

Antibodies to Factor VIII: Specificity and Kinetics of Iso- and Hetero-antibodies in Hemophilia A

By J. P. Allain and D. Frommel

Time course inactivation of isologous and heterologous AHF preparations induced by anti-factor VIII antibodies from hemophiliacs followed two major patterns: one was consistent with a second-order reaction, the other more complex. The second-order type, characterized by a plateau of residual factor VIII activity occurring after 2–4 hr of incubation, was observed in the interaction of factor VIII with its corresponding and specific antibody. The com-

plex type, showing a gradual decline of factor VIII activity following a rapid initial fall, was detected in antigen–antibody reactions relying predominantly on cross-reactions. It is proposed that reactions of the second-order type reflect the formation of stable antigen–antibody complexes, while reactions of the complex-order type express a tendency to spontaneous dissociation of antigen–antibody complexes.

IN HEMOPHILIA A, the occurrence of antibodies inactivating factor VIII activity has been frequently reported, and the characteristics of these antibodies have been recently reviewed.¹ The formation of factor VIII–anti-factor VIII complexes, stable in fluid phase, has been well documented,^{2,5} but the mode of association of factor VIII molecules with their corresponding antibodies and the kinetics of the resulting factor VIII inactivation is still open to debate.^{4,6–8}

As recently described by Biggs et al.,^{4,8} two types of kinetics may be observed when antibodies react with factor VIII. When the logarithm of residual factor VIII concentration is plotted against time, some antigen–antibody mixtures show a rapid decrease of factor VIII activity during the first 2–4 hr of incubation, which then reach a plateau of stable residual factor VIII. Other antigen–antibody mixtures show a more complex pattern of reaction, characterized initially by a rapid loss of factor VIII activity which is then followed by a continual gradual decline in residual factor VIII. These two kinetic patterns

From the Institut Médico-Pédagogique pour Jeunes Hémophiles de la Croix-Rouge Française, 78940 La Queue-lès-Yvelines, the Unité 56 de l'I.N.S.E.R.M., and the Centre National de Transfusion Sanguine, 75015 Paris, France.

Submitted December 10, 1973; accepted March 29, 1974

Supported in part by a special grant of the Caisse Régionale d'Assurances-Maladie de Paris.

Address for reprint requests: Dr. D. Frommel, C.N.T.S., 6 rue A. Cabanel, 75739 Paris Cedex 15, France.

© 1974 by Grune & Stratton, Inc.



will be designated the second-order type and the complex-order type, respectively.

In the present investigation, anti-factor VIII antibodies from 12 hemophiliacs, transfused with human or both human and animal blood derivatives, were studied. Each inhibitor was tested against human, porcine, and bovine factor VIII and the time course inactivation of these three factor VIII preparations was compared.

MATERIAL AND METHODS

Venous blood was collected in plastic tubes (9 parts of blood for 1 part of 0.13 *M* sodium citrate). Samples were centrifuged for 1 hr at 4°C (5500 *g*). The platelet-poor plasma was adsorbed unless stated otherwise, with aluminum hydroxide (0.1 ml of a 25% Al(OH)₃ solution for 1 ml of citrated plasma) for 5 min at 37°C. After centrifugation, the plasma was stored in capped polystyrene tubes at -20°C.

Source of Antibodies to Factor VIII

Antibodies from 12 severe hemophiliacs were studied. Seven of them (patients 1-7) were treated with human blood derivatives only and were tested at the peak of their anamnestic response (Tables 1 and 2). Three of these seven patients (1, 5, and 6) were also studied shortly before a stimulation. Two patients (8 and 9) had to be treated consecutively with human and porcine AHF as previously reported.⁹ They were thus studied during an anamnestic response to human factor VIII and after their primary immune response to animal factor VIII. Patient 10, whose treatment was alike, could be studied before and after a 6-day course of therapy with porcine AHF. Patient 11 was treated at 1-wk intervals with human, porcine, and once again with high doses of human AHF. He was studied three times: twice during the antiporcine immune response and once at the peak of the last antihuman response. Patient 12 was treated over a 15-day period with human, porcine, and bovine AHF.⁹ His antibodies were studied 6 mo later (Table 1).

