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Research Article

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The Immunologic Character of Acquired Inhibitors of Antihemophilic Globulin (Factor VIII) and the Kinetics of Their Interaction with Factor VIII *

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Summary. Investigations were undertaken of the chemical nature and kinetics of interaction of three acquired inhibitors of Factor VIII. The inhibition was stoichiometric, one molecule of inhibitor (or one site on a molecule) being required to inactivate one molecule of Factor VIII. All three inhibitors were found to be monotypic antibodies of class IgG, Type K (γ_2 κ_2). This appears to be the second syndrome due to the production of monotypic antibody, and the first instance involving IgG immunoglobulins.

Introduction

The sudden appearance of a circulating inhibitor of antihemophilic globulin (Factor VIII) is an uncommon, but well-documented phenomenon (1). Most inhibitors are found in individuals belonging to one of four clinical groups: *1*) patients with classical hemophilia A (2, 3); *2*) normal postpartum females, within the first 6 months after delivery (4, 5); *3*) individuals suffering from a wide variety of disease states having, or thought to have, an immunologic setting (e.g., rheumatoid arthritis, penicillin reactions) (6–8); and *4*) elderly persons, of both sexes, in prior good health (9–11). The nature of these inhibitors has not been clearly defined. Kinetic studies have led some investigators to conclude that Factor VIII is destroyed enzymatically (9), whereas others interpret their results to indicate stoichiometric inactivation (12, 13). Chemical studies are consistent with the assumption that Factor VIII inhibitors are antibodies (2, 3, 13), but, with one exception (14), direct evidence for their identification as immunoglobulins is lacking.

For the present investigation three cases, representative of the first three clinical categories, were compared. The interaction of Factor VIII with each inhibitor was studied by a general kinetic approach that will be described in detail; physicochemical and immunological properties of each inhibitor were delineated with appropriate techniques. Results demonstrate that all three inhibitors are IgG-immunoglobulins of pure kappa type, which inactivate Factor VIII by a mechanism consistent with one-to-one stoichiometry.

Methods

Coagulation methods

For the preparation of plasma, blood was collected into plastic bags, with acid-citrate-dextrose solution as anticoagulant.[1] Occasional blood samples were drawn with silicone-coated equipment[2] into $\frac{1}{9}$ vol of 0.1 M sodium citrate or 0.1 M sodium oxalate. After centrifugation at 4° C, the platelet-poor plasma was removed, quick-frozen in acetone-dry ice, and stored at −20° C in small aliquots in silicone-coated tubes.

Serum was prepared from blood collected into uncoated glassware without anticoagulant and incubated

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[1] Fenwal Laboratories, Morton Grove, Ill.

[2] GE Drifilm SC87, General Electric Co., Schenectady, N. Y.

TABLE I

Representative coagulation data of the three patients studied

	Hemophilia A	Postpartum	Ulcerative colitis
Coagulation time, *minutes*			
Glass	60	62	90
Silicone	200	>420	260
Platelet count	224,000	398,000	170,000
Prothrombin time, *% of normal*	90	100	80
Factor VIII, *% of normal*	<0.5	<0.5	<0.5
Factor IX, *% of normal*	82	98	66
Factor XI, *% of normal*	104	120	82

at 37° C for 18 hours. The clot was removed by centrifugation and the serum stored frozen.

Assays for Factors VIII and IX were performed with the kaolin-activated partial thromboplastin time (15, 16). Substrate plasma samples were prepared with plastic equipment [1] from individuals deficient in Factor VIII or Factor IX, quick-frozen in acetone-dry ice, and stored in small aliquots in silicone-coated tubes at −20° C. Normal plasma, for use as a daily standard, was collected and stored in a similar fashion. To minimize any inactivation of substrate plasma during performance of the test, contact activation was completed before the addition of inhibitor-containing plasma, in a manner similar to that employed by Breckenridge and Ratnoff (9): 0.1-ml aliquots of substrate plasma, 15 mg per ml kaolin suspension, and crude brain cephalin (17) were incubated in glass tubes at 37° C for 8 minutes; at the end of this time 0.1 ml of test sample was added, incubation was allowed to proceed another 30 seconds, and finally 0.1 ml of M/30 $CaCl_2$ was added and the time for clot formation measured. In this system 8 minutes' incubation was found sufficient for maximal activation of the contact system. Standard normal plasma dilution curves were run with every set of experiments. Normal and deficient plasma samples, tested daily in this manner, showed excellent stability over periods of at least 2 to 3 months. Forty standard curves for Factor VIII assays, performed over this period of time, demonstrated the error of the assay to be ± 15%, that is, at all plasma dilutions tested (2% to 50%) the coefficient of variance was less than 7.5%. Twenty normal donors were assayed for Factors VIII and IX, and the mean values of these determinations were used to define 100%, or 100 U per ml. This "unit" has been utilized primarily to avoid ambiguity in the presentation of kinetic data.

