Immunoglobulin G from Patients with Antiphospholipid Syndrome Impairs the Fibrin Dissolution with Plasmin

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Keywords

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Summary

Immunoglobulin G (IgG) isolated from normal human blood plasma stabilizes the structure of perfused crosslinked fibrin and prolongs the time for its dissolution with plasmin, when the fibrin surface is exposed to 500 s\(^{-1}\) shear rate flow. The IgG from patients suffering in antiphospholipid syndrome with thrombotic complications exerts even stronger antifibrinolytic effect. A patient, whose IgG does not affect the fibrin dissolution with plasmin, displays a bleeding tendency. The shear stress-induced disassembly of the fibrin clots containing IgGs with antifibrinolytic potency occurs at a much more advanced stage of fibrin digestion, as evidenced by the electrophoretic pattern of the urea-treated samples. The antifibrinolytic effects are also produced under static conditions and these are caused by the variable portion of the IgG molecules (fragment F\(_{\, v}\)), whereas the constant part (fragment F\(_{\, c}\)) has no inhibitory effect. The IgGs with antifibrinolytic properties do not affect directly the plasmin activity in amidolytic assay, but the IgGs from APS patients obliterate the competition of the fibrin and the peptideyl-p-nitroanilide for the protease in the same assay system suggesting interference of the IgGs with the plasmin action on the fibrin substrate. Thus, the correlation of the clinical symptoms with the effect of the isolated IgG on the dissolution of perfused fibrin clots supports a physiological and a pathological role of IgG in the fibrinolytic process related to the variability of the cross-reactions of immunoglobulins with fibrin, fibrin degradation products or fibrin-plasmin complexes.

Introduction

The primary antiphospholipid syndrome (APS) is an autoimmune condition characterized by recurrent venous and arterial thrombotic events in various regions of the circulatory system (including the cerebral vasculature) or recurrent fetal loss associated with laboratory diagnosis of lupus anticoagulants (LA) or anticardiolipin antibodies (aCL) in the absence of another autoimmune disease, such as systemic lupus erythematosus (1, 2). The exact mechanism of thrombosis is uncertain despite the evidence for interference of the antibodies with the phospholipid-dependent anticoagulant processes (1, 3). Data in the literature suggest that the IgG isotype of aCL is predominantly associated with the thrombotic complications of the APS. The aCL detected by the conventional assays actually binds to epitopes of \(\beta_2\)-glycoprotein-1 or oxidized lipids (4). There is also literature to support aCL of other specificities, some of which interfere with the function of the plasminogen/plasminogen activator system considered to be the major mechanism for the dissolution of thrombi (5, 6). Despite some evidence for fibrinolytic abnormalities in patients with APS (7) and in animal models of APS (8) the potential effect of aCL on the solubilization of fibrin has not been addressed directly so far. In this report we present evidence that the dissolution of fibrin clots with plasmin is impaired by IgG from plasma of patients with APS.

Materials and Methods

Patients

Ten patients (6 men and 4 women, mean age: 49; range: 28-72) with APS diagnosed according to Hughes (9) were examined. Seven patients had ischaemic stroke, one pulmonary embolism, one deep vein thrombosis with recurrent abortion and one thrombotic occlusion of central retinal vein. IgG and IgM aCL were measured by enzyme-linked immunosorbent assay (ELISA) (levels of IgG or IgM aCL higher than 16 GPL/MPL indicated the diagnosis) (10). LA were detected using recommendations of the International Society on Thrombosis and Haemostasis (11). All patients showed LA negativity and IgM aCL were in normal range.

The plasma of a male patient (age 50 years) with Q-fever (patient Q) and severe bleeding following liver biopsy was also examined. At the time of biopsy this patient had normal prothrombin time, activated partial thromboplastin time, platelet count and fibrinogen level, the LA and aCL tests were negative, polycanal elevation of the plasma IgG was measured (35.4 g/L versus normal < 15 g/L).

Normal pooled human plasma provided by the Hungarian Blood Supply Service and plasma from 5 healthy volunteers was also tested. All subjects gave their informed consent for participation in the study in accordance with the requirements of the Hungarian laws.

In all cases the plasma samples were obtained after centrifugation at 2500 \(\times\) g for 20 min of the citrated blood from venous puncture and stored at \(-70^\circ\) C until the assay.

