Differences between Type I Autoimmune Inhibitors of Fibrin Stabilization in Two Patients with Severe Hemorrhagic Disorder

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ABSTRACT Inhibitors of fibrin stabilization of apparently autoimmune origin, found in two severely bleeding unrelated patients (W. G. and G. A.), were compared with regard to their biological target specificities, potencies and immunological characteristics. Both interfered only with the activation of fibrin stabilizing factor (coagulation Factor XIII) and, while totally preventing the conversion of this zymogen to the functional transamidating enzyme, fibrinoligase (Factor $XIII_a$), they showed very little inhibition toward the enzyme itself. Thus, according to the classification of Lorand concerning biological specificities, both can be characterized as Type I inhibitors of fibrin stabilization. Potencies of the two inhibitors were quite similar when measured in conjunction with the plasma zymogen, but they differed remarkably in tests with platelet Factor 13. The inhibitor of patient W. G. prevented the activation of the zymogen from platelets, but that of G. A. had no effect on the platelet factor. It may therefore be concluded that the inhibitor of W. G. is directed exclusively against the *a* subunit which is a common constituent of plasma as well as platelet factors. The inhibitor of G. A., however, must be targeted against determinants uniquely characteristic for the *ab* ensemble of the plasma zymogen including the *b* subunit. On the basis of this difference in target specificity, the inhibitor of W. G. is designated as Type I-1 and that of G. A. as Type I-2.

The inhibitors of both patients were isolated as immunoglobulins, and neutralization tests revealed that the antibody of W. G. comprised mainly heavy chains of the IgG1 and light chains of the κ class. The antibody of G. A. proved to be considerably more heterogeneous and contained IgG1 and IgG3 heavy chains as well as κ- and λ-light chains.

The finding that the antibody of W. G. inhibited conversion of platelet Factor 13 and also its thrombin-modified form, but had no effect on the thrombin and Ca^{2+}-activated factor, is an indication that antigenic determinants existing both on the native zymogen and on its hydrolytically modified form become buried in the Ca^{2+}-dependent step of activation. This is clear evidence for the occurrence of a significant conformational change in the protein structure attendant to the process of unmasking of its enzymic activity.

INTRODUCTION

Although disorders of fibrin stabilization that are a result of the appearance of inhibitors in the circulation are quite rare, studies of such diseases are particularly challenging from the point of view of molecular analysis. Suitable quantitative assays are now available for obtaining rather precise definitions as to the targets of the various inhibitors, and it has already been possible to differentiate three major forms within this family of bleeding diseases. According to the classification of

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Lorand (1, 2), the Type I inhibitor prevents the activation of the fibrin stabilizing factor zymogen (Factor XIII); the Type II inhibitor interferes with the transamidase function of the fully activated enzyme, fibrinoligase or Factor $XIII_a$; the Type III inhibitor is directed against the fibrin substrate itself and alters the reactivities of its cross-linking sites vis à vis fibrinoligase.

The present study deals with the detailed characterization of Type I inhibitors of Factor XIII from two unrelated individuals. One of these is the case pertaining to the appearance of the inhibitor in a 50-yr-old man after prolonged therapy with isoniazid (3). The other is a hitherto unreported case of a 9-yr-old boy who developed a severe hemorrhagic condition shortly after the onset of allergy to penicillin. These two inhibitors were compared with regard to biological target specificities, potencies and immunological characteristics.

CASE HISTORIES

G. A. is an adult male who, as has already been described (3), at the age of 50 yr developed an IgG antibody to Factor XIII which specifically interfered with the activation of this zymogen.