Factor XI was measured with artificially depleted substrate plasma, essentially as described by Horowitz, Wilcox, and Fujimoto (18). Other coagulation tests were performed by standard methods (19, 20).

Patients

1) Hemophilia A. This 15-year-old white male with classical Factor VIII deficiency developed a potent circulating anticoagulant at age 14. Since that time it has not been possible to produce measurable levels of Factor VIII in his circulation with infusion of fresh plasma or concentrated Factor VIII preparations.

2) Postpartum. This 36-year-old white female developed a severe life-threatening hemorrhagic diathesis, secondary to a circulating anti-Factor VIII, 2 months after the normal delivery of her third child. She had never received transfusions, and her previous pregnancies had been normal. Three months after the onset of the disease her inhibitor concentration began to decrease spontaneously; after 6 months no inhibitor was present, and her Factor VIII level had returned to normal.[3]

3) Ulcerative colitis. This 25-year-old white male with ulcerative colitis has received corticosteroid therapy since the onset of his disease at age 16. At age 20 he suddenly developed a severe hemorrhagic syndrome, characterized by a circulating inhibitor to Factor VIII. Since then it has not been possible to produce significant levels of Factor VIII with replacement therapy, although bleeding is usually least when his intestinal disease is well controlled.[3]

Some pertinent coagulation tests are summarized in Table I.

Chemical and immunological methods

Sephadex gel filtration was performed according to Flodin (21). Starch block electrophoresis was run by the method of Kunkel and Slater (22). Protein concentration was measured either as absorbance at 280 mμ or by the Folin-Lowry procedure (23), with bovine plasma albumin as standard.

Antisera against the three major immunoglobulin classes were purchased from a commercial source.[4] Light chain type-specific antisera were produced by immunizing rabbits with Bence Jones proteins of either kappa or lambda type and subsequently absorbing the antisera with Bence Jones protein of opposite type. Specificity and potency of all five antisera were verified by immunoelectrophoresis (24) and double diffusion in agar (25).

To determine the immunologic nature of the Factor VIII inhibitors, mixtures of antiserum and inhibitor fractions purified by starch block electrophoresis were incubated for 1 hour at 37° C, then overnight at 4° C. Precipitates were removed by centrifugation, and supernates were assayed for residual anti-Factor VIII activity. Controls of antiserum alone and inhibitor alone were

[3] Details of this patient will be reported elsewhere.

[4] Hyland Laboratories, Los Angeles, Calif.

always included, and all experiments were performed at least in duplicate.

Inhibitor measurement

Inactivation of Factor VIII was studied at 37° C by incubating inhibitor-containing materials with standard normal plasma or with more purified Factor VIII preparations (26) in plastic tubes.[5] Samples were removed for Factor VIII assay with plastic pipettes [5] every 2 to 3 minutes during the first 15 minutes of incubation, in order to obtain accurate estimates of the initial velocity of the reaction. Sampling was continued at 15- to 30-minute intervals for a further 105 minutes. Controls at 37° C of Factor VIII alone and inhibitors alone showed no loss of Factor VIII activity for 90 minutes, and only a 10% loss at 2 hours. No corrections have been applied to 2-hour values. Inhibitors were stable for the entire period. The relationship of reaction velocity to temperature was studied in the range of 7° to 40° C. At temperatures below 37° C controls showed no measurable loss of Factor VIII in 2 hours; at 40° C some decrease of Factor VIII was measurable by 1 hour, and all results were corrected for these losses. All kinetic experiments were performed at least twice; most were run in triplicate.

Inhibitor concentrations were expressed in units in a manner similar to that of Biggs and Bidwell (12), except that the definition is based more closely on initial velocities: One unit of inhibitor is that amount which will inactivate 30% of a 50 U per ml solution of Factor VIII in 15 minutes at 37° C. The source of Factor VIII can be standard normal plasma (diluted 1:2) or a concentrated preparation (26). The definition assumes similar time curves for the inactivation of Factor VIII by all inhibitors. That exceptions occur can be documented from the literature (14, 27) and from data in this report. It must be emphasized, therefore, that measurements in "units" may not be comparable for different inhibitors, although very useful in the study of any given case.