Table 1. Overview of the Treatments Received by 12 Hemophiliacs Having a High Titer of Anti-Factor VIII Antibodies

Patient	Age (yr)	Cumulative Days of Administration of Factor VIII			Highest Titer of Antihuman Factor VIII Antibody*	Interval Between the Last Stimulation and Study (days)
		Human Origin	Animal Origin			
			Porcine	Bovine		
1 L.L.	11	30	—	—	44	See Table 2
2 M.L.	10	2	—	—	225	10
3 P.H.	9	16	—	—	13	10
4 L.H.	6	14	—	—	10	10
5 J.M.C.	8	3	—	—	1900	See Table 2
6 J.C.L.	14	80	—	—	360	See Table 2
7 H.V.C.	14	Unknown	—	—	>10†	>360
8 B.L.	17	48	6	—	>20†	180
9 A.B.L.	22	Unknown	6	—	800	30
10 E.J.	23	20	6	—	240	25
11 P.E.	15	40	8	—	20	13
12 G.Y.	16	60	6	3	>30†	180

*The titers given in the sixth column represent the mean of duplicate measurements; they do not necessarily correspond to the plasmas under study in Figs. 1, 2, and 3.

†Highest titer unknown.

Table 2. Analysis of Cross Reactions of Antibodies From Patients 1, 5, and 6 Taken at Different Intervals Before and After Treatment With Human Factor VIII Concentrates.

Patient		Time of Sampling (days) Before, After Stimulation Respectively	Titer of Antibody (U)		
			Human Factor VIII	Porcine	Bovine
1	a	- 1	4	1.2 (30%)	1.0 (25%)
	b	+ 8	16	1.4 (9%)	3.2 (20%)
	c	+ 10	44	5.5 (13%)	8.4 (19%)
5	a	- 90	4	1.3 (32%)	0.5 (13%)
	b	+ 8	1200	150 (12%)	95 (8%)
	c	+ 17	1900	250 (13%)	225 (12%)
	d	+ 40	620	68 (11%)	75 (12%)
6	a	- 90	10	7.6 (76%)	3.8 (38%)
	b	+ 20	360	140 (39%)	130 (35%)
	c	+ 50	210	N.D.*	45 (21%)
	d	+ 80	135	16 (12%)	19 (14%)

The titers (mean of two determinations) were calculated after 2 hr of incubation at 37°C with human, porcine, and bovine factor VIII. The cross-reactivity to animal AHF is expressed in per cent of the anti-human titer. The transfusion preceding collection of sample 6 a was 7 mo previously.

*N.D., no determination available.

Source of Antigens

The source of human factor VIII was a pool of citrated, adsorbed plasma from three healthy male blood donors. The sources of animal factor VIII were citrated, adsorbed porcine or bovine plasma, or lyophilized concentrates (porcine antihemophilic globulin, lot A 1116, bovine antihemophilic globulin, lot 7654, Maws Pharmacy Supplies Ltd., Barnet, England). One unit of factor VIII was defined arbitrarily as that contained in 1 ml of a pool of normal human plasma,¹⁰ and 1 U of animal factor VIII was defined as the dilution which gave a clotting time identical to human plasma in a one-stage factor VIII assay. Porcine and bovine plasma were diluted in buffer and concentrates in buffer or in CRM-negative hemophilic plasma.

Factor VIII Assay

Factor VIII was tested by a one-stage method in the presence of cephalin and kaolin and using a citrated plasma of a severe hemophiliac as substrate. All dilutions were prepared with Veronal-acetate buffer.

