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INVESTIGATIONS ON THE RELATIONSHIP OF FACTOR VIII RELATED ANTIGEN, FACTOR VIII PROCOAGULANT ACTIVITY AND VON WILLEBRAND FACTOR ACTIVITY USING INSOLUBILIZED RABBIT ANTISERUM.

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ABSTRACT

Insolubilized rabbit antifactor VIII sera removed factor VIII activity and factor VIII related antigen almost completely from normal plasma. This reduction was accompanied by a parallel decrease of the factors correcting the abnormal platelet retention and ristocetin aggregation in Von Willebrand's Disease. This indicated that rabbit antifactor VIII sera are indeed directed not only against factor VIII but also against the correcting factors on the abnormal platelet retention and ristocetin aggregation in Von Willebrand's Disease.

In fluid phase assays antifactor VIII sera inhibited factor VIII activity only partly. Non inhibitory antibodies may be responsible for this. Prolonged immunization of a rabbit with factor VIII resulted in an antiserum, which inhibited factor VIII activity very potently This can be explained by assuming that the functional site of the factor VIII molecule has a very conservative structure that has little changed in the course of evolution.

INTRODUCTION

Factor VIII purified by gelchromatography was shown to correct the abnormal platelet retention and ristocetin aggregation in Von Willebrand's Disease (VWD)[1-4]. Antibodies raised in rabbits against factor VIII not only inhibited factor VIII activity in

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normal plasma, but also reduced the platelet retention of normal blood and the ristocetin aggregation of normal platelets[1,2]. In immunodiffusion only one precipitin line was observed when these antisera were tested against normal plasma, whereas no line was observed against VWD plasma. This suggested that factor VIII and Von Willebrand Factor are identical or closely associated. Recently it was demonstrated that purified factor VIII indeed corrected the prolonged bleeding time in VWD[5].

The observation that some antifactor VIII sera inhibited factor VIII activity only partly or not at all, together with the discrepancy between factor VIII activity and factor VIII related antigen (FVIIIRA) levels in different situations (e.g. VWD plasma, supernatant of cryoprecipitate and after transfusion in VWD) led to the suggestion that antifactor VIII sera were in fact anti-Von Willebrand factor sera[6-9]. Zimmerman and Edgington[10] and Hougie et al[11] suggested that factor VIII activity and FVIIIRA are present in plasma as 2 independent entities. On the other hand Hoyer[12] presented evidence that precipitates formed with rabbit antifactor VIII sera include that portion of the molecule which is responsible for the factor VIII procoagulant activity and Kernoff[13] showed in ultracentrifugal studies that in plasma most of the activity is carried in association with FVIIIRA.

The purpose of our study was to investigate the relationship between factor VIII activity, FVIIIRA and also the Von Willebrand Factor activity using affinity chromatography with insolubilized antibodies. In addition we have studied the influence of prolonged immunization upon the factor VIII neutralizing capacity of antisera directed against factor VIII.

MATERIALS AND METHODS

Antisera

Antisera against factor VIII and low ionic strength components were prepared as described before[2,14,15]. The antisera were absorbed according to Zimmerman et al[16]. The γ-globulin fractions of the absorbed antisera were precipitated with Na_2SO_4 (18% w/v) and purified on Sephadex DEAE-A50 (Pharmacia, Uppsala, Sweden).

One rabbit was immunized in the footpads with 1 ml (20-50 µg protein/ml) of purified factor VIII solution and an equal volume of Freund's Adjuvant Complete (Difco, Detroit). The rabbit was boosted intramuscularly every 2 weeks during one year with different factor VIII preparations (1 ml) containing approximately 20-50 µg protein/ml.

Immunoadsorbentia

The immunoadsorbentia were prepared in 2 different ways:

a) The purified γ-globulin fractions of antifactor VIII serum (7 mg of protein) was incorporated into 40 ml of a gel (T-C=8-25) by polymerization of acrylamide (Fluka, Buchs) in 0,1 M Tris HCl (pH 7.5) containing 5,1 mmol. sodiumcitrate in the presence of the crosslinking agent N,N'-methylenbisacrylamide (Fluka, Buchs) and riboflavin (Sigma, St.Louis) according to the method of Carell and Barandum[17]. After homogenization of the gel by pressing through injection needles of decreasing diameter, the gel was mixed with Sephadex G 25 (Pharmacia, Uppsala, Sweden) and the mixture was poured into a column (Pharmacia, Uppsala, Sweden, K 15/30). Acrylamide and Bis acrylamide were recrystallized before use in respectively chloroform and aceton.

b) Purified γ-globulin fractions of antifactor VIII sera or of the antisera against the low ionic strength components of factor VIII were coupled to CNBR-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the method of Axen, Porath and Ernbach[18]. 15 gram of gel was used, to which 13 mg of protein/gram was coupled. The gel was resuspended in a 0,1 M Tris HCl buffer (pH 7,5) containing 5,1 mmol sodiumcitrate and packed into a chromatography column (K 15/30, Pharmacia, Uppsala, Sweden).