Kinetic methods

After qualitative data had demonstrated that the interaction between inhibitor and Factor VIII was nonenzymatic (see Results), the exact stoichiometry of the reaction became of interest. Since one of the inhibitors did not obey time-dependent first-order kinetics at any concentration tested (Figure 1), a meaningful kinetic analysis required some other approach. For these purposes the method of initial velocities, originally stated by Van't Hoff (28, 29), was used:

In a reaction of the type under investigation,

$$\text{Factor VIII} + \text{inhibitor} \rightarrow \text{products}, \quad [1]$$

one can usually, though by no means always, write the velocity as

$$v = k\,[A]^m[I]^n, \quad [2]$$

where [A] = concentration of Factor VIII at time t, in moles per liter; [I] = concentration of inhibitor at time t, in moles per liter; k = reaction constant; and m + n = whole number, or fractional, constants.

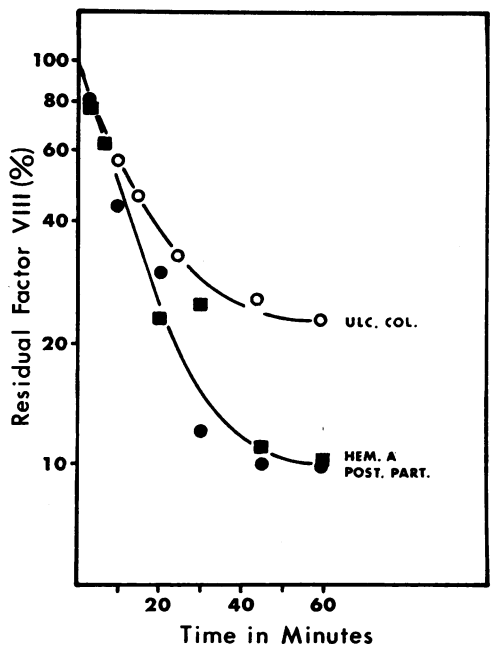

FIG. 1. INACTIVATION OF FACTOR VIII PLOTTED AS A FIRST-ORDER REACTION. Initial concentration of Factor VIII was 50 U per ml. Initial dilution of inhibitor plasma was 1:4 (postpartum patient), 1:60 (hemophilia A patient), and 1:100 (ulcerative colitis patient). Temperature was 37° C. Note that although the initial velocity is the same for all three reactions, the reaction in the ulcerative colitis patient is curvilinear throughout the entire course. Similar results were obtained for all dilutions of plasma from the patient with ulcerative colitis.

The initial velocity of the reaction, before significant amounts of reactants disappear, can be approximated closely by

$$v_{initial} = k\,[A]_0^m\,[I]_0^n, \quad [3]$$

where the subscript zero denotes initial concentration.

If a series of kinetic curves is obtained by varying $[I]_0$, while keeping $[A]_0$ constant, the initial velocities are described by

$$v_{initial} = K\,[I]_0^n. \quad [4]$$

If one takes logarithms of both sides, Equation 4 becomes

$$\log v_{initial} = \log K + n \log [I]_0. \quad [5]$$

Thus a plot of $\log v_{initial}$ vs. $\log [I]_0$ should give a straight line with slope n. In similar fashion, varying $[A]_0$ while holding $[I]_0$ constant allows calculation of m.

The problem of units of measurement of the reactants, whose molar concentrations are quite unknown, is more apparent than real: In any plasma sample the inhibitor concentration bears a constant relationship to the "plasma

[5] Falcon Plastics, Los Angeles, Calif.

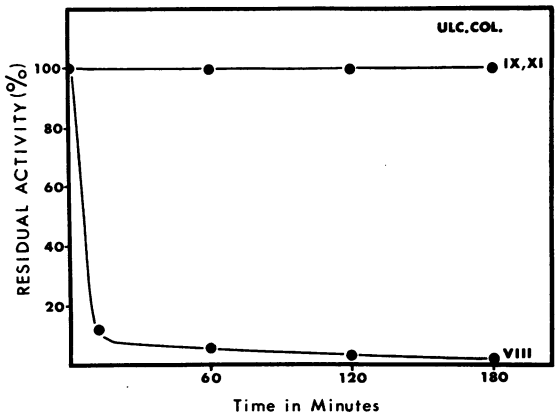

Fig. 2. SPECIFICITY OF INHIBITOR OF THE ULCERATIVE COLITIS PATIENT FOR FACTOR VIII. Initial dilution of ulcerative colitis patient's plasma was 1 : 10. Initial concentration of Factor VIII was 50 U per ml.

concentration," defining undiluted plasma as 100%. Thus

$$[I] = C \times [\text{plasma } \%], \qquad [6]$$

where C = proportionality constant, or

$$\log [I] = \log C + \log [\text{plasma } \%]. \qquad [7]$$

Since log C is a constant, the use of serial dilutions of plasma in Equation 5, instead of molar concentrations of $[I]_o$, will alter the intercept of the line on the log v axis, but not its slope; hence the value of n calculated will be unchanged. A similar argument holds for the determination of m. The values obtained for m and n represent the order of Reaction 1 with respect to Factor VIII and inhibitor, respectively.