Assay of Antifactor VIII Activity

The method initially was described by Biggs and Bidwell¹¹ was modified. The plasma with inhibitor was diluted with buffer to contain approximately 1 U of anti-factor VIII antibody, defined as the amount of antibody which inactivated, after 2 hr of incubation at 37°C, 75% of the factor VIII present in an equal volume of normal plasma (ratio of inhibitor to normal plasma, 1:1) incubated under the same conditions. The residual factor VIII activity was measured in duplicate by the one-stage method, and the titers of the inhibitors were determined using the dilutions of neutralizing plasma at which 10%, 30% of factor VIII remained active; thus, all assays were performed with antigen in excess of antibody. Sources of factor VIII were defined above.

Kinetics Studies

The kinetics of the factor VIII inactivation by specific antibodies were studied by the method of prolonged incubation.^{4,8,12} The mixtures of antigen and antibody (1:1) were incubated for 7-8 hr in glass tubes at 37°C. When required the antibodies were diluted with buffer as necessary in order to obtain a slight to moderate antigen excess (10%, 50% of residual factor VIII). Residual factor VIII was measured initially after 30 min and again after each hour

of incubation by removals of aliquots from the mixture; an aliquot of the control mixture (factor VIII and buffer, 1:1) withdrawn at the same time was used for the reference curve, reprepared for each set of incubation.

Prolonged incubations were performed with human, porcine, and bovine AHF antigens for plasmas with antifactor VIII activity.

Control Experiments

Since it was reported that if antigen (AHF-containing solutions) was adsorbed before interaction with antibodies, titers of antifactor VIII were higher than after incubation with unadsorbed plasma,⁷ experiments were undertaken so as to evaluate these differences and to compare inactivation of the AHF preparations under study. Neutralizing activity was significantly enhanced using human and porcine plasma which had been adsorbed; no such differences were observed between native and adsorbed bovine plasma.

No real variations could be detected whether the inhibitory plasmas were adsorbed or not. For practical reasons, mostly because in our experience adsorption improves reproducibility and increases sensitivity, adsorbed plasma was used throughout this study.

During incubation at 37°C, spontaneous inactivation of animal AHF occurs more rapidly in concentrates than in plasma (percentage of remaining factor VIII activity in plasma versus concentrate, after 2 hr, 96 ± 5 versus 76 ± 6 ; after 6 hr, 68 ± 7 versus 48 ± 12). This spontaneous decay of factor VIII activity was taken into consideration since all titers were expressed in reference of a control curve, prepared from a mixture containing the same source of AHF, diluted with hemophilic plasma or buffer, and incubated likewise. The comparatively lower titers obtained after incubation with concentrates probably reflect the decrease of the amount of antigen available for reaction with antibodies.

In prolonged incubation studies, determinations of factor IX were also performed; the differences between the results obtained from mixtures containing human unadsorbed plasma and anti-factor VIII antibodies and those containing buffer were not significant.

Dissociation Studies

As previously described,⁵ antigen-antibody complexes formed in vitro were dissociated by heat at 56°C. The recovery of the anti-factor VIII activity was measured by the factor VIII inhibitor assay using normal human plasma or animal AHF preparations as source of factor VIII.

RESULTS

Time Course Inactivation by Antibodies of Patients Treated Only With Isologous Antigen, Human Factor VIII

The action on human factor VIII of the antibodies of these seven patients resulted in second-order kinetics (Fig. 1). The graphs showed an initial phase of rapid inactivation followed, after 2–3 hr of incubation, by a plateau of stable factor VIII activity; with the antibodies of patients 1, 5, and 6, the plateaus were, however, not strictly horizontal. In patients 5 and 6, studied before and after a renewed antigenic challenge, this type of kinetics remained unchanged. Conversely different types of inactivation were found using heterologous antigens (animal AHF). Rapid decreases with linear slopes were observed in the inactivation of porcine and bovine factor VIII by antibodies of patient 1, of bovine factor VIII by antibodies of patient 6 (Fig. 1A and 1D). In the other cases, the curves of inactivation, of animal AHF were biphasic; the slope of the second phase was variable but never formed a plateau completely (Fig. 1B and 1C).

