

FACTOR VIII COAGULANT ACTIVITY AND FACTOR VIII-LIKE ANTIGEN: INDEPENDENT MOLECULAR ENTITIES*[†]

BY THEODORE S. ZIMMERMAN[§] AND THOMAS S. EDGINGTON

(From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037)

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Factor VIII is a requisite molecule in the intrinsic pathway of blood coagulation and is functionally deficient in hemophilia A. Factor VIII coagulant activity (VIII_C) and Factor VIII-like antigen, or von Willebrand's disease antigen (vW-Ag), appear to be biologically linked. They show a direct quantitative relationship in normal individuals, both are decreased to a comparable degree in most individuals with von Willebrand's disease, and they are isolated together under a variety of conditions (1-4). These observations have suggested that the molecule expressing vW-Ag may indeed be the same molecule that possesses VIII_C, namely the Factor VIII molecule. This unitary hypothesis, invoked to explain the observed biologic linkage, has been brought into question by the observation of a marked disparity between VIII_C and vW-Ag in plasma of patients with von Willebrand's disease after infusion of plasma or Factor VIII concentrates (5, 6).

In this report we provide direct evidence that VIII_C and vW-Ag do not reside on the identical molecule in plasma. We here demonstrate that antibodies, covalently coupled to agarose beads, are capable of binding and segregating these two entities one from the other. These data are consistent with a two-molecule hypothesis in which VIII_C resides on the Factor VIII molecule and vW-Ag resides on a biologically linked but different molecule, tentatively referred to as the vW molecule.¹

Materials and Methods

Factor VIII Preparations.—Partially purified Factor VIII containing both VIII_C and vW-Ag was isolated by subjecting Factor VIII concentrates (American National Red Cross Blood Research Laboratory, Bethesda, Md.) to molecular sieve chromatography on 5 × 45 cm columns of Bio-Gel A-15 M, 200-400 mesh (Bio-Rad Laboratories, Richmond, Calif.), and collecting the void volume as described previously (1).

Antisera.—Antiserum against Factor VIII preparations was prepared in New Zealand White rabbits (1). A polyvalent antiserum was constituted by pooling multiple bleedings of

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¹ This material was presented in part at the 30th Annual Meeting of the American Federation for Clinical Research (7), Atlantic City, N. J., 29 April 1973.

four animals, each with demonstrable antibody to vW-Ag by Ouchterlony analysis (1) and to VIII_C by neutralization assay (2 Oxford units) (8). The pool was absorbed with 0.1 vol of supernatant plasma from a 3% cryoethanol precipitate (1) and with 0.02 vol of a pool of sera from patients with IgM myeloma proteins. The absorbed antisera formed a single precipitin line in Ouchterlony gel double diffusion with crude cryoethanol concentrates, and exhibited identity with the precipitin line formed by the originally described antiserum to Factor VIII-like antigen (vW-Ag) (1). The gamma globulin fraction was isolated by ammonium sulfate fractionation and coupled to Sepharose 2B (agarose) beads with cyanogen bromide (9). Gamma globulin concentration varied from 3 to 4 mg/ml beads with various lots, and these are referred to as rabbit antibody beads. Rabbit control beads, possessing comparable amounts of protein, were prepared with gamma globulin of unimmunized rabbits.

The gamma globulin fraction of a human anti-Factor VIII (with an VIII_C neutralizing potency of 235 Oxford units [8]), obtained from an individual with hemophilia A, was prepared and comparable quantities of protein were coupled to Sepharose beads and are referred to as human antibody beads. Human control beads were prepared with normal human gamma globulin.

Coagulant Factor and vW-Ag Assays.—Factor VIII and Factor IX coagulant activities were assayed by partial thromboplastin time assays employing genetically deficient plasma substrates (10). vW-Ag was assayed by quantitative immunoelectrophoresis as described previously (1); however, the sensitivity of the latter assay was increased so as to permit assay of as little as 1.5% of the antigen concentration in normal plasma. This was accomplished by reduction of anti-vW antiserum concentration in the agarose gel, the use of 20- μ l sample vol, and staining of washed and fixed slides.² A pool of plasma from 20 normal healthy individuals, anticoagulated with citrate and stored frozen at -70°C , was used as the standard plasma.