Initial velocities, expressed as Factor VIII units per milliliter per minute inactivated, were measured graphically from the initial slopes of kinetic runs. Most points earlier than 10 minutes have been omitted from the graphs, however, for the sake of clarity in reproduction.

Temperature dependence of the reaction has been expressed by means of the Arrhenius equation (30),

$$k = Ae^{-E_a/RT}, \qquad [8]$$

where k = reaction constant, R = gas constant, A = "frequency factor" (a constant), E_a = activation energy, and T = absolute temperature. Thus

$$\log k = \log A - (E_a/2.3R)\ (1/T). \qquad [9]$$

Substituting $v_{initial}$ for k and plotting log $v_{initial}$ vs. (1/T) should produce a straight line of slope $-E_a/2.3R$, allowing the calculation of E_a.

All dilutions of plasma and test reagents were made with imidazole-buffered saline, pH 7.4, 0.04 M in imidazole, total ionic strength 0.165.

TABLE II

*Effect of inhibitor plasma concentration upon the initial velocity of inhibition**

	Inhibitor concentration (plasma dilution)	Initial velocity
		U/ml/min
Ulcerative colitis	1:10	20
	1:20	10
	1:40	5
	1:80	4.5
	1:160	1.5
Postpartum	1:2	8
	1:4	4
	1:10	2
	1:16	1.2
Hemophilia A	1:20	25
	1:40	11.5
	1:60	7.5
	1:80	5.2
	1:120	3.6
	1:160	3.2

* Reaction mixture: 0.7 ml inhibitor plasma + 0.7 ml standard normal plasma. Initial concentration of Factor VIII was 50 U per ml. Plasma dilution is that in reaction mixture proper.

Results

Mechanism of inhibition. Specificity of the inhibitors for Factor VIII was demonstrated by the results of standard coagulation tests (Table I) and by incubation studies: Admixture of inhibitor plasma and standard normal plasma resulted in a progressive loss of Factor VIII activity, whereas concentrations of Factor IX and Factor XI remained unchanged. Representative data are depicted in Figure 2.

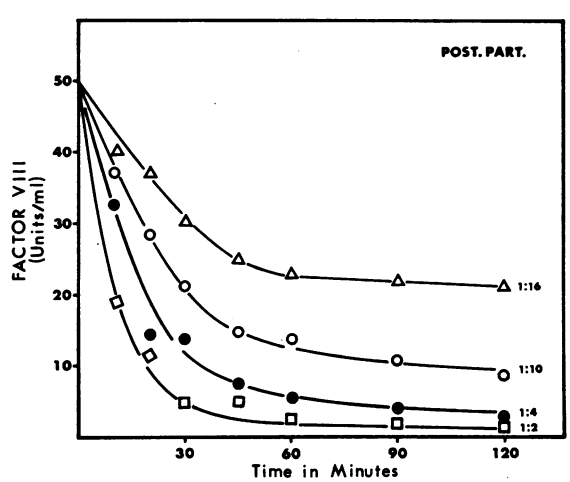

FIG. 3. INHIBITION OF FACTOR VIII AS A FUNCTION OF INHIBITOR CONCENTRATION.

The effects of varying initial concentrations of reactants upon the initial velocity of the reaction are shown in Tables II and III. The initial velocity was proportional both to the amount of inhibitor and Factor VIII added to incubation mixtures. Representative data for one inhibitor are illustrated in Figures 3 and 4. All such curves are averages of triplicate runs. Six Factor VIII determinations were made during the first 15 minutes of the reaction, allowing considerable accuracy in estimating initial velocities. However, accurate kinetic data could not be obtained when more than 80% or less than 15% of the initial Factor VIII was destroyed within this time period. As demonstrated in these Figures, equilibrium concentrations of Factor VIII varied directly with initial Factor VIII levels and inversely with concentrations of inhibitor.

All three inhibitors could be completely neutralized by the serial addition to reaction mixtures of sufficient Factor VIII; a representative experiment is illustrated in Figure 5. Since the inhibitor plasmas were quite potent, this saturation phenomenon was most easily demonstrated by diluting patient plasmas to inhibitor concentrations of 0.5 to 1.0 U per ml. Results were identical when purified inhibitor fractions were tested. Such saturation phenomena could not be demonstrated after an equal number of additions of cryoprecipitate prepared from hemophilic plasma.