W. G. is a male born in Poland in 1960 with normal delivery. The patient has twin sisters and there is no family history of bleeding. In March 1969 two adult teeth were extracted without undue bleeding. However, by November 1969 hemorrhagic episodes were noted, with painful swelling in the left thigh. On surgical exploration, a deep hematoma of the quadriceps muscle was found and, in spite of repeated transfusions, the bleeding continued well beyond the operation; wound healing occurred only some 3 mo later. In the next 2 yr, repeated hospitalization was necessitated by other deep hematomata in the thigh, calf, and forearm regions. The patient remained unresponsive to transfusions, and it was also observed that he bruised very easily. In 1972 he was referred to the Institute of Hematology in Warsaw for diagnosis. Physical examination revealed superficial bruises, atrophy of the left quadriceps muscle with an oblong and deformed cicatrix, and a systolic murmur at the apex of the heart. He had a past history of myocarditis and probably rheumatic fever in 1966, for which he received salicylates and prednisone for 3 wk. He had also undergone treatment with penicillin G for 1.5 yr at a dose of 1,200,000 U every 3rd wk. In May 1969 he had pharyngitis, and a skin test for penicillin allergy caused a severe local erythematous reaction which took 10 days to resolve. No further treatment with penicillin was given.

Results of routine laboratory blood tests on W. G. were within normal limits. Serum electrophoresis and immunoglobulin determinations were also normal. Tests for lupus cells, antinuclear antibodies and the direct Coomb's test were negative. Bleeding time (Ivy) was 4 min; platelet count, ADP-induced platelet aggregation, prothrombin time, thrombin time, and activated partial thromboplastin time were normal. Fibrinogen concentration ranged from 230 to 290 mg/100 ml. Activity assays for clotting Factors II, V, VII, VIII, IX, X, XI, and XII were within normal ranges. The patient's plasma had no fibrinolytic activity when tested on either unheated or heated fibrin plates.

The only definitely abnormal finding was that the clot formed in the plasma of patient W. G. could be readily and fully dispersed by the addition of 30% urea (4, 5) or 1% monochloroacetic acid (6). Mixing experiments with normal plasma indicated that an inhibitor to fibrin stabilization was present.

On readmission to the Institute of Hematology in Warsaw in 1973 with hemarthrosis of the knee, W. G. was placed on immunosuppressive therapy with prednisone and azothioprine. This did not produce any appreciable clinical improvement, and therapy was discontinued after 2.5 mo. The bleeding disease in the patient remains severe and recurrent hemorrhages into the calf muscle have caused a fixed equinus deformity of the left foot.

METHODS

The IgG inhibitor from the serum of patient G. A. was prepared as previously described (3). Purification of the immunoglobulin of patient W. G. was carried out by precipitation with Rivanol and ethanol (7). As judged by immunodiffusion (8) against rabbit antibodies to Factor XIII, or against antibodies to the separate *a* and *b* subunits of this zymogen, the IgG fractions of the two patients were free of Factor XIII contamination. Both preparations contained a strong and a minor (<2% of protein) band by sodium dodecylsulfate disc gel electrophoresis under nonreducing conditions (9). The major band corresponded to IgG with mol wt of about 160,000. The purified inhibitors were dissolved in 50 mM Tris-HCl buffer of pH 7.5.

The two inhibitors of Factor XIII were also characterized by subjecting 5 ml of the patients' plasmas to preparative zone electrophoresis on polyvinyl copolymer (Pevikon; Mercer Chemical Co., Amityville, N. Y.) and to chromatography on agarose (Bio-Gel A-1.5m) as previously described (10, 11). Fractions collected from Pevikon were assayed for protein (12) as well as IgG content (8) and for Factor XIII inhibitor activity as given below. The Factor XIII inhibiting fractions were pooled and applied to Bio-Gel A-1.5m (Bio-Rad Laboratories, Richmond, Calif.). The eluant was monitored for absorbance at 280 nm and was assayed for IgG as well as inhibitor activity.

Purified immunoglobulins of the immunoglobulin classes (IgG, IgA, IgM, IgD, IgE), of the IgG heavy chain subclasses (IgG1, IgG3, IgG4) and of the light chain types (κ and λ) were obtained from patients with multiple myeloma or macroglobulinemia, and rabbit antisera to each of the immunoglobulins were prepared as previously described (11). In preparing the antiserum to IgG1, aggregate-free immunoglobulins of IgG2 and IgG3 subclasses were injected for the sake of immune tolerance against the latter two 5 days before immunization

with IgG1. Antisera were precipitated by 50% saturation of ammonium sulfate, adsorbed with barium sulfate, and heated at 56°C for 30 min.

