. Since the genetic X-linked locus for factor VIII is normal in von Willebrand's disease the present results may represent FVIIIC not stabilized due to the complete lack of FVIIIRAG. Treatment of intermediate vWd with cryoprecipitate (Table II) produced an increase in FVIIICAG very similar to that of FVIIIC. In a more detailed study DDAVP was administered (Mannucci *et al*, 1977) and a rise in the FVIIIC, FVIIICAG, FVIIIRAG and FVIIIR:RistoCof was observed. Apart from an increase in FVIIICAG greater than that of the other three activities after the first administration of DDAVP, all four activities behaved very similarly during the course of treatment, suggesting a close relationship between them.

The FVIIICAG results with normal and haemophilic serum led to studies on fetal serum samples. Full-term cord plasmas and sera showed very similar results close to the normal range. In the few post termination fetal samples tested five normals had FVIIICAG levels ranging from 10% to 23%. In the two male fetuses from obligate carriers of haemophilia no serum FVIIICAG was measurable. On the basis of this data it seems reasonable to suggest that the assay of FVIIICAG may be useful in the diagnosis of haemophilia in the fetus. To do this a suitable sample of fetal serum must be obtained by fetoscopic techniques. Although 50 μl of serum is sufficient, contamination with maternal blood must be avoided although some dilution with amniotic fluid may be acceptable. Results on other affected family members should also be available, if possible, in order to show if the fetus could be CRM+. Finally, a range of normal levels of FVIIICAG in the developing fetus must be determined by the assay of

as many normal fetal serum samples as possible. However, the validity of the assay for prenatal diagnosis will depend in the shorter term on its efficacy in demonstrating the expected distribution of abnormal (presumptive haemophilic) male fetuses after termination of pregnancy in a substantial number of CRM− obligatory carriers and eventually on the predictive results in clinical practice.

FVIIICAG was shown to be relatively stable in plasma at 37°C, and also to be present at only slightly (15–20% less) reduced levels in serum. In view of this it may be possible, therefore, to assess the original level of FVIIIC in samples which, for one reason or another, are thought to have lost some of their procoagulant activity. This may be important where a sample has travelled some distance, for instance by post, and particularly in the detection of carriers of haemophilia, where the FVIIIC/FVIIIRAG ratio is critical (Zimmerman *et al*, 1971a). Indeed because of the low FVIIICAG:FVIIIC activity ratio seen in many haemophiliacs, and hence possible in carriers, the assay may even offer increased discrimination in carrier detection.

The development of assays for FVIIICAG has several important ramifications. It may enable the FVIIIC part of the FVIII complex to be studied and estimated unaffected by the lability problems associated with the FVIIIC assay and it may be invaluable in determining the site of synthesis of factor VIII (Piovella *et al*, 1978). Diagnosis of haemophilia is now possible using stored plasma or serum in the majority of cases, and prenatal diagnosis seems to be a possibility. Finally, the assay may alleviate some of the sampling problems and improve the discriminant function associated with coagulation assays in the detection of haemophilia carriers and may permit a valid postal service. These and many other applications remain to be assessed.

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