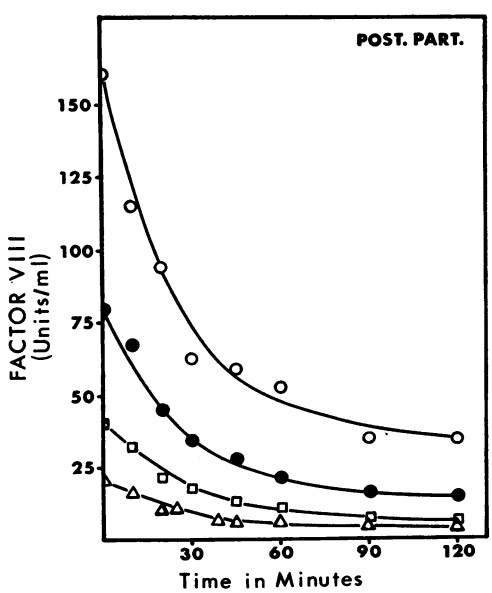

FIG. 4. INHIBITION OF FACTOR VIII AS A FUNCTION OF FACTOR VIII CONCENTRATION.

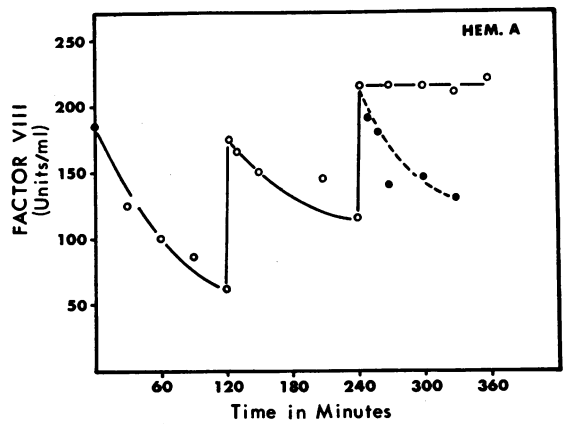

FIG. 5. NEUTRALIZATION OF HEMOPHILIA A PATIENT'S INHIBITOR BY FACTOR VIII. Incubation mixture: 3.2 ml hemophilia A patient's plasma diluted 1:30 + 0.8 ml cryoprecipitated Factor VIII (26). Small volumes of cryoprecipitate were added at 2 and 4 hours to raise Factor VIII to initial values. A control mixture of hemophilia A patient's plasma and buffered saline was incubated 4 hours and tested for inhibitor activity after addition of cryoprecipitate (dashed line, solid circles).

Chemical properties. All three inhibitors were present in serum as well as plasma and were not absorbed onto $BaSO_4$. They were precipitated from plasma by ammonium sulfate between 30 and 50% saturation, and all traveled in the inter-

TABLE III

*Effect of Factor VIII concentration upon the initial velocity of inhibition**

	Factor VIII	Initial velocity
	U/ml	*U/ml/min*
Ulcerative colitis	160	7
	80	3.4
	40	2.2
	20	1.0
Postpartum	160	2.8
	80	1.4
	40	0.8
	20	0.3
Hemophilia A	160	9.6
	80	5.6
	40	2.8
	20	0.9

* Reaction mixture: 0.7 ml inhibitor plasma + 0.7 ml concentrated Factor VIII (26) appropriately diluted. Inhibitor plasma concentrations were 1:20, 1:4, 1:40 for ulcerative colitis, postpartum, and hemophilia A patients, respectively.

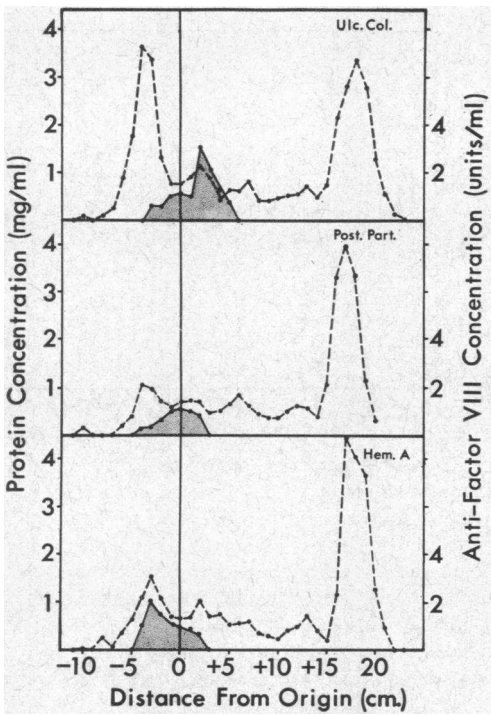

FIG. 6. STARCH BLOCK ELECTROPHORESIS OF INHIBITOR PLASMAS. Sample volume: ulcerative colitis, hemophilia A, 2 ml; postpartum, 4 ml. Dotted line: protein concentration; solid line: inhibitor concentration.

mediate (7 S) peak in gel filtration experiments on Sephadex G-200. When whole inhibitor plasma was gel filtered, no free Factor VIII could be detected under any of the three peaks, although it was easily measured under the first peak in runs with normal plasma. Thus there was no evidence that the inhibitor-Factor VIII complex could be easily dissociated.