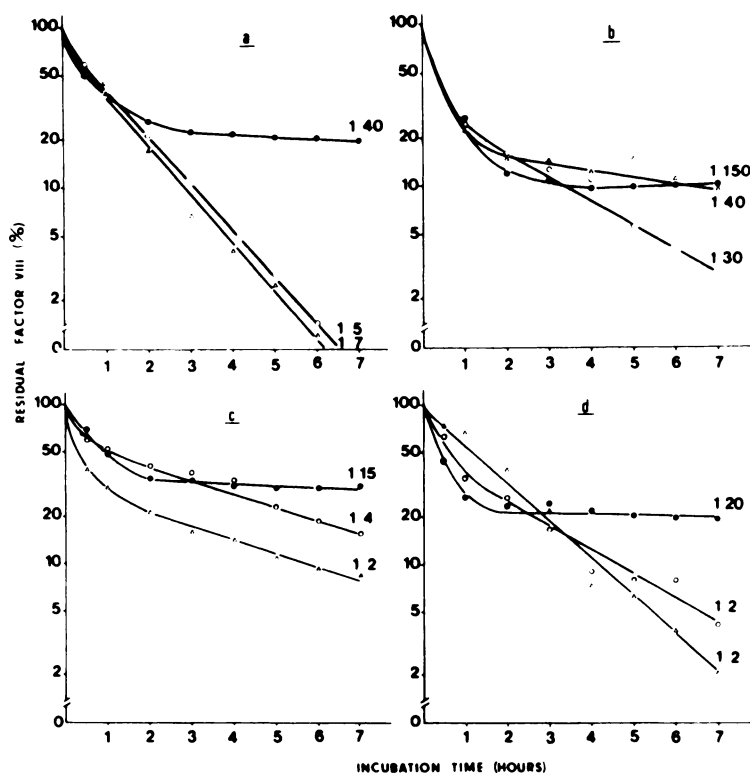


Fig. 1. Time course inactivation of human (●—●), porcine (○—○), and bovine (Δ—Δ) factor VIII by plasma of patients 1, 2, and 3 (A, B, C) studied 8–10 days after treatment with human cryoprecipitate. In D, patient 6 was studied for more than 1 yr after stimulation. Dilutions of antibodies in buffer are as indicated.

Time Course Inactivation by Antibodies of Patients Treated With Isologous and Heterologous Antigens, Human and Animal Factor VIII

Two patients (8 and 9) had a high antibody response after sequential stimulation with both human and porcine AHF preparations, and samples were drawn 6 and 1 mo, respectively, after treatment. A second-order curve was observed for the inactivation of the two, human and porcine, antigens, whereas neutralization of bovine factor VIII followed complex-order curves (Fig. 2A and 2B). Patient 12 was treated with porcine and bovine concentrates, but to a much lesser extent than with human cryoprecipitates. Inactivation of human and porcine factor VIII followed second-order kinetics while that of bovine was of a more complex order (Fig. 2D).

Time Course Inactivation by Antibodies Studied After Sequential Stimulations With Human and Porcine AHF

Patient 10, after 3 days of ineffective replacement therapy with human AHF, was found to have anti-factor VIII antibodies at a titer of 19 U. He was then transfused with porcine AHF (1000 U/kg/day) for 6 days. The analyses of the