Fibrin stabilizing factor (Factor XIII) was purified from human plasma by the procedure of Lorand and Gotoh (13, 14) and was dissolved in 50 mM Tris-HCl buffer of pH 7.5, containing 1 mM EDTA. Platelet factor 13 was prepared from washed human platelets by ammonium sulfate fractionation followed by chromatography on organomercurial-agarose (15). The purified platelet protein showed a single band by sodium dodecylsulfate electrophoresis, corresponding to *a* subunit alone (16). Furthermore, as measured by immunodiffusion, the platelet factor contained no *b* subunit.

Tests for analyzing Factor XIII activities were performed either by an isotope method of incorporating [^{14}C]putrescine (14, 17) (Amersham Corp., Arlington Heights, Ill.) or by a fluorescence assay of incorporating dansylcadaverine (Sigma Chemical Co., St. Louis, Mo.) into casein (13, 18). When working with systems containing plasma, rather than purified protein components, the heat-desensitization step was employed to prevent clotting of fibrinogen. Solutions of dithiothreitol (0.2 M; Calbiochem, San Diego, Calif.), Hammarsten casein (2%; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), [1-4-^{14}C]putrescine dihydrochloride (approximately 11.6 mM) and $CaCl_2$ (40 mM) were made up in 50 mM Tris-HCl buffer of pH 7.5. Thrombin (Parke, Davis & Co., Detroit, Mich.; topical) was dissolved to a strength of 500 National Institutes of Health U/ml in 25 mM Tris-HCl of pH 7.5, containing 25% of glycerol. Titrations of the patients' inhibitors were carried out by methods previously described (3, 11). In typical experiments, normal plasma, purified Factor XIII or platelet Factor 13, before or after exposure to thrombin or after activation by thrombin and Ca^{2+}, was mixed with an aliquot of the patients' plasma, serum, or IgG fraction and was allowed to incubate for a period of about 75 min at 37°C, before testing for amine incorporation.

Neutralizations of the patients' inhibitors with specific anti-immunoglobulin antisera were carried out by a two-step procedure, as previously described (11). In the actual neutralization phase (phase A), the patients' sera were incubated with antisera to specific immunoglobulin classes, subclasses, or chain types. Correct volume ratios between the patients' sera and antisera insuring an excess by the latter were determined by Ouchterlony diffusion (8), and the following actual ratios were used: for establishing immunoglobulin class, 1:30; IgG subclass, 1:40; light chain type for patient G. A., 1:40; for patient W. G., 1:60. The mixtures were allowed to stand for 1 h at 37°C and subsequently for 12 h at 4°C. Precipitates were removed by centrifugation, and the supernates were analyzed for residual inhibitory potencies against Factor XIII in normal plasma in the second phase of the experiment. In studying patient W. G., phase A supernates were mixed with an equal volume of normal plasma; for patient G. A., volume ratios for phase A supernates were doubled. The mixtures were incubated for 90 min at room temperature (about 22°C) and centrifuged. Finally, 0.2-ml aliquots were withdrawn from the resulting supernates for assays of dansylcadaverine incorporation into casein. Results (see Table I and II) are expressed in terms of micromoles of amine incorporated in 30 min of reaction. To determine expected Factor XIII activities when the patients' inhibitors did not become neutralized by antisera, experiments were carried out by replacing the antisera in phase A mixtures with either 0.15 M NaCl or with nonimmune rabbit serum. On the other hand, replacement of patients' plasmas in phase A mixtures by saline made it possible to evaluate the Factor XIII levels expected if total neutralization of the inhibitors occurred. As a further check on the specificities of the neutralization reactions, whenever a given antiserum was found to neutralize the patients' inhibitors, the antiserum was absorbed with its specific purified immunoglobulin antigen and was retested with the patients' inhibitors.