Starch block electrophoresis revealed differences in mobility for the three inhibitors (Figure 6), peak activity varying from a β position (ulcerative colitis) to a slow γ mobility (hemophilia A). Peak positions were very reproducible, although the degree of trailing varied somewhat from run to run.

Direct evidence of the immunoglobulin nature of the inhibitors was sought in neutralization experiments. Peak inhibitor tubes isolated by starch block electrophoresis were allowed to react overnight with specific antisera to human immunoglobulins. Reactions were carried out in the region of relative antibody (antiserum) excess, as previously determined by maximal precipitation.

TABLE IV

*Neutralization of Factor VIII inhibitors by antisera against human immunoglobulins**

Test reagent		Inhibitor concentration: Ulcerative colitis	Postpartum	Hemophilia A
		U/ml	*U/ml*	*U/ml*
Saline		3.1	1.0	2.1
	Anti-IgG	0.0	0.0	0.0
Saline		3.1	1.0	2.1
	Anti-IgA	3.1	1.0	2.1
Saline		3.1	1.0	2.1
	Anti-IgM	3.1	1.0	2.1
Saline		2.1	1.0	1.5
	Antikappa	0.0	0.0	0.0
Saline		2.1	1.0	1.5
	Antilambda	2.1	1.0	1.4

* Reaction mixture: 0.2 ml inhibitor + 0.2 ml imidazole-buffered saline or 0.2 ml antiserum. Residual inhibitor measured after 18 to 24 hours (see Methods).

Results are depicted in Table IV. All three inhibitors were completely and specifically neutralized by anti-IgG antiserum, as well as by antikappa antiserum.

Kinetic analysis. Plots were made from the kinetic data in Tables II and III, as described under Methods, and the results are shown in Figures 7 and 8. In all three cases inhibition is clearly first order both with respect to Factor VIII and inhibitor.

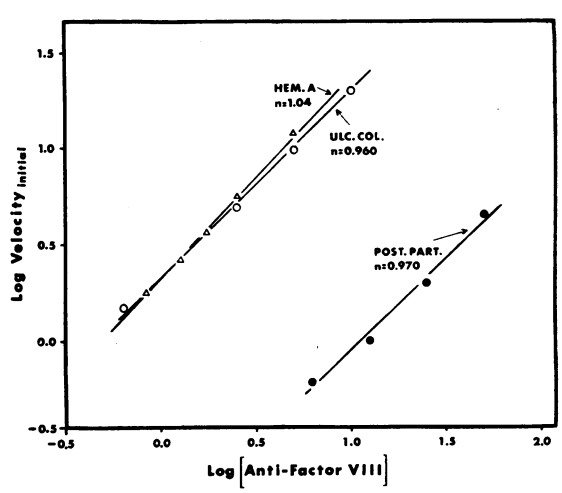

FIG. 7. PLOT OF REACTION VELOCITY AS A FUNCTION OF INHIBITOR CONCENTRATION, ACCORDING TO EQUATION 5. Inhibitor concentrations are plotted as per cent plasma (see Methods).

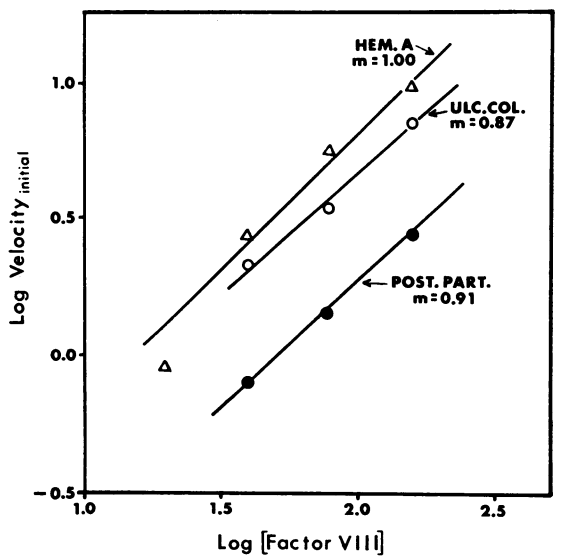

FIG. 8. PLOT OF REACTION VELOCITY AS A FUNCTION OF FACTOR VIII CONCENTRATION (SEE METHODS).