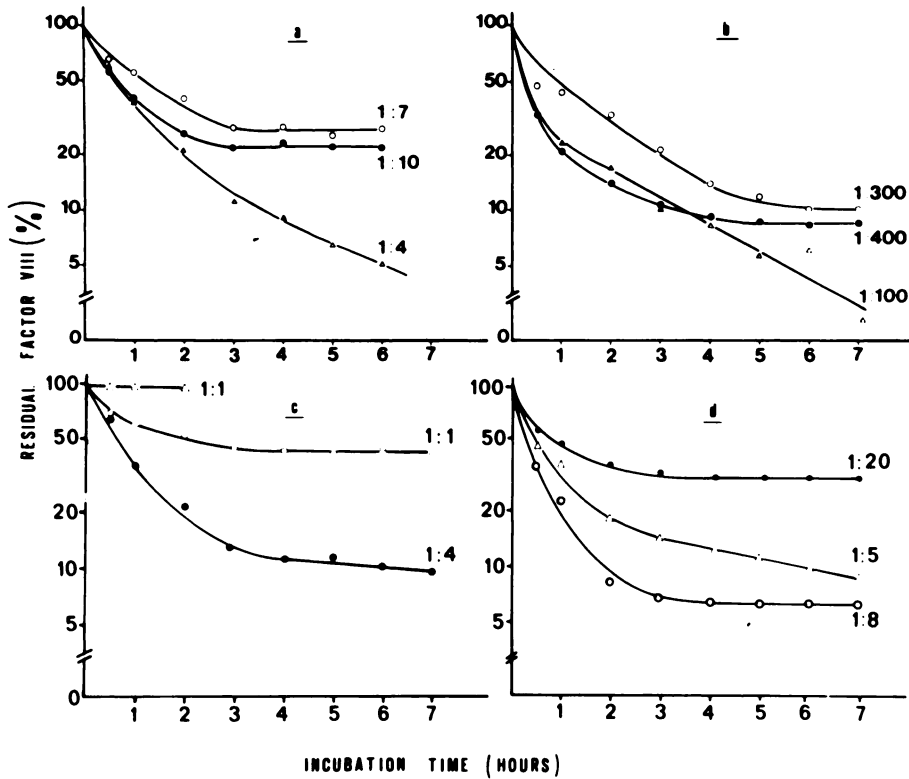


Fig. 2. Time course inactivation of human (●—●), porcine (○—○), and bovine (△—△) factor VIII by plasma of patients 8 (A), 9 (B), 11 (C), and 12 (D). Patients 8, 9, and 11 were treated with human and porcine AHF and patient 12 also with bovine concentrates. Samples of patients 8, 9, and 12 were collected 30–180 days after stimulation. Plasma of patient 11 was obtained 13 days after transfusion with porcine AHF.

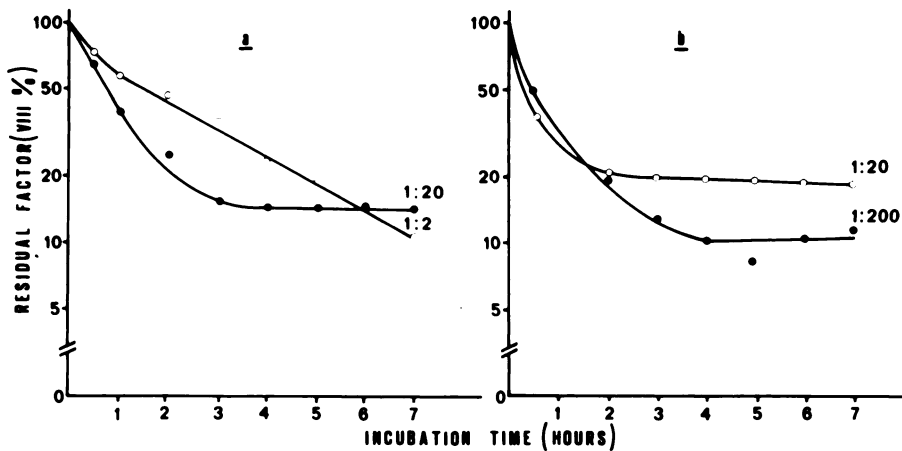


Fig. 3. Time course inactivation of human (●—●) and porcine (○—○) factor VIII by plasma of patient 10 before (A) and after (B) stimulation with porcine AHF.

samples obtained before and after treatment with porcine concentrates illustrate the change occurring in the inactivation patterns of porcine factor VIII as a result of specific antibody response, the kinetics shifting from complex order to second order (Fig. 3). However, the titer against human AHF being tenfold higher than against porcine AHF (240:23), no definite plateau was formed in the presence of porcine antigen as a consequence of some inactivation due to cross-reactivity.

