be of wider significance as a cause of cerebellar disease. Whether the destruction of cerebellar granule cells is due to a cytocidal effect of LCM virus on a proliferating and migrating cell population, or is mediated by an immunological mechanism, is not presently known.

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Factor VIII Detection by Hemagglutination Inhibition: Hemophilia A and von Willebrand's Disease

Abstract. *Factor VIII activity was detected immunologically in both the serums and plasmas of 14 normal individuals and 14 patients with hemophilia A. A hemagglutination-inhibition test with rabbit antibody to highly purified (10,000-fold) factor VIII from humans was used. Serums and plasmas from eight patients with von Willebrand's disease showed little or no factor VIII activity in this test, an indication that the test may serve as a specific assay for differentiation between von Willebrand's disease and hemophilia A.*

The genetic defect in classic hemophilia is manifested by a lack of factor VIII (antihemophilic factor) activity in plasma. The molecular basis for this defect has not been established. The diminished activity may be due to an inability to synthesize factor VIII or to synthesis of an "abnormal" factor VIII which lacks biological activity. In von Willebrand's disease, the low level of factor VIII activity could be the result of either of these mechanisms or of other still-undefined mechanisms.

The presence of a biologically inactive molecule might be detectable immunologically. However, obstacles to the application of an immunologic assay for factor VIII have included the lack of pure antigen and the difficulty in demonstrating precipitation with either human or rabbit antibody to factor VIII; hence, there is general dependence on tests for procoagulant activity. Recently, Hershgold *et al.* have produced 10,000-fold concentrated human factor VIII from plasma cryoprecipitate (*1*). This material is physically, chemically, and immunologically essentially homogeneous. We now describe the immunologic detection of factor VIII by means of a passive hemagglutination-inhibition technique in which a rabbit antibody to this highly purified factor VIII is used (*2*). Our results indicate that material either antigenically related or identical to factor VIII is present in similar amounts in both normal and hemophilic plasma and serum. In contrast, little or no such material was detected in specimens from patients with von Willebrand's disease.

Factor VIII was purified from an extract of a cryoprecipitate of human plasma by a succession of adsorbing agents, followed by precipitations with ammonium sulfate and polyethylene glycol, and by agarose-gel filtration. Per unit volume, the final product contained 10,000 times the factor VIII activity of plasma and had less than 10 percent impurities (*1*). An antibody was prepared in rabbits by serial subcutaneous injections of this preparation combined with Freund's incomplete adjuvant. In immunoelectrophoresis with normal plasma, this antiserum precipitated (as evidenced by precipitin arcs) small amounts of immunoglobulin and a protein with the expected mobility of factor VIII. The antiserum was inactivated by heat and absorbed with washed human O+ erythrocytes.

The purified factor VIII was coupled to human O+ erythrocytes by the chromic chloride method. Equal parts of factor VIII concentrate (0.7 mg/ml of normal saline), 0.0375*M* $CrCl_3 \cdot 6\ H_2O$ (1:15 or 1:20 dilution in saline), and washed red blood cells were mixed for 4 minutes and washed in saline. The titer of the rabbit antibody to factor VIII was determined by hemagglutination in V-shaped microtiter plates (*3*). The antibody agglutination titers averaged 1:1000. For inhibition assays, a series of twofold dilutions of test plasma

Table 1. Inhibition of agglutination of factor VIII–coated red cells by various plasma and serums. Red blood cells (O+) were sensitized with factor VIII (0.7 mg per milliliter of saline); a 1:20 dilution of 0.0375*M* $CrCl_3$ was used. Rabbit antibody to factor VIII was used at a 1 : 400 dilution; 0, inhibition of agglutination; +, agglutination.

Test material	Reciprocal of dilution of test material								
	2	4	8	16	32	64	128	256	512
Factor VIII	0	0	0	0	0	0	0	+	+
Human serum albumin	+	+	+	+	+	+	+	+	+
Buffer	+	+	+	+	+	+	+	+	+
Normal plasma	0	0	0	+	+	+	+	+	+
Normal serum	0	0	0	0	+	+	+	+	+
Hemophilic plasma	0	0	0	0	+	+	+	+	+
Hemophilic serum	0	0	0	0	+	+	+	+	+
Von Willebrand plasma*	+	+	+	+	+	+	+	+	+

* Two of eight samples consistently showed inhibition in the first two wells.

or serum were made, and the antiserum was added in a dilution one-fourth that of the agglutination titer. The indicator red cells (0.2 percent suspension) coated with factor VIII were then added to the wells. Controls included human serum albumin, purified homologous factor VIII, and buffer. After 1 hour at room temperature, the inhibitory titer of the various test specimens was determined by visual inspection of the plates after centrifugation.