Purification of IgG

The IgG fraction of the plasma samples was isolated using affinity chromatography on Protein A Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) according to manufacturer’s instructions. It represented a single band when visualized with silver staining after SDS electrophoresis on...
polyacrylamide gel under non-reducing conditions. Protein concentration was determined using extinction coefficient of 0.24 μmol/L·cm⁻¹ at 278 nm (12). The isolated IgGs were tested for aCL activity by ELISA: all preparations from APS patients retained the aCL positivity after the purification procedure.

Preparation of IgG fragments (Fₐ and F₇)

The IgG, isolated as described above, was digested for 3 h at 37° C with papain immobilized on agarose (Sigma, St. Louis, MO) in 100 mmol/L HEPES-NaOH pH 7.0 buffer containing 150 mmol/L NaCl, 10 mmol/L cysteine and 2 mmol/L EDTA (0.4 U papain/1 mg IgG). Using the nomenclature introduced by the pioneer work of Porter (13), the digest products could be identified with non-reducing sodium-dodecylsulfate polyacrylamide gel-electrophoresis as Fₐ₆ (Mₚ ~50 kDa), F₇ (Mₚ ~60 kDa) and partially digested IgG (Mₚ ~110 kDa). Following dialysis against 50 mmol/L TRIS pH 7.0 buffer, the digest products were applied to 5 mL Protein-A-Sepharose equilibrated with the same buffer. The F₇ and the partially degraded IgG fragments bind to the Protein-A-Sepharose, whereas the Fₐ₆ passes through the column (14). The bound fragments were eluted with 0.1 mol/L glycine pH 3.0 and following a concentrating step with polyethylene-glycol 20 000 (Fluka Chemie, Buchs, Switzerland) the F₇ and the partially degraded IgG were separated with gel-filtration on Sephadex G-100 column (1.5 × 55 cm, repeated twice). Protein concentration was determined using extinction coefficients of 0.08 μmol/L·cm⁻¹ at 278 nm for Fₐ₆ and 0.084 μmol/L·cm⁻¹ for F₇ (12).

Monitoring the Fibrinolytic Process under Flow Conditions

The perfusion circuit described previously was used (15). Briefly: fibrinogen (from human plasma, Calbiochem, LaJolla, CA) or mixtures of fibrinogen and IgG containing plasmin (10 mmol/L) and thrombin (1 NIH unit/mL) were cast into plastic syringes with a needle along their long axis. After clotting (in approximately 2 min) the needle was pulled out. Thus, a cylindrical fibrin clot was gained with a central channel of 1.2 mm diameter. With appropriate tubing this channel was attached to a peristaltic pump and a UV detector with a flow cell. The circuit was filled up with 10 mmol/L HEPES-NaOH pH 7.4 containing 150 mmol/L NaCl and 2 mmol/L CaCl₂, which was recirculated by the pump producing 500 s⁻¹ initial shear rate on the fibrin surface. The recorder attached to the UV monitor continuously registered the extinction at 280 nm measured in the circulating fluid phase.

Plasmin, thrombin and fibrinogen were purified as previously described (15). The amidolytic activity of plasmin was measured on the chromogenic substrate Spectrozyme-PL (H-D-norleucyl-hexahydroxytyrosyl-lysine-p-nitroanilide) (American Diagnostica, Hartford, CT) (16). Plasmin concentration is reported as active site titrated enzyme (17). Student’s unpaired t-test was used for statistical evaluation.

Fibrinolytic Activity under Static Conditions

Turbidometric assay was used as described previously (18). Fibrinogen (6 mmol/L in 10 mmol/L HEPES pH 7.4 buffer containing 150 mmol/L NaCl, 3 mmol/L CaCl₂, and the examined additive proteins) was added to thrombin and plasmin (final concentrations 1 NIH U/mL and 3 mmol/L, respectively) in microplate wells at 37° C. The course of clot formation and dissolution was monitored by measuring the light attenuation at 340 nm with a Dynatech microplate reader.