The columns were equilibrated during one night at 4°C with 0.1 M Tris HCl (pH 7,5) 5,1 mmol sodiumcitrate. Normal plasma was applied on the column and subsequently eluted with a speed of 3 ml/hr. In order to remove aspecifically adsorbed protein it was followed by 0.1 M Tris HCl (pH 7,5) containing 5,1 mmol sodiumcitrate until no protein could be detected in the effluent. The antigen-antibody complex was dissociated with 20-30 ml of

3 M NaCNS (pH 6,0) (elutionspeed 3 ml/hr). The columns were re-equilibrated with 0,1 M Tris HCl 5,1 mmol sodiumcitrate (pH 7,5) and could be used again. Several columns were used up to 10 times without a significant loss of binding capacity. In the effluent NaCNS was detected by means of a drop of $FeCl_3$ added to a drop of the effluent. The NaCNS positive fractions including the fraction before the first positive NaCNS fraction were either dialysed against Tris-barbital-sodium-barbital (ionic strength 0.028, pH 8,8) and tested for the presence of FVIIIRA or submitted to $(NH_4)_2SO_4$ (50%) precipitation. The precipitated FVIIIRA was dissolved in Tris-barbital-sodium-barbital (ionic strength 0.028, pH 8,8) and applied to a Sepharose 6B column equilibrated in the same Tris-barbital buffer. FVIIIRA was eluted at the void volume.

Control experiments

For control experiments the γ-globulin fraction of normal rabbit serum was either incorporated into polyacrylamide gel or coupled to CNBR-activated Sepharose 4B. Using columns packed with these materials, control plasma was prepared by elution of normal plasma through these columns in an identical way as described for the insolubilized antifactor VIII columns.

Factor VIII activity and FVIIIRA

The determination of factor VIII activity was performed with a one stage assay using diluted haemophilic plasma as a reagent and Hyland standard as reference[19]. Quantitation of FVIIIRA was performed by the Laurell technique as described previously[20] and modified[21]. FVIIIRA was defined as the amount present in 1 ml of pooled normal plasma.

Platelet function tests

The correcting activity of plasma fractions on the abnormal platelet retention in VWD was tested on cryoprecipitates prepared of 20 ml plasma as described before[20]. The ristocetin aggregation (Lundbeck, Copenhagen) was performed in a Payton Aggregometer (Payton, Canada). To 0,5 ml of platelet rich plasma of a patient with VWD was added 0,1 ml of the plasma fraction. Final platelet count was 200.000/µl platelet rich plasma. After 3 min incubation at 37°C ristocetin (2 mg/ml final concentration) was added. Stirring speed was 1500 rpm. The initial velocity

of aggregation was expressed by calculating the tangent drawn to the steepest part of the aggregation curve on a potentiometric chart recorder. The initial velocities of the tested fractions were expressed as percentages of the initial velocity of aggregation obtained with a pool of normal plasma.

Normal plasma pool

Pooled normal plasma was prepared from 40 healthy subjects. All blood samples were drawn on a 1/10 vol of a 0.129 M citrate solution. The plasma separated by centrifugation at 6000 g for 30 min was stored at -70°C.

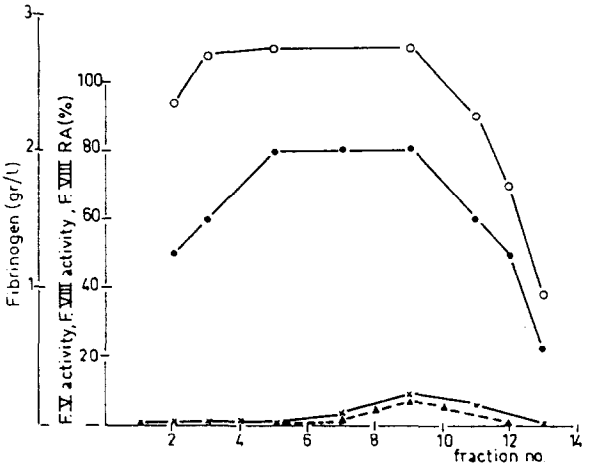

FIG 1

Immunoadsorption of normal plasma. Elution pattern obtained after passage of 70 ml of normal plasma through a column of insolubilized antifactor VIII globulin by entrapment in polyacrylamide gel (see methods) (×) Factor VIII activity (▲) FVIIIRA (●) Factor V activity (o) Fibrinogen