Plasma and serums from 14 normal individuals, 14 patients with hemophilia A, and 8 unrelated individuals with von Willebrand's disease (*4*) were tested for their ability to inhibit the agglutination in this assay. As shown in a representative experiment (Table 1), serums and plasma from both hemophiliacs and normal subjects produced comparable inhibition of agglutination. In contrast, plasma from six of eight patients with von Willebrand's disease produced no inhibition, and the remaining two produced inhibition (*5*) only in the first two wells. Serums from three patients with spontaneously occurring antibodies to factor VIII produced inhibition at titers comparable to those of normal serum, and produced no agglutination of red blood cells coated with factor VIII.

Previous reports have suggested that human factor VIII can cross-react with that of various animal species (*6*). However, in this assay only the serums of gorillas and chimpanzees produced inhibition of approximately the same magnitude as human serum. On the other hand, serums from the spider monkey, baboon, cow, fetal calf, goat, pig, rabbit, dog, guinea pig, and chicken produced no inhibition at all. In addition, a purified porcine factor VIII fraction produced no inhibition in our assay.

Since the rabbit antiserum to factor VIII contained traces of antibody to IgG and IgM, as shown by immunoelectrophoresis against pooled human plasma, the specificity of the inhibition reactions was examined at the dilution of antiserum used in our assays. Isolated human serum proteins, including β-lipoprotein, transferrin, fibrinogen, α-globulins, albumin, IgG, IgA, and IgM, and pooled light chains produced no inhibition in the assay system. The purified factor VIII itself generally inhibited the reaction to a titer of 1 : 128 (equivalent to about 6 μg of factor VIII per milliliter).

Our results indicate that material antigenically identical or closely related to factor VIII is present in the plasma and serum of both normal subjects and patients with hemophilia A. These results also suggest that the deficiency in procoagulant activity noted in hemophilic plasma is not due to the absence of factor VIII but probably results either from alteration of an active moiety of the substance or from the failure to synthesize a portion of the molecule needed for biological activity (*7*). Similar results have been reported by Shanberge and Gore (*8*) who used a precipitation reaction with a rabbit antibody to partially purified human factor VIII. Goudemand *et al.* (*9*) found that human antibodies to factor VIII from two different persons were inhibited by normal serum and plasma and by hemophilic plasma. Recently, Zimmerman *et al.* (*10*), as a result of their experiments in which semiquantitative immunoelectrophoresis was used to detect factor VIII, suggested that the immunologically reactive material present in the serums and plasmas of hemophilic subjects was present in quantitatively reduced amounts in plasmas from patients with von Willebrand's disease.

A similar dichotomy between biological and antigenic reactivity has also been reported in factor IX deficiency (hemophilia B) (*11*), in catalase deficiency in mice (*12*), and in the tryptophan synthetase found in *Neurospora* (*13*).

Serums from humans who have spontaneously developed autoantibodies to factor VIII have been used by others to define variants of hemophilia A (*14*). Plasma from cross-reacting material (CRM)–positive (A+) patients inactivates these antibodies, whereas CRM-negative (A−) plasma does not. It seems likely that the different specificity of these autoantibodies is due to the recognition of selected determinants on factor VIII, which are possibly allotypic and probably in close proximity to the site necessary for biologic activity. In our assay, human antiserums to factor VIII did not agglutinate cells coated with our purified factor VIII preparation (*15*).

In six of eight plasmas from the patients with von Willebrand's disease, no inhibition was detected, indicating that no material related immunologically to purified factor VIII was present. Despite our inability to detect the molecule antigenically, these patients have low levels of factor VIII activity in their plasma (*16*). One can speculate that failure to detect factor VIII in this disease may reflect a gross distortion of the molecule; alternatively, the procoagulant activity may be carried on some unrelated substance. It is of interest that in two of eight patients, small amounts of factor VIII were detected, suggesting that heterogeneity may be present in this disease.

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