Monitoring of Plasma Clot Lysis

Plasma clots were prepared by mixing 100 μl citrated plasma with 100 μl solution “B” composed of 10 mmol/L HEPES pH 7.4 buffer containing 150 mmol/L NaCl, 25 mmol/L CaCl₂, 3 NIH U/mL thrombin and 1 μmol/L plasmin or 1.2 μg/mL tissue-type plasminogen activator (tPA) (Genentech Inc., South San Francisco, CA, USA). For the experiments with plasma supplemented with immunoglobulins the IgG was added as an additional component of solution “B” at final concentration of 40 μmol/L. The formation and dissolution of the plasma clots was monitored at 37° C by measuring the light attenuation at 340 nm as above.

Eu-labeling of IgG and its Binding to Fibrinogen

The IgG was labeled with Eu-chelate (N-(p-isothiocyanatobenzyl)-diethyl-l-lysine-p-tetraacetic acid chelated with Eu³⁺) according to manufacturer’s instructions (Wallac, Turku, Finland) with efficiency of 1.5 Eu/molecule protein (determined using a europium standard solution from Wallac). The Eu-label binds covalently to primary aminogroups of the proteins. The detection sensitivity of the label is similar to that of radioisotopes (19). Microtiter plate wells were coated with fibrinogen as previously described (17). For estimation of the binding of IgG and fibrinogen, IgG solutions containing constant amount of Eu-labeled IgG (20 mmol/L) and varying amounts of non-labeled IgG (total concentration in the range of 0.02-12 μmol/L) were applied in triplicate to the fibrinogen-coated wells. Following incubation of 20 min the wells were washed quickly (in less than 20 s) three times with 300 μL 10 mmol/L HEPES 150 mmol/L NaCl pH 7.4 buffer, the fibrin bound Eu was released in 100 μL of Enhancement Solution (Wallac, Turku, Finland) and the long-lived and sharp emission fluorescence of the lanthanide was measured with a time-resolved microplate fluorimeter Victor² (Wallac, Turku, Finland) (excitation wavelength 340 nm, emission wavelength 615 nm, counting delay 400 μs).

Results

Turbidimetric evaluation of the dissolution of plasma clots (Fig. 1A) shows that the course of fibrinolysis initiated by plasmin or tPA in the samples from APS patients is definitely slower than in the control normal plasma samples. Seeking the background of this phenomenon the IgG has been isolated from the blood plasma and added to normal plasma. The IgG from APS patients retards the dissolution of clots prepared from such supplemented plasma samples, whereas the addition of normal IgG (elevating the IgG concentration by 30% over the basal level) does not affect essentially the course of fibrinolysis (Fig. 1B). All IgG fractions have been screened in a turbidimetric fibrinolytic assay under static conditions using fibrin clots, too (Fig. 2). When fibrin is being dissolved with plasmin embedded in the clot, the presence of 18 μmol/L IgG (from any examined type except the one from patient Q) prolongs the lysis time. The prolongation with the IgG from normal pooled plasma and healthy subjects is by at least 25%, up to 2-fold compared to the pure fibrin, whereas with the IgG fraction from the APS patients – at least 60% up to 6-fold (Fig. 2A presents the lower and upper limits of the lysis-time range with 18 μmol/L IgG from APS patients and the median representative sample with normal IgG). According to the data in Fig. 2B the mean prolongation of the lysis time caused by the APS IgG (3.12 ± 1.31) is significantly higher than the one caused by normal IgG (1.45 ± 0.23, p <0.001 with Student’s unpaired t-test). Figure 2A also precludes the non-specific volume exclusion effects of inert macromolecules as a background of the retarded fibrinolysis in the presence of IgG: albumin at 40 μmol/L concentration affects the course of fibrinolysis to a negligible degree compared to the effect of normal IgG at the same concentration. The antifibrinolytic effect of the normal IgG can be reproduced by two-order magnitude higher concentration of albumin.

The antifibrinolytic effect is apparently related to the variable region of the immunoglobulin molecule (Fig. 3). Equimolar amounts of Fₐ₆ fragments (and also partially digested IgG, not shown) exert always stronger inhibitory effect on the fibrin dissolution than the respective IgG molecules, from which they are derived, whereas the corresponding F₇ fragments do not affect the lysis-time. This trend is confirmed by measurements carried out at higher molar excess of IgG or derivative
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For the studies reported in the next sections, which require larger amounts of IgG, the 3 normal samples with the highest prolongation of the turbidimetric lysis time (2 healthy subjects and normal pooled plasma), the 3 APS samples with the highest prolongation and the sample of patient Q have been selected.