RESULTS

Since the case of one of the patients (W. G.) discussed in this paper has not been analyzed before, it was essential to examine whether the new inhibitor was of the Type I variety, targeted selectively against the fibrin stabilizing factor (Factor XIII) zymogen, as with the previously described (3) patient (G. A.). When W. G. serum was mixed in varying dilutions with 10 μl of pooled normal citrated plasma, and Factor XIII determinations were carried out with the [^{14}C]putrescine-casein assay (17), results such as those given in Fig. 1 were obtained. A marked reduction in the apparent Factor XIII content of the test plasma was seen (curve

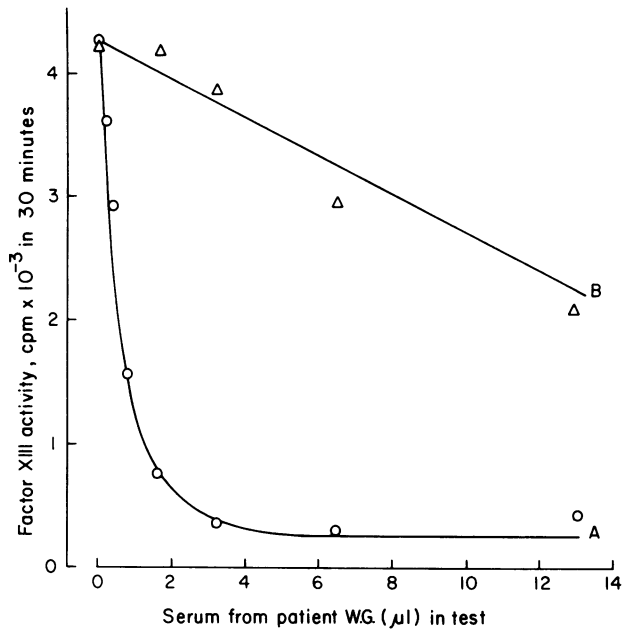


FIGURE 1 Inhibition of Factor XIII conversion in normal plasma by the serum of patient W. G. For each experimental point in curve A, 10 μl of pooled normal citrated plasma was desensitized by addition of 5 μl of 50% glycerol and a 4-min treatment at 56°C. After cooling to 37°C, 20 μl of the patient's serum (in various dilutions with 50 mM Tris-HCl of pH 7.5; abscissa) and 5 μl of 0.2 M dithiothreitol were admixed, followed by the addition of 5 μl each of 40 mM $CaCl_2$ and thrombin (2.5 National Institutes of Health U) solutions. 10 min later, 20 μl of casein (2%) and 5 μl of [^{14}C]putrescine (11.6 mM) were added, and amine incorporation was allowed to proceed for 30 min when an aliquot of 5 μl was spotted onto filter paper, washed with TCA, dried, and counted for protein-bound isotope (17). Conditions for the experiments in curve B were similar except that the order of mixing of the initial reagents was such that the desensitized normal plasma was incubated with dithiothreitol, $CaCl_2$ and thrombin for 10 min before adding the patient's serum.

A) whenever the patient's serum was added to the normal plasma before the admixing of thrombin and Ca^{2+}, which is necessary for activating the zymogen. However, when the patient's serum was added after mixing normal plasma with thrombin and Ca^{2+}, and thus having converted its Factor XIII to $XIII_a$, relatively little inhibition was found (curve B). In its action on the zymogen (curve A), the patient's serum lowered Factor XIII values in a dose-dependent manner, and approximately 3 μl of the patient's serum completely neutralized the Factor XIII equivalent in 10 μl of normal plasma.

The IgG fraction obtained from W. G. by precipitation with Rivanol and ethanol (7) gave a very similar pattern of inhibition when assayed either in conjunction with normal plasma or with pure human Factor XIII. Testing with the latter provided the most clear-cut definition of selectivity for the patient's inhibitor and showed that its potency towards the thrombin and Ca^{2+}-activated factor (curve B) was only about 1.5% of that seen with the zymogen itself (curve A). Furthermore, it could be shown for both W. G. and G. A., that the critical element in obtaining inhibition curves such as A in Fig. 1, lies in the order in which the patient's IgG is added to Factor XIII in relation to the other two essential reaction components, thrombin and Ca^{2+}. Even when the Factor XIII was incubated with thrombin first (10 min) and IgG was added next, followed by Ca^{2+}, the inhibitory pattern remained as in curve A. However, changing this order of mixing to Factor XIII, thrombin, Ca^{2+} (10 min), and adding IgG last, caused a shift to the behavior shown by curve B with hardly any inhibition at all.