Reaction velocity was moderately dependent on temperature: The velocity tripled when the reaction temperature changed from 7° C to 37° C. Activation energies, calculated from the data in Figure 9, were approximately 6 kcal per mole for all three inhibitor reactions.[6]

Discussion

Although many cases of circulating anticoagulants directed against Factor VIII have been reported in recent years (1–14, 27), there is little agreement concerning their nature. Those authors who have examined the kinetics of Factor VIII inactivation have reached opposing conclusions (9, 12, 13); part of this discrepancy can be explained by the nature of the kinetic approach employed. Biggs and Bidwell (12), in a study of six inhibitors, found a first-order loss of added Factor VIII with time. They felt their data supported the hypothesis of a bimolecular reaction between Factor VIII and inhibitor, in which the inhibitor was present in great excess. However, similar first-order kinetics would result from the enzymatic destruction of Factor VIII. To differentiate these two situations, one must follow the reactions to completion and measure equilibrium concentrations of Factor VIII as a function of initial inhibitor concentration, a study these authors did not perform. Furthermore, several instances have been described (14, 27, and the patient with ulcerative colitis in this report) in which first-order time-dependent kinetics simply do not apply. Leitner, Bidwell, and Dike (13), in an investigation of an inhibitor in a hemophilic patient, added important information when they demonstrated complete neutralization of the inhibitor by added Factor VIII. Breckenridge and Ratnoff, on the other hand, have presented data indicating no loss of inhibitor activity after serial additions of Factor VIII in their three patients (9).

In all of the present patients both the rate of the reaction and the equilibrium concentration of Factor VIII were functions of the initial concentrations of reactants. In addition, all three inhibitors could be completely neutralized by sufficient Factor VIII, whereas inhibitors incubated alone for similar periods of time retained full activity. It is unlikely that exhaustion of inhibitor is due to a material in the cryoprecipitates other than Factor VIII, since similar preparations from hemophilic plasma did not have a measurable effect on the inhibitors. These inhibitors clearly inactivate Factor VIII by a nonenzymatic mechanism. The data of Breckenridge and Ratnoff raise the possibility that other inactivation mechanisms may

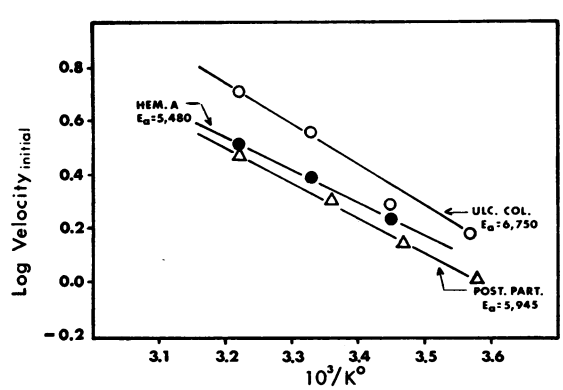

FIG. 9. TEMPERATURE DEPENDENCE OF FACTOR VIII INHIBITION, PLOTTED ACCORDING TO EQUATION 9 (SEE METHODS). E_a = activation energy. Initial dilution of inhibitor plasma was 1:2 (postpartum patient), 1:40 (hemophilia A patient), and 1:20 (ulcerative colitis patient).

[6] In a preliminary communication (J. clin. Invest. 1966, 45, 1072) values of E_a were reported that differed somewhat in two cases from the present figures. It is probable that the present data, derived from plasma drawn when the patients were not receiving any form of Factor VIII replacement therapy, are the more reliable.

exist, but it is also possible that the apparent resistance of their inhibitors to neutralization is related to the addition of insufficient quantities of Factor VIII. In the present patients very large amounts of Factor VIII could be inactivated by inhibitor plasmas: 5,160, 1,110, and 7,360 U of Factor VIII were required to neutralize 1 ml of plasma from ulcerative colitis, postpartum, and hemophilia A patients, respectively.