Immunochemical Segregation of Molecules.—The capacity of antibody beads to bind and remove VIII_C and/or vW-Ag from the fluid phase was assayed by incubating varying volumes of antibody beads (from 25 to 400 μ l) with 500 μ l of pooled plasma. The beads were incubated with the plasma by rocking at 37°C for 4 h and then sedimented by centrifugation at 5000 g for 5 min. The supernatant plasma was assayed for residual VIII_C and vW-Ag. In all cases supernatant plasma, similarly incubated with control beads, showed no significant loss of VIII_C or vW-Ag when corrected for dilution. Control supernatant plasma was used as the standard for assessing specific immunologic removal of VIII_C and vW-Ag, and the results are expressed relative to this control.

RESULTS AND DISCUSSION

Solid-phase antibodies provide a means of segregating molecules bearing antigenic markers. Rabbit antibody beads, prepared from antiserum exhibiting both the capacity to neutralize VIII_C and precipitate vW-Ag, do bind and remove both VIII_C and vW-Ag as illustrated in Fig. 1. The binding and removal of each is proportional to the quantity of antibody beads. However, the relative quantity of each remaining in the plasma differs after incubation with antibody beads, a finding inconsistent with a unitary hypothesis. Such an hypothesis would require that the ratio of VIII_C to vW-Ag remain constant. After exposure to dilute rabbit antibody beads, the VIII_C/vW-Ag ratio increases from 1.0 of the initial plasma to 1.47, and it progressively increases with increasing concentration of antibody beads. These observations indicate differential binding

² Edgington, T. S., L. Dickson, and T. S. Zimmerman. Assay of von Willebrand's disease antigen in plasma. Manuscript in preparation.

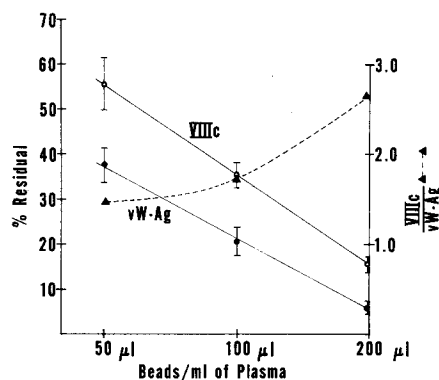


FIG. 1. Immunologic binding and removal from plasma of Factor VIII coagulant activity (VIII_C, ○—○) and von Willebrand's disease antigen (vW-Ag, ●—●) by solid-phase rabbit antibodies. Rabbit gamma globulin exhibiting both anti-VIII_C and anti-vW-Ag properties was coupled to agarose beads. Various amounts of antibody beads were incubated with normal human plasma. After centrifugation the supernatant plasmas were assayed for residual VIII_C and for vW-Ag. (Results are expressed relative to control supernatants from plasmas that had been incubated with similar quantities of rabbit control beads. Control beads had no effect beyond that of dilution.) The brackets include the mean and 1 SD for $n = 4$. Consistently less vW-Ag than VIII_C remained in the plasma. The ratio of residual VIII_C to vW-Ag (▲-----▲) increased as increasing quantities of both were removed by the antibody beads

and partial segregation of vW-Ag from VIII_C, a finding inconsistent with a unitary hypothesis. A unitary hypothesis would require that VIII_C and vW-Ag be bound to an equal degree.

Direct evidence that rabbit antibody beads removed VIII_C from the standard plasma by immunologic binding was obtained in the following manner. After incubation of rabbit antibody beads with plasma they were washed and assayed for residual VIII_C. 31 times more VIII_C was found on these beads as compared with rabbit control beads similarly exposed to plasma. In order to evaluate the specificity of the beads for VIII_C they were also assayed for Factor IX coagulant activity. No significant increase in Factor IX activity could be detected on the antibody beads as compared with the control beads (ratio of 1.2:1).

The effect of human antibody beads on plasma is also inconsistent with a unitary hypothesis. The results of 13 experiments illustrated in Fig. 2 demonstrate binding and removal of 58% of VIII_C from normal human plasma; whereas, no significant removal of vW-Ag was observed. The VIII_C to vW-Ag ratio, 1.0 in the initial plasma exposed to human control beads, decreased to 0.42 after exposure to the insolubilized human anti-VIII_C antibody.