Once it was demonstrated that the inhibitor in W. G. was of the Type I variety, a quantitative comparison with the inhibitor previously reported in patient G. A. (3) could be performed with isolated IgG fractions. Various amounts of these were incubated with normal citrated plasma before the addition of thrombin and Ca^{2+}, and residual Factor XIII activities were measured by the method of incorporating dansylcadaverine into casein (13, 18). As illustrated in Fig. 2, the preparations from the two patients inactivated the Factor XIII component of normal plasma quite similarly, though at equivalent concentrations of IgG protein, the inhibitor from W. G. appeared to be slightly more potent. The similarity between the two, however, broke down when they were tested on pure platelet Factor 13. Curve A in Fig. 3 shows that the IgG preparation from W. G. was effective in preventing activation of the platelet factor (as long as it was added to this zymogen or to its thrombin-treated form before adding Ca^{2+}) but that the fraction obtained from G. A., even when it was applied at concentrations at which the W. G. inhibitor reduced measurements of Factor 13 activities to virtually zero, had no effect on the platelet zymogen.

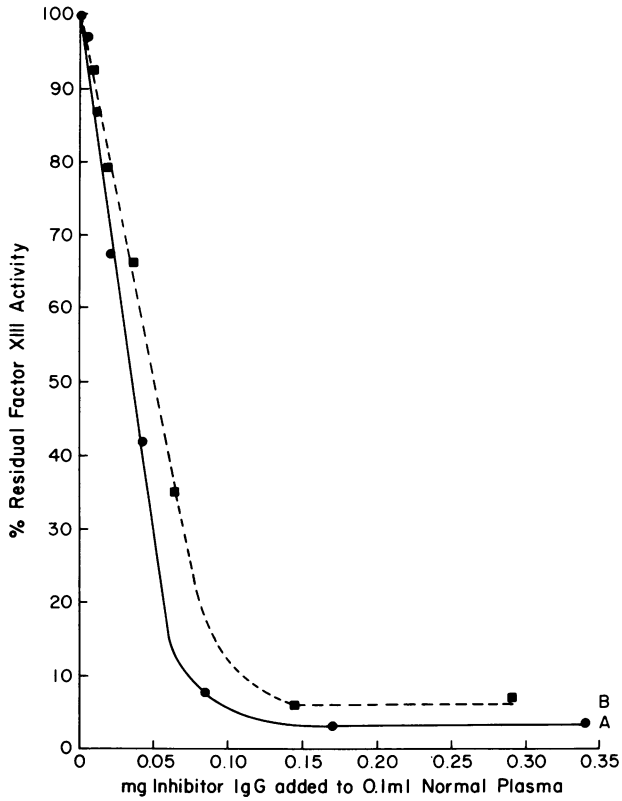


FIGURE 2 Comparison of the potencies of W. G. and G. A. inhibitors with respect to plasma Factor XIII. Patients' purified IgG fractions (0.1 ml) were incubated with normal plasma for 90 min before thrombin treatment. Residual Factor XIII activity in the pooled plasma was measured by the incorporation of dansylcadaverine into casein. Results were recorded as micromoles dansylcadaverine incorporation/30 min and expressed as a percentage of residual activity in the plasma. A: purified IgG from patient W. G.; B: purified IgG from patient G. A.

After 30 min reaction of amine incorporation into casein, it could be shown that 17 μg of the W. G. inhibitor produced a drop of 1 μmol in the quantity of incorporated dansylcadaverine when tested in conjunction with pure platelet Factor 13, and essentially the same amount of the inhibitor (16 μg) gave an identical result when assayed on pure plasma Factor XIII.