The IgGs from the selected samples do not affect the amidolytic activity of plasmin measured on a peptidyl-\(\beta\)-nitronilide substrate in the absence of fibrin (sample “IgG” of Table 1). This result precludes the presence of any direct inhibitor of plasmin in the studied IgG fractions and any direct inhibiting effect of the IgG itself. However in the presence of fibrin, which as an alternative competing substrate reduces the amidolytic activity of plasmin (16, 18), the IgGs from the normal and the APS samples behave differently: the normal IgG does not affect the plasmin activity in the fibrin-Spectrozyme-PL substrate mixture (samples “fibrin” and “fibrin+N”), whereas the IgG from the APS patients restores the plasmin activity to the level of the fibrin-free system (there is no statistically significant difference between samples “IgG” and “fibrin+APS”, \(p < 0.11\)). The IgG from patient Q produces effects similar to the normal IgG in this assay (not shown). When the \(F_{ab}\) fragments of normal and APS IgGs are added instead of whole IgG to the same assay system, unexpectedly the APS \(F_{ab}\) inhibits the amidolytic activity of plasmin, whereas the normal \(F_{ab}\) does not. Because of this direct inhibiting effect, which can be attributed to the exposure of a cryptic plasmin-binding site following the papain-digestion of the IgG, this assay system is not suitable to examine the interference of \(F_{ab}\) with the action of plasmin on fibrin.

The plasmin-catalyzed dissolution of crosslinked fibrin clots perfused with buffer is affected by the examined IgG samples in a way similar to that seen in the static fibrinolytic assay (with the exception of

Fig. 1 Formation and dissolution of clots prepared from blood plasma. A) Clots are prepared as described in “Materials and Methods” from normal blood plasma (dotted line, dashed-and-dotted line) or from an APS patient’s plasma (solid line, dashed line) and fibrinolysis is induced by 0.5 \(\mu\)mol/L plasmin (solid line, dotted line) or 0.6 \(\mu\)g/mL tPA (dashed line, dashed-and-dotted line). The light attenuation at 340 nm is monitored. The ascending phase of the curve reflects the generation of fibrin, the descending phase its dissolution. B) Clots are prepared as in A) from normal pooled citrated blood plasma without any supplementation (dotted line) or supplemented with 40 \(\mu\)mol/L IgG isolated from normal plasma (dashed and dotted line) or 3 APS patients’ plasma (dashed and double-dotted, continuous, dashed line). Fibrinolysis is induced by 0.5 \(\mu\)mol/L plasmin.

over fibrin (5:1 and 10:1), with higher plasmin concentration (4 \(\mu\)mol/L) and with IgG fragments from another APS patient (because of the large amounts of starting material needed for the fragment preparation only 2 patients’ samples could be worked up for the effect of \(F_c\) and \(F_{ab}\)).

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the patient Q’s sample, which has no effect) (Fig. 4). A similar trend, but with even more expressed differences is observed with crosslinked fibrin prepared from more concentrated fibrinogen (9 μmol/L) or at lower plasmin activity (5 nmol/L) (not shown). When the molecular weight distribution of the soluble fibrin degradation products from crosslinked fibrin is examined (Fig. 5), the perfusate at complete dissolution of the fibrin without any additive contains mainly fragments of 200 kDa or larger size in agreement with recent data (21). In the presence of normal IgG, however, much more extensive degradation of the fibrin structure is necessary for the solubilization (fragments of size
Fig. 3 Effect of IgG and its fragments (F_{ab} and F_{c}) on the dissolution of fibrin. The experimental conditions are the same as in Fig. 2. The bars represent the values of the lysis-time, measured as shown in Fig. 2. The source of IgG in panels A and C is normal human plasma, whereas in panels B and D it is plasma from APS patient 10 (the IgG fragments are prepared from the same source as the whole IgG molecules). In panels A and B the molar ratio of IgG or fragments to fibrin monomers is 1, whereas in panels C and D it is 3. The values are the mean of 5 independent measurements in the absence of additive and the mean of 2 independent measurements in the presence of the indicated additives.