The antibodies of patient 11, studied 2 days after the end of treatment with porcine AHF, neutralized both human and porcine factor VIII according to complex-order kinetics. Three days later, at a time which corresponded to the theoretical peak of the immune response to porcine AHF and using the same antibody concentrations as on day 10, a change towards second-order kinetics of porcine factor VIII inactivation was observed (Fig. 2C). After renewed stimulation with large doses of human AHF, antibodies against human factor VIII rose to a titer of 18 U and inactivated isologous factor VIII following second-order kinetics, a stable plateau being present from the fourth hour on.

Dissociation Upon Heating of Antibodies Bound to Human, Porcine, and Bovine Factor VIII

Patient 6 was repeatedly sensitized with human AHF only. His plasma was incubated 2 hr at 37°C with human, porcine, and bovine AHF. At the end of this 2-hr period, residual factor VIII was present in each of the mixtures. These mixtures were then further incubated at 56°C. Upon heating, anti-factor VIII antibodies were rapidly released from the mixtures containing animal AHF as antigen, whereas after 60 min, less than 20% of the antibodies could be recovered from the mixtures formed by human factor VIII-antihuman factor VIII complexes (Fig. 4). Patient 5 was also immunized solely with human AHF but over 3 days only. His antibodies, although at a high titer, bound less

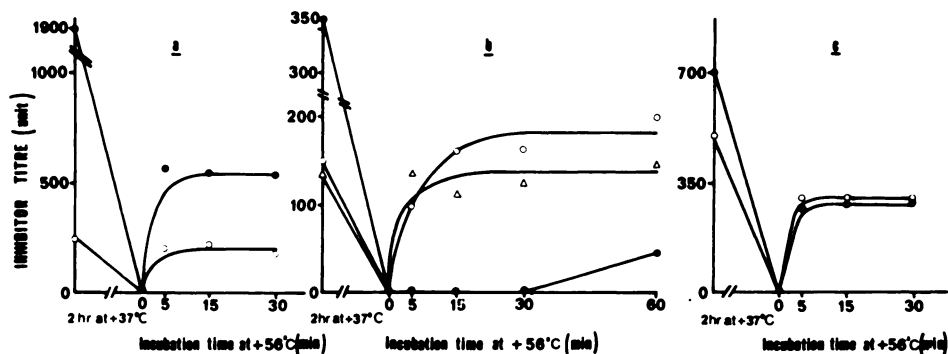


Fig. 4. Recovery of antifactor VIII activity upon heating of antibodies bound to human (●—●), porcine (○—○), and bovine (Δ—Δ) AHF. The antibody titers against the different antigens are shown on the ordinates. Dilutions of inhibitory plasma were conventionally incubated with human and animal AHF 2 hr at 37°C. At the end of this 2-hr period, residual factor VIII activity was detectable in all mixtures. These mixtures were then incubated at 56°C, and the inhibitory activity towards each of the two or three antigens, released by heating, was titrated in aliquots withdrawn at 5, 15, 30, and 60 min intervals. A, patient 5; B, patient 6; C, patient 8.

firmly human factor VIII than did those of patient 6, since 30% of the anti-factor VIII activity was released after a very short incubation at 56°C (Fig. 4A). Patient 9 was immune to human and porcine AHF, having been transfused with heterologous concentrates for 6 days. Dissociation of complexes formed with porcine factor VIII was not, as in cases 5 and 6, complete, indicating that this patient developed antibodies against porcine AHF that could form specific, heat-resistant complexes (Fig. 4C).

DISCUSSION

In 1967, Denson⁶ described that the pattern of time course inactivation of factor VIII preparations could differ depending on the source of the antibodies to AHF. In further analyses, Biggs et al.^{4,8} distinguished two major types of interactions of human factor VIII with anti-factor VIII antibodies: a reaction resulting in inactive factor VIII-anti-factor VIII complexes and a reaction in which complexes would still exert some procoagulant activity.