In an effort to characterize further the inhibitors of the two patients, plasma samples were subjected to preparative zone electrophoresis on Pevikon, and fractions were assayed for protein and IgG content as well as for Factor XIII inhibition by the dansylcadaverine-casein system of analysis. For both W. G. and G. A. regions of inhibitory activities were found with mobilities corresponding to IgG, but, especially with G. A. plasma, the spread of distribution indicated that the population of inhibitory antibodies was rather heterogeneous in electrophoretic terms. The fractions ob-

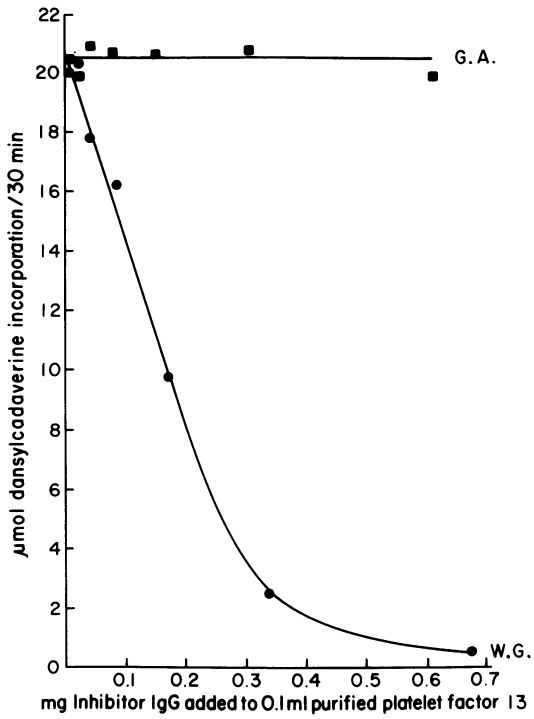


FIGURE 3 Comparison of the potencies of W. G. and G. A. inhibitors with respect to platelet Factor 13. Patients' purified IgG fractions were incubated with purified platelet Factor 13 before thrombin treatment. Residual platelet Factor 13 activity was measured by incorporation of dansylcadaverine into casein, and results are expressed as micromoles incorporation/30 min. A: purified IgG from patient W. G.; B: purified IgG from patient G. A.

tained from zone electrophoresis which contained inhibitory activities were pooled, concentrated and analyzed by means of gel filtration on Bio-Gel A-1.5m. Both the inhibitor of W. G. and of G. A. eluted in peaks characteristic for the elution volume of IgG. These experiments suggested that the IgG fractions contained all the inhibitory activities in the patients' plasma.

Neutralization experiments with specific antisera to the major immunoglobulin classes, IgG subclasses, and light chain types (11) were carried out for the further identification of the inhibitors (Tables I and II). When the patients' sera were incubated with monospecific antisera raised against IgG, IgA, IgM, IgD, and IgE, only anti-IgG was able to abolish their inhibitory activities. The specificity of the reaction was confirmed by absorbing anti-IgG with purified IgG, which destroyed the effectiveness of the antiserum to neutralize the patients' inhibitors.

Further experiments were performed to determine the IgG subclass and light chain type of the inhibitors. Certain neutralization (59%) of the serum of W. G. could only be achieved by using antiserum against IgG1 subclass, and this neutralization was specific in the sense that it did not occur when the antiserum was absorbed with purified IgG1 myeloma proteins (Table I). In similar experiments with antisera to κ- and λ-light chains, 84% of the W. G. inhibitor could be neutralized by antiserum to κ-chains, and this could be abolished by prior absorption of the antiserum with purified κ-type Bence-Jones protein. There was no neutralization with anti-λ-antiserum. On the basis of these studies, the Factor XIII inhibitory antibody in W. G. was classified as comprising mainly IgG1 heavy chains and κ-light chains.

Classification of the inhibitor in G. A. was determined in an analogous manner (Table II). This inhibitor was sensitive to neutralization to the extent of 54% by anti-IgG1 and to 31% by anti-IgG3. In both cases neutralization was blocked if the antisera were absorbed with their specific purified myeloma proteins. As far as the light chain type of the G. A inhibitor is concerned, 77% of its activity could be neutralized by anti-κ- and 23% by anti-λ-antiserum. Again, the specificity of these reactions could be shown by prior absorption of antisera with their specific purified Bence-Jones proteins. The findings indicate that the Factor XIII inhibitor in G. A. was a polyclonal IgG antibody, containing both IgG1 and IgG3 heavy chains, and κ- as well as λ-light chains.