RESULTS

Rabbit antifactor VIII sera were used which only partly inhibited factor VIII activity in normal plasma[2]. The γ-globulin fraction of these antisera was either entrapped in polyacrylamide gel or insolubilized by coupling to Sepharose particles. Passage of normal plasma through columns prepared of these materials reduced factor VIII activity to below 5%. The reduction of the activity was accompanied by a parallel decrease in FVIIIRA (Fig 1). The correcting activities on the abnormal

platelet retention (Table 1) and ristocetin aggregation (Table 2) in VWD were also reduced. In the control experiments factor VIII activity, FVIIIRA and correcting activities were not retained in the column.

TABLE 1

Effect of Immunoadsorption on the Correcting Activity of Normal Plasma on the Abnormal Platelet Retention in VWD

		Normal value	VWD patient	Immunoadsorbed plasma	control plasma
Platelet retention (%)	1.		10	25	75
	2.		13	6	73
	3.		0	13	35
	4.		10	11	75
	5.		13	29	71

The correcting activity on the abnormal platelet retention in VWD was tested by adding cryoprecipitate prepared of 20 ml immunoadsorbed- or control plasma to 8 ml of 5 patients with VWD[20]. For details see Methods.

By recycling of the eluted plasmas it was possible to reduce the factor VIII activity to below 1%. These plasmas could be used as a reagent in the determination of the factor VIII activity.

Dissociation of the antigen-antibody complex with 3M NaCNS resulted in a recovery of FVIIIRA, which was however devoid of factor VIII activity and correcting activity on the abnormal ristocetin aggregation in VWD. It was demonstrated that dialysis of purified factor VIII against 3M NaCNS resulted also in a striking decrease in factor VIII activity and correcting activity on the abnormal ristocetin aggregation in VWD. Other dissociating agents (e.g. Glycine-HCl (pH 2,5), Glycine-HCl (pH 3,5)) had the same effect. The FVIIIRA eluted from the

TABLE 2

Effect of Immunoadsorption on the Correcting Activity of Normal Plasma on the Abnormal Ristocetin Aggregation in VWD.

		immunoadsorbed plasma	control plasma
Ristocetin aggregation (%)	1.	13	100
	2.	0	75
	3.	8	100

The correcting activity on the abnormal ristocetin aggregation in VWD, was tested by adding 0,1 ml of plasma to 0,5 ml of platelet rich plasma of 3 patients with VWD. For details see Methods.

column was concentrated by ammoniumsulphate precipitation and applied to gelchromatography on Sepharose 6B. The void volume fraction containing FVIIIRA was injected into a rabbit and the antiserum obtained inhibited the factor VIII activity in normal plasma and gave a precipitation line when tested in immunodiffusion against purified factor VIII (Fig 2).

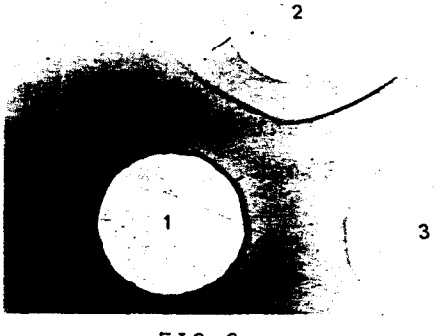

FIG 2

Agarose gel immunodiffusion
Well no 1 contained antifactor VIII; Well no 2 purified factor VIII and Well no 3 the antiserum against the eluted Factor VIII related antigen.

Individual rabbit antisera against factor VIII vary in their factor VIII activity inhibiting capacity. To test whether prolonged immunization of rabbits would result in a more potent factor VIII inactivating capacity a single rabbit was immunized during one year and the antiserum was tested from time to time on factor VIII inhibiting properties. A steady increase in factor VIII neutralizing capacity was observed. Absorption of antifactor VIII serum obtained after one year of immunization with plasma (1:5 and 1:10 v/v) caused a loss of visible precipitation when tested in immunodiffusion against normal plasma (Table 3). The inhibiting property of the antiserum on the platelet retention of normal blood was reduced in parallel but the absorbed antiserum still inhibited the factor VIII activity. The same result was obtained when cryoprecipitates were used for the absorption of this antiserum.