In the study reported herein, the nature and the schedule of the patients' immunizations with AHF preparations were taken into consideration. Kinetics of factor VIII inactivation were all performed choosing antigen-antibody ratios so that factor VIII was in slight excess of antibody. When antibodies were tested against the antigen which had caused the primary or the anamnestic immune response, the kinetics of factor VIII inactivation approached or reached second order. Occurrence of a plateau of stable residual factor VIII activity was observed in most cases with both isologous and heterologous AHF antigens, providing that the antibodies were from a patient immune to that antigen. These patterns were found with antihuman AHF antibodies collected more than 1 yr after the preceding transfusion (cases 5 and 6, Fig. 1D) and also with antiporcine AHF antibodies studied as long as 180 days after a single course of treatment with porcine concentrate (patient 8, Fig. 2A). Antibodies from hemophiliacs sensitized to human AHF only inactivated heterologous factor VIII preparations according to linear (patients 1 and 6, Fig. 1A and 1D; patient 10, Fig. 3A) or curvilinear time course graphs (patients 2 and 5, Fig. 1B and 1C), as did antibodies from patients also immune to porcine AHF when interacting with bovine factor VIII (patients 8 and 9, Fig. 2A and 2B). Thus, antibodies inactivate cross-reacting factor VIII preparations, the AHF antigens not involved in the process of immunization, according to complex-order reactions.

Antigen-antibody reactions relying predominantly or exclusively on cross-reaction cannot form complexes with as strong cohesion forces as those present in complexes resulting from specific interactions. In the first situation, conformational complementarity will be inferior to that realized in the second. The fact that specific factor VIII-anti-factor VIII complexes were relatively resistant to dissociation procedures⁵ can be taken as an evidence that second-order kinetics is based on an irreversible factor VIII-anti-factor VIII reaction. This interpretation is also supported by the findings of Leitner et al., Shapiro, and Green,^{2,3,13} who have shown that antibodies from hemophiliacs could be neutralized by addition of sufficient factor VIII. In addition to neutralization induced solely by antigen excess,³ the demonstration that nonspecific complexes

dissociated easily and at a fast rate upon heating (Fig. 4A and 4B) offers some evidence of the lability of complexes based on antigenic cross-reactivities. In our experience, most of the anti-factor VIII antibodies other than those occurring in classic hemophilia also displayed a tendency to spontaneous dissociation.¹⁴

Hemophiliacs who had to be treated with both human and animal AHF preparations developed a double or triple population of antibodies, each one specific for a given immunogen. Their plasma interacted with the factor VIII preparations in terms of specificity and cross-reactivity. With antibodies of low affinity, the plots of time course inactivation departed somewhat from the expected profiles (e.g., patients 11 and 12, Fig. 2C and 2D).

Our findings suggest that accurate titrations of inhibitor to factor VIII can only be obtained at the time at which the antigen-antibody reaction has been completed and has resulted in the formation of stable complexes. Incubations longer than 2 hr are often required to obtain stable residual factor VIII activity, and our results concur with the proposal of Rizza and Biggs¹⁵ recommending a 4-hr incubation for the determination of titer of anti-factor VIII antibodies. Respecting these conditions, it is possible in classic hemophilia to predict from the titer of the inhibitor the amount of human AHF necessary to neutralize in vivo a given antibody. On the contrary, requirement of heterologous AHF is essentially unpredictable. Despite the usually low degree of cross-reactivity in in vitro neutralization assays, the results obtained by analyses of in vivo recovery after administration of animal factor VIII preparations are below the levels expected from theoretical considerations.¹⁶ Dissociation of nonspecific complexes, occurring in vivo and enhanced by antigen excess, is probably responsible for the inactivation of a larger number of factor VIII molecules than that expected from calculations.