These studies provide direct evidence that VIII_C and vW-Ag can be physically segregated by heterologous as well as homologous antibodies, and thus they cannot uniformly reside on one and the same molecule in plasma. The presence of vW-Ag cannot be considered as evidence, per se, for an inactive form of Factor VIII in classic hemophilia. However, antigenic material capable of

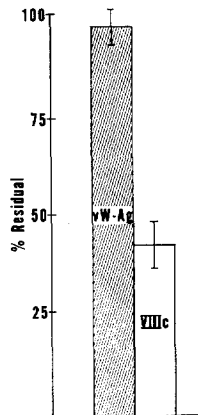


FIG. 2. Removal of Factor VIII coagulant activity (VIII_C) from plasma, independent of von Willebrand's disease antigen (vW-Ag), by insolubilized human isoantibodies to VIII_C. Human antibodies to VIII_C were coupled to agarose beads, incubated with normal human plasma, and removed by centrifugation. The supernatant plasma was assayed for residual VIII_C and vW-Ag and results are expressed as in Fig. 1 except that human control beads were used.

blocking the Factor VIII-neutralizing capacity of rabbit antibodies has been found in the great majority of either hemophilic plasmas (10) or cryoethanol concentrates (1) or cryoprecipitates³ from hemophilic plasmas. This antigenic material would appear to represent inactive Factor VIII. In addition, a solid-phase immunologic assay for Factor VIII⁴ also detects antigenic material in the plasma of hemophiliacs.

Recently, an additional factor with biologic linkage to the vW-Ag has been described (11-15). This factor, designated the von Willebrand's Factor (see Glossary of Definition of Terms), confers glass bead adhesiveness and ristocetin aggregability to human platelets. Like the vW-Ag, it is present in hemophilia but often decreased in von Willebrand's disease. As is the case with VIII_C, vW-Ag and the von Willebrand's disease factor are isolated together under a variety of conditions. These considerations suggest that the molecule expressing vW-Ag may also possess von Willebrand's Factor activity. However, attractive as it may appear, this hypothesis remains to be proven.

GLOSSARY OF DEFINITION OF TERMS

Factor VIII Coagulant (Antihemophilic Factor, VIII_C).—An activity that corrects the coagulation abnormality of hemophilic blood or plasma. Measured by coagulation assays. Prob-

³ Fienstein, D. I., S. I. Rapaport, and M. M. Y. Chong. The differing effects of the 2 subtypes of hemophilia A(A⁺ and A⁻) upon the neutralizing activity of a rabbit antiserum to human Factor VIII. Manuscript in preparation.

⁴ Zimmerman, T. S., L. de la Pointe, and T. S. Edgington. A solid phase immunologic assay for antigenic determinants residing on the Factor VIII molecule. Manuscript in preparation.

ably resides on a molecule distinct from that expressing the von Willebrand's antigen, though both are biologically linked. Decreased in both classic hemophilia and von Willebrand's disease.

The von Willebrand's Factor.—An activity that confers ristocetin aggregability and glass bead adhesiveness to human platelets. Shows a close biologic correlation with the molecule expressing the von Willebrand's antigen though residence on the same molecule has not been established at present. Often decreased in von Willebrand's disease but normal in classic hemophilia.

The von Willebrand's Antigen (Factor VIII-Like Antigen, vW-Ag).—A precipitating antigen that is often decreased in von Willebrand's disease. Usually normal in classic hemophilia.

The relationship of the Factor VIII molecule to the molecule bearing vW-Ag is not resolved. The nature of the biological linkage is open to a number of hypothetical speculations including the possibility that the two molecules exist in plasma as a reversible complex; the vW molecule may be the precursor of Factor VIII; or the vW molecule may govern synthesis or release of Factor VIII from tissue sites. Elucidation of the biologic linkage of these two molecules should provide a sound molecular approach to the pathobiology of those diseases associated with decreased or abnormal function of Factor VIII.

SUMMARY

Factor VIII coagulant activity (VIII_C) and the von Willebrand's disease antigen (Factor VIII-like antigen, vW-Ag) are biologically linked, and it has been suggested that they reside on the same molecule. However, insolubilized human isoantibody to VIII_C and rabbit antiserum containing antibodies to VIII_C and vW-Ag differentially bind and remove these entities from plasma, thus physically segregating one from the other. These findings indicate that Factor VIII coagulant activity resides on a molecule distinct from that expressing the von Willebrand's antigen.

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