Rabbit antibodies against the low ionic strength components of factor VIII[14] inhibit factor VIII activity only partly[15]. These antibodies were insolubilized by coupling to sepharose particles. The insolubilized antibodies were capable to adsorb factor VIII activity and FVIIIRA completely from normal plasma.

TABLE 3

Properties of Antifactor VIII Serum obtained after One Year of Immunization.

antiserum	influence on platelet retention of normal blood (%)	inhibition of Factor VIII activity (%)	immuno precipitation
unabsorbed	15	3	++
1 vol. + 5 vol. normal plasma	23	14	+
1 vol. + 10 vol. normal plasma	69	22	–
normal rabbit serum	64	100	–

The influence of the antisera on the platelet retention of

normal blood was tested as described before[2]. For these experiments 10 µl of antiserum or normal rabbit serum was added to 8 ml of normal blood.
The influence of the antisera on factor VIII activity was tested by incubation of 0.2 ml of antiserum with 1 ml of normal plasma during 20 min. at 37°C. The residual factor VIII activity was tested and expressed as percentage of the control experiment in which normal rabbit serum was added instead of the antiserum. The antisera and the normal rabbit serum were heated for 1 hr. at 56°C before use.
The immunoprecipitating properties were tested in immunodiffusion against normal plasma.

DISCUSSION

Direct evidence that the antifactor VIII sera are directed against both factor VIII and Von Willebrand factor was obtained from the observation that factor VIII activity, FVIIIRA and the correcting factors on the abnormal platelet retention and ristocetin aggregation were removed by the insolubilized antisera (Fig 1, Table 1 and 2). Using insolubilized antibodies against factor VIII Zimmerman and Edgington[10] provided evidence that factor VIII activity and FVIIIRA (Von Willebrand factor) were 2 independent entities. In contrast our immunoadsorption experiments did not demonstrate any differential binding or partial segregation of FVIIIRA which may be due to the excess of antibody used in our experiments.

The mechanism by which antifactor VIII sera inhibit factor VIII activity is not clear. The antibodies might interfere with the adsorption of factor VIII to phospholipids or with the interaction with factor IX, calcium and factor X. Another possibility is interference with the interaction of thrombin with factor VIII which is thought to be necessary before factor VIII can take part in the intrinsic pathway. Antibodies directed to antigenic determinants more remote from the functional site(s) are expected to be less potent inhibitors of factor VIII activity. These antibodies are called noninhibitory antibodies.

In a fluid phase assay inhibitory antibodies are detected. In a solid phase system antibodies directed to antigenic determinants residing anywhere on the factor VIII molecule, can bind and remove the molecule from plasma. The immobilized antifactor VIII sera used in our experiments indeed removed factor VIII activity and FVIIIRA completely from normal plasma. The residual factor VIII activity in plasma in the presence of excess of antifactor VIII or of the anti-low ionic strength components in fluid phase assays can therefore be explained by the presence of non inhibitory antibodies. The non inhibitory antibodies may protect factor VIII from inactivation by inhibitory antibodies by sterical hindrance, or by inducing a conformational change. Similar effects have been described for antibodies directed against enzymes[22,23].

The residual factor VIII activity in antibody excess decreased during the course of immunization (Table 3). This might be explained by assuming that the functional site(s) of the factor VIII molecule has a conservative structure that has little changed in the course of evolution. The functional site(s) therefore will be a (very) weak immunogen and consequently antibodies will be formed preferentially against antigenic structures not related to the functional site(s). Prolonged immunization may however lead to the formation of antibodies directed against antigenic determinants near or adjacent to the functional site. Such antibodies might be expected to interfere more with the functional site(s) than antibodies will do that are directed against structures located at a greater distance from the functional site as might be the case earlier in the immunization procedure.

Absorption of antifactor VIII serum obtained after one year of immunization, with plasma or cryoprecipitate caused a loss of visible precipitation when tested in immunodiffusion against normal plasma (Table 3). The inhibiting property of the antiserum on the platelet retention of normal blood was decreased in parallel but the absorbed antiserum still inhibited the factor VIII activity. These results are comparable with those of Thomson et al[24] and indicate that the titers of antibodies directed against different antigenic determinants of the factor

VIII molecule can vary in antifactor VIII sera. The variation may depend on the immunization procedure, the purification of the antigen and on the absorption procedure applied.

In conclusion, this study emphasizes the importance of the specificity of antisera used in studies concerning the relationship between structure and function of factor VIII.

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