TABLE I
Neutralization of the Inhibitor in W. G. Serum by Specific Antisera

Antisera	Factor XIII activity: Expected if inhibitor was not neutralized	Expected if inhibitor was neutralized	Observed	Factor XIII inhibitor neutralized
	µmol of dansylcadaverine incorporated in 30 min			%
To IgG	0.2	2.3	2.1	90
IgG (absorbed)	0.2	2.3	0.2	0
IgA	0.2	2.3	0.1	0
IgM	0.2	2.3	0.2	0
IgD	0.2	2.3	0.2	0
IgE	0.2	2.3	0.2	0
(W. G. serum:antisera = 1:30)				
κ	0.4	2.3	2.0	84
κ (absorbed)	0.4	2.3	0.4	0
λ	0.4	2.3	0.4	0
(W. G. serum:antisera = 1:40)				
IgG1	0.6	2.3	1.6	59
IgG1 (absorbed)	0.6	2.3	0.6	0
IgG2	0.6	2.3	0.6	0
IgG3	0.6	2.3	0.6	0
IgG4	0.6	2.3	0.6	0
(W. G. serum:antisera = 1:60)				

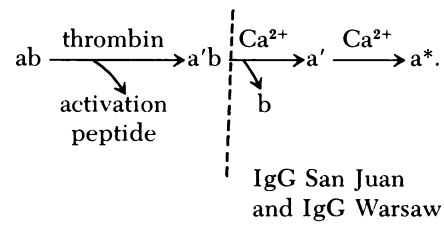
TABLE II
Neutralization of the Inhibitor in G. A. Serum by Specific Antisera

Antisera	Factor XIII activity: Expected if inhibitor was not neutralized	Factor XIII activity: Expected if inhibitor was neutralized	Factor XIII activity: Observed	Factor XIII inhibitor neutralized
	μmol of dansylcadaverine incorporated in 30 min			%
To IgG	0.2	1.6	1.7	100
IgG (absorbed)	0.2	1.6	0.2	0
IgA	0.2	1.6	0.2	0
IgM	0.2	1.6	0.2	0
IgD	0.2	1.6	0.1	0
IgE	0.2	1.6	0.2	0
(G. A. serum:antisera = 1:30)				
κ	0.3	1.6	1.3	77
κ (absorbed)	0.3	1.6	0.4	8
λ	0.3	1.6	0.6	23
λ (absorbed)	0.3	1.6	0.3	0
(G. A. serum:antisera = 1:40)				
IgG1	0.3	1.6	1.0	54
IgG1 (absorbed)	0.3	1.6	0.3	0
IgG2	0.3	1.6	0.4	8
IgG3	0.3	1.6	0.7	31
IgG3 (absorbed)	0.3	1.6	0.3	0
IgG4	0.3	1.6	0.4	8
(G. A. serum:antisera = 1:40)				

DISCUSSION

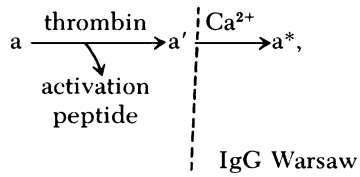
Fibrin stabilizing factor (Factor XIII) circulates as an inactive precursor in plasma (18), serving as the zymogen for the last enzyme (fibrinoligase or Factor $XIII_a$) of the coagulation cascade (19), and requiring both thrombin and Ca^{2+} ions (20) for activation. The physiological role of fibrinoligase is to catalyze an amide exchange reaction (transamidation) between γ-glutamine and ε-lysine side chains of fibrin molecules and to stiffen the clot structure by inter-fibrin γ-glutamyl-ε-lysine peptide bridging (1). As illustrated by the two patients presented in this paper, malfunctioning of the fibrin stabilizing system is often associated with very severe bleeding (2). These two patients suffered from acquired abnormalities, and their sera contained IgG antibodies which selectively interfered only with the conversion of the Factor XIII zymogen (Fig. 1 [3]). As such, these inhibitors should be classified as Type I within the context of inhibitors giving rise to hemorrhagic disorders of fibrin stabilization (2).