Table 1 Effect of IgG on the amidolytic activity of plasmin

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<th>F+N</th>
<th>F+APS</th>
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<td>0.63</td>
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<td>0.03</td>
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<tr>
<td>n</td>
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<td>5</td>
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Plasmin (20 nmol/L) is added to 100 μmol/L Spectrozyme-PL in 10 μmmol/L HEPES-NaOH pH 7.4 containing 150 mmol/L NaCl and 5 mmol/L glycyl-prolyl-arginy1-proline (GPRP, a peptide inhibitor of fibrin polymerization) (20) and the change in the absorbance at 405 nm is measured with a Beckman 2500 DU spectrophotometer. Results are presented in relative units (the activity of additive-free plasmin is 1) with the standard error of mean (each sample is measured in duplicate). Abbreviations: IgG, 16 μmol/L IgG of any type (2 from healthy subjects, 1 from pooled human plasma, 3 from APS patients, 1 from patient Q); F, 6 μmol/L fibrin (prepared from fibrinogen treated for 30 min with 0.5 NIH U/mL thrombin in the presence of 5 mmol/L GPRP); N, 16 μmol/L IgG from normal pooled plasma and 2 healthy subjects; APS, 16 μmol/L IgG from 3 APS patients.

Discussion

The pathophysiology of the thrombosis in APS still remains controversial despite the abundant evidence for interference of the antibodies with the phospholipid-dependent anticoagulant mechanisms (protein C-protein S system, annexin V) (1, 3). The concept of impaired fibrinolysis is gaining support with the identification of antibodies to tissue-type plasminogen activator (tPA) and fibrin-bound plasminogen in patients with APS (6, 22), as well as with the findings of blunted tPA response and increased plasminogen activator inhibitor-1 levels in blood plasma from patients with APS (7). In addition it has recently been shown that in a mouse model the anti-prothrombin antibodies cross-react with plasminogen or tPA (23). Our present report provides direct evidence that IgG isolated from the blood of APS patients inhibits the dissolution of fibrin with plasmin both under static conditions and when exposed to shear stress (Figs. 2 and 4) suggesting a mechanism contributing to the retarded fibrinolysis in the plasma clots of APS patients (Fig. 1). The IgG from healthy donors also impairs the fibrinolysis, but to a significantly lesser degree, and certain pathological states can be identified, in which the IgG fraction does not exert such an antifibrinolytic effect (e.g. the reported patient Q, Fig. 4). These results support the concept that the effects of IgG on the fibrin dissolution are related to the variability of these proteins under physiological and...
pathological conditions. Such a conclusion is reinforced by the effects of the IgG fragments on the course of fibrinolysis (Fig. 3). The F₃₄ fragments, which carry the antigen specificity of the IgG, are even more potent in the inhibition of fibrin dissolution than the respective source IgG molecules at similar molar ratios over fibrin monomers, whereas the invariable F₁ fragments do not affect the fibrinolysis. The reported effects correlate well with the clinical symptoms of the patients. The IgG isolated from the blood of the patient with bleeding tendency and elevated IgG plasma level does not stabilize the perfused clot against plasmin degradation (Fig. 4). In contrast, the IgGs from patients with APS and thrombotic complications (ischemic stroke, retinal vein thrombosis) render the perfused fibrin more resistant to plasmin.

Earlier studies, in which fibrin dissolution was initiated with plasminogen and tPA, have provided evidence that IgG from healthy subjects and from patients with myeloma multiplex prolong the lysis time and have correlated this effect to the changed size of the fibrin fibers formed in the presence of IgG (24). In order to preclude the role of the fibrin fiber size on the rate of plasminogen activation (25), we use plasmin to catalyze the digestion of fibrin. Although it is well documented that the IgG significantly changes the structure of the fibrin gel (26, 27) and the fiber size profoundly affects not only the tPA-, but also the plasmin-induced fibrinolysis (28, 29), the variations in the structure of the fibrin clots used in the present study are too small to account for the changes in the lysis-time. According to our earlier studies (28) the observed variation of 10-15% in the maximal clot turbidity [which reflects the mass-length ratio of the fibrin fibers (30)] can not account for the several-fold changes in the dissolution time (Fig. 2). In addition there is no straightforward correlation between the maximal turbidity and the course of dissolution (lower and higher turbidity is accompanied by retardation of the dissolution, Fig. 2). Although we report here saturating binding of IgG to fibrinogen, the lack of difference in the behavior of the normal and the APS IgG suggests that the IgG impact on fibrinolysis is not based on interference with the stage of fibrin formation (when the spatial structure of the fibrin network is settled), but with events occurring in the pre-formed fibrin. Furthermore, the antifibrinolytic effect of the IgG is clearly not based on direct inhibition of plasmin, as evidenced by the amidolytic activity of plasmin in the presence of IgG (Table). In the same assay only the APS IgG (and not the normal one) eliminates the competition between the fibrin and the peptidyl-p-nitroanilide substrate. This effect raises the possibility for interaction between the APS IgG and fibrin or between the APS IgG and the fibrin-bound plasmin that makes the fibrin less susceptible for the protease or changes the kinetic properties of the fibrin-bound enzyme.