ACKNOWLEDGMENT

We wish to thank Miss A. Gaillandre and Mr. L. Meunier for their technical assistance and Mrs. J. François for secretarial help. We are indebted to Professor F. Josso and Dr. A. Cosson who kindly provided plasma of patients 8, 9, 10, and 12, and to Dr. R. Biggs and Dr. Charles Jenkins who reviewed our manuscript. We also express our gratitude to Professor B. Cinader, Professor S. Shapiro, and Professor J.P. Soulier for stimulating discussions.

REFERENCES

1. Feinsein DI, Rapaport SI: Acquired inhibitors of blood coagulation, in Spaet T (ed); *Progress in Hemostasis and Thrombosis*. New York, Grune & Stratton, 1972, p 75
2. Shapiro SS: The immunologic character of acquired inhibitors of antihemophilic globulin (factor VIII) and the kinetics of their interaction with factor VIII. *J Clin Invest* 46:147, 1967
3. Green D: Spontaneous inhibitors of factor VIII. *Br J Haematol* 15:57, 1968
4. Biggs R, Austen DEG, Denson KWE, Rizza CR, Borrett R: The mode of action of antibodies which destroy factor VIII. I. Antibodies which have second-order concentration graphs. *Br J Haematol* 23:125, 1972
5. Allain JP, Frommel D: Antibodies to factor VIII. I. Variations in stability of antigen-antibody complexes in hemophilia A. *Blood* 42:439, 1973
6. Denson KWE: The use of antibodies in the study of blood coagulation, chapter 4. Oxford, Blackwell, 1967
7. Pool JG, Miller RG: Assay of the immune inhibitor in classic haemophilia: application of virus-antibody kinetics. *Br J Haematol* 22:517, 1972
8. Biggs R, Austen DEG, Denson KWE,

Borrett R, Rizza C: The mode of action of antibodies which destroy factor VIII. II. Antibodies which give complex-concentration graphs. *Br J Haematol* 23:137, 1972

9. Josso F, Sultan Y, Dommergues JP, Alagille D: Surgery in haemophiliacs with circulating inhibitor. Proceedings of the Twelfth International Congress of the Society for Blood Transfusion, Moscow 1969, Basel, Karger, *Bibl haematol* No 38, part 1, 1971, p 817-821

10. Ratnoff OD, Botti RE, Breckenridge RT, Little AS: Some problems in the measurement of antihemophilic activity, in Brinkhous KM (ed): *The Hemophilias*. Chapel Hill, Univ of North Carolina Pr, 1964, p 3

11. Biggs R, Bidwell E: A method for the study of anti-haemophilic globulin inhibitors with reference to six cases. *Br J Haematol* 5: 379, 1959

12. Kernoff PBA: The relevance of factor

VIII inactivation characteristics in the treatment of patients with antibodies directed against factor VIII. *Br J Haematol* 22:735, 1972

13. Leitner A, Bidwell E, Dike GWR: An anti-haemophilic globulin (factor VIII) inhibitor: purification, characterization and reaction kinetics. *Br J Haematol* 9:245, 1963

14. Frommel D, Gaillandre A, Allain JP: Antibodies to factor VIII. II. Studies of complexes formed in vitro as index of immune reactivity. *Fed Proc* 32:1034, 1973

15. Rizza CR, Biggs R: The treatment of patients who have factor VIII antibodies. *Br J Haematol* 24:65, 1973

16. Rizza CR: The management of patients with coagulation factor deficiencies, in Biggs R (ed): *Human Blood Coagulation, Haemostasis and Thrombosis*. Oxford, Blackwell, 1972, p 345



blood[®]

1974 44: 313-322

Antibodies to Factor VIII: Specificity and Kinetics of Iso- and Hetero-antibodies in Hemophilia A

J. P. Allain and D. Frommel

Updated information and services can be found at:

<http://www.bloodjournal.org/content/44/3/313.full.html>

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:

http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:

<http://www.bloodjournal.org/site/misc/rights.xhtml#reprints>

Information about subscriptions and ASH membership may be found online at:

<http://www.bloodjournal.org/site/subscriptions/index.xhtml>