To study the stoichiometry of Factor VIII inhibition quantitatively, one must limit the kinetic complexities introduced by back reactions and possible intermediary reactions, both of which may become more significant as the over-all reaction progresses. These considerations are especially important in dealing with antigen-antibody reactions, in which both reactants are multivalent, and are presumably operative in the "exceptional" cases of Factor VIII inhibitors that do not follow simple time-dependent kinetics. In such situations the chemical reaction in incubation mixtures actually may be changing with time, and an explicit mathematical expression of the entire time curve may be nearly impossible to obtain. By utilizing initial velocity measurements one may avoid these pitfalls. He is, of course, assuming that little of the initial reactant (with respect to the total present) has disappeared during the very early course of the reaction. The limits of this assumption can be estimated from the kinetics (Figures 3 and 4). In the present experiments it is possible to conclude that less than 10% of any reactant has disappeared during the time necessary to measure initial reaction velocities. Thus the coefficients m and n in Equation 2 have a probable error of approximately $\pm$ 10%. This approach is especially advantageous in that it allows the analysis of reactions in which concentrations of reactants can only be expressed in arbitrary terms such as "per cent plasma" or biological "units."

The results of this kinetic analysis indicate that inactivation of Factor VIII is first order with respect to inhibitor and with respect to Factor VIII. Although it rarely is permissible to deduce a molecular mechanism from reaction kinetics, the simplest mechanistic conclusion consistent with the observed data is that the inactivation of one molecule of Factor VIII requires its combination with one molecule (or one site on a molecule) of inhibitor. No statement can be made concerning the possibility of subsequent steps, such as the combination of the complex with still another molecule of Factor VIII or inhibitor, even though such may occur.

The chemical nature of Factor VIII inhibitors has not been investigated systematically. Inhibitory activity generally has been found in γ-globulin-rich fractions isolated by ammonium sulfate precipitation (1, 13), alcohol fractionation (3, 8, 9), and electrophoresis (1, 13, 31, 32); however, one patient has been reported in whom the electrophoretic γ-globulin fraction was devoid of activity (10). A sedimentation constant of 7 S has been estimated by density gradient centrifugation in a single case (13), and a similar, or smaller, size can be inferred from the report of transplacental transfer of a circulating anticoagulant (33).

In the present study, in addition to fractionation techniques, specific immune neutralization was employed to identify directly the chemical nature of the inhibitors. Results demonstrate convincingly that these materials are immunoglobulins of the class IgG. The different electrophoretic mobilities of the three inhibitors, reminiscent of similar findings in myeloma, suggested that they might represent relatively homogeneous subfractions of IgG. Light chain typing (34, 35) confirmed this possibility: All three inhibitors appeared to be exclusively of kappa type.

Normal circulating human immunoglobulins are composed of both Type K (kappa) and Type L (lambda) molecules (36). Investigations of specific blood group agglutinins (37, 38), antithyroglobulins (37, 39), and experimentally produced antidextrans (39) have shown them to be mixtures of active antibody of both types. Multiple myeloma and Waldenström's macroglobulinemia, on the other hand, are characterized by the production of monotypic immunoglobulins (40), but only rarely has antibody activity been associated with these paraproteins (14, 41, 42). The syndrome of chronic hemolytic anemia due to cold agglutinins (43) is the only condition known in which monotypic antibody is regularly produced. In 59 reported patients the antibody has been found to be an IgM exclusively of Type K (37, 38, 44). One case has recently been described due to an IgA cold agglutinin; this antibody, too, was of Type K (45).

The present report may represent the second syndrome due to production of a monotypic antibody, and the first instance involving IgG immunoglobulins. In none of these patients could cold hemagglutinins be demonstrated.

The production of antibodies in at least one of these patients (hemophilia A) probably is in response to immunization, since this patient has received numerous transfusions from a variety of donors since birth. Antibody production in the postpartum female may reasonably be ascribed to immunization during pregnancy. In both instances the implication is clear that genetic polymorphism for Factor VIII may exist. The fact that these antibodies are monotypic may reflect important antigenic characteristics of the Factor VIII molecule. Experiments are in progress in this laboratory designed to detect allelic forms of Factor VIII.

The lack of reports of monotypic Type L antibodies may have biological significance, since it is known that major differences exist between kappa and lambda light chains (46). However, in analogy with the myelomas, in which Type K paraproteins are twice as frequent as the Type L variety (40), it is possible that other monotypic antibody syndromes may be discovered due to Type L immunoglobulins.

Temperature dependence of the present inhibitors is similar to that reported by others (9, 12, 13) although even at 7° C the rate of Factor VIII inactivation was appreciable. Leitner and his colleagues (13) remarked on the unusually great effect of temperature on the inhibitor Factor VIII reaction. But, as these authors point out, similar behavior has been observed in other antibody-antigen reactions (47, 48) in which the rate of the reaction was measured by loss of biological activity. Furthermore, some antibody-hapten reactions, although much more rapid, have shown temperature dependence of the same degree (49), resulting in activation energies of 4.0 to 4.5 kcal per mole. This figure is quite close to the 6 kcal per mole activation energy calculated for the Factor VIII-inhibitor reaction.

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