Fig. 4 Effect of IgG on the dissolution of fibrin with clot-embedded plasmin under flow conditions. Fibrin clots are prepared from fibrinogen (6 μmol/L in 10 mmol/L HEPES-NaOH pH 7.4 containing 150 mmol/L NaCl and 2 mmol/L CaCl₂) containing 10 mmol/L plasmin and 10 or 12 μmol/L IgG from various samples, and buffer is perfused through the central channel of the clot as described in “Materials and Methods”.

The factor XIII content of the fibrinogen yields cross-linked fibrin (no γ-chains are detected in reduced electrophoretic samples 20 min after clotting). The release of soluble products is continuously monitored in the perfusate as absorbance at 280 nm (A₂₈₀). The value of A₂₈₀ presented in the figure is normalized as a fraction of the maximal A₂₈₀ at the final plateau stage of complete dissolution to compensate for the presence of IgG in some of the samples. IgG samples in the clots: none (solid line), from normal pooled plasma (dashed line), from APS patient (dotted line), from patient Q (dashed and dotted line). The 3 IgG preparations from APS and control plasma samples produce consistent patterns of dissolution.

Fig. 5 Effect of IgG on the molecular mass distribution of the degradation products of crosslinked fibrin digested with plasmin. Experimental conditions are as in Fig. 4. When the A₂₈₀ of the perfusate reaches the plateau values, samples are taken, denatured in 2% Na-dodecysulfate, separated with electrophoresis under non-reducing conditions on 4-15% polyacrylamide gradient gels and visualized with silver staining. Lane labels: F, fibrin without IgG; APS, fibrin with APS IgG; P, fibrin with IgG from patient Q; N, fibrin with normal IgG; APS-U, the APS sample denatured with 8 M urea in addition to the 2% Na-dodecysulfate (the urea does not affect the pattern of the F sample, not shown).
The results from the monitoring of fibrinolysis under flow conditions (Figs. 4 and 5) suggest that the IgG from normal subjects stabilizes the larger degradation products within the structure of the clot preventing their removal by the shear forces acting on the interface of the fibrin and the circulating fluid. Thus, plasmin should digest more extensively the fibrin structure prior the disassembly of the clot and more advanced degradation products are detected in the fluid phase (Fig. 5). The IgG from APS patients has similar impact on the digestion pattern of the disassembling fibrin clots, but the smaller digestion products are seen only after urea-treatment of the samples. The urea effect implies a different type of fibrin-IgG interaction, which results in greater stabilization of the clot compared to the normal IgG.

Our present results provide direct evidence that normal IgG fractions modify the plasmin dissolution of perfused fibrin clots. In addition to this novel physiological effect we report two pathological abnormalities that correlate well with the clinical symptoms. The IgG isolated from the blood of a patient with bleeding tendency and elevated IgG plasma level does not stabilize the perfused clot against plasmin degradation. In contrast, the IgGs from patients with APS and thrombotic complications (ischemic stroke, retinal vein thrombosis) render the perfused fibrin more resistant to plasmin. The antifibrinolytic effect of the IgG is confined to the variable, antigen-recognizing portion of the molecule. The recently described cross-reactions of anti-prothrombin antibodies (commonly detectable in APS patients) with plasminogen (23) could interfere with the plasmin action on fibrin. Thus, the background of the reported effects could be the variability of the cross-reactions of immunoglobulins produced in different clinical conditions with fibrin, fibrin degradation products or fibrin-plasmin complexes.

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