Conversion of the plasma zymogen to the active transamidase is known to occur in two distinct steps (21–24). The first is catalyzed by thrombin and the second depends rather specifically upon the presence of Ca^{2+} ions. Modification of the *ab* ensemble in the zymogen by thrombin affects only *a* and, while converting this subunit to a somewhat smaller *a'* component (25, 26), it allows the two different subunits to remain associated within an *a'b* protomer (21–24). Furthermore, the hydrolytically altered species still has no measureable transamidase activity (21, 22). Formation of the catalytic center in *a'* requires the presence of Ca^{2+} ions which bring about the heterologous dissociation of subunits ($a'b \xrightarrow{Ca^{2+}} a' + b$) and also cause an unmasking of the active center cysteine residue ($a' \xrightarrow{Ca^{2+}} a^*$ transition). Thus, the factor XIII $\xrightarrow{\text{thrombin}} XIII' \xrightarrow{Ca^{2+}} XIII_a$ conversion, in terms of protomeric formulas, can be illustrated as:



Both patients' sera were found to inhibit generation of the enzyme as long as they were added to the plasma zymogen before the Ca^{2+}-dependent steps (see vertical broken line). As such, it is clear that neither the inhibitor of patient G. A. (designated as IgG San Juan), nor that of W. G. (IgG Warsaw) could be directed against the activation peptide region which is removed from the *a* subunit in the thrombin-catalyzed step.

Since platelet Factor 13 preparations do not contain the carrier *b* subunit (16), it was of considerable interest also to compare the two patients' inhibitors in relation to the conversion of this zymogen,



and while IgG Warsaw produced a complete inhibition (as long as it was applied before adding Ca^{2+}; vertical broken line), remarkably IgG San Juan had no effect whatsoever on the platelet factor (Fig. 3). Thus the two patients' inhibitors are really quite different from the point of view of precise biological target specificities. In fact, this difference warrants that IgG Warsaw be designated inhibitor Type I-1 and IgG San Juan as Type I-2. Further evidence for the separate antigenic specificities of the two inhibitors was ob-

tained[1] when they were tested against plasma from 15 mammalian and avian species.

The inhibitory potency of IgG Warsaw was just as great against platelet Factor 13 as against plasma Factor XIII. Therefore, this antibody seems to be directed exclusively against the *a* (and also the *a'*) type of subunit occurring in both zymogens. By contrast, IgG San Juan must be targeted against a different set of determinants which seem to be uniquely characteristic for the *ab* and *a'b* ensembles of the plasma zymogen only. The finding that IgG Warsaw inhibited platelet Factor 13 and its thrombin-modified form, but had no effect when applied after activation by thrombin and Ca^{2+}, is a definite indication that antigenic determinants existing both on the native zymogen (*a*) and on its hydrolytically altered (*a'*) form become buried in the Ca^{2+}-dependent step during enzyme generation. This is a clear sign of a conformational change in protein structure during the process of unmasking of the active center in the $a' \xrightarrow{Ca^{2+}} a^*$ transition.

Results of fractionation as well as antibody neutralization experiments revealed that both patients' inhibitors belonged to the IgG class of immunoglobulins. With antisera with various heavy chain subclass and light chain specificities, it could also be shown that the inhibitor of patient W. G. (IgG Warsaw) could be neutralized only with antisera against IgG1 heavy chain and κ-light chain (Table I). By contrast, IgG San Juan (the inhibitor of patient G. A.) was more polyclonal in comprising IgG1 and IgG3 heavy chains as well as κ- and λ-light chains (Table II).

The clinical picture in the two patients with the Type I inhibitors of fibrin stabilization is dominated by life-threatening bleeding episodes which cannot be alleviated by conventional plasma transfusions. Failure to respond to transfusions is, of course, explainable by the fact that in both patients the circulating inhibitors are present in large excess (Fig. 1 and [3]), effectively neutralizing the Factor XIII zymogen content of normal plasma transfusions. Appearance of the circulating inhibitors in the two patients may well have been drug related. Both penicillin and isoniazid are known for their reactivities with proteins (27–30) and also for their autoimmunizing side effects (31–33). Thus, the possibility exists that the covalent modification of Factor XIII by these drugs may have led to a breakdown of immunological tolerance to the native coagulation factor itself.

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[1] Lopaciuk, S., J. M. McDonagh, and R. P. McDonagh. Unpublished observations.

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