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Anti-plasminogen Autoantibodies from Plasma of Patients with Systemic Lupus Erythematosus Having Anti-phospholipid Antibody Syndrome: Isolation and Some Immunochemical Properties

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Abstract—Blood plasma samples from patients with systemic lupus erythematosus having the anti-phospholipid antibody syndrome were found to contain anti-plasminogen antibodies of the IgG class. The titers of anti-plasminogen autoantibodies of the IgG class were elevated in these patients compared with normal controls. Part of the pool of IgG anti-plasminogen antibodies reacts with an epitope in the lysine-binding sites of plasminogen. Anti-plasminogen IgG isolated from patients' blood plasma is specific only for a native epitope of human plasminogen passively adsorbed on immunosorbent micro-titration plate. As shown by enzyme immunoassay, autoantibodies to plasminogen of the IgG class cross-react with human fibrinogen.

Key words: plasminogen, autoantibodies, IgG, systemic lupus erythematosus, anti-phospholipid antibody syndrome

Systemic lupus erythematosus (SLE) is generally considered to be a multisyndrome autoimmune disease as signified by the occurrence of pathogenic autoreactive autoantibodies [1]. Anti-phospholipid antibodies, which are strongly associated with SLE, have become an increasing focus of clinical study and laboratory research of SLE in recent years. Anti-phospholipid antibodies have been associated with thrombotic complications in patients with SLE (known as secondary anti-phospholipid syndrome) or without any underlying disease (primary anti-phospholipid syndrome). Anti-phospholipid syndrome (APS) is characterized by a variety of clinical phenomena, including arterial and venous thromboses, thrombocytopenia, and obstetric complications [2].

Thrombosis is a relatively common complication in patients with SLE and is strongly associated with the presence of anti-phospholipid antibodies [3].

The molecular mechanism involved in the pathogenesis of this prothrombotic state remains obscure. However, there is a plausible connection between the prevalence of thrombotic complications and elevated levels of autoantibodies targeted to human serum proteins combined with anionic phospholipids. Among them are β_2-glycoprotein I, prothrombin, annexin V, protein C, and protein S [4].

The assumption that impaired fibrinolysis could contribute to the hypercoagulable state in SLE patients have made an experimental validation of existence of autoantibodies directed to plasminogen [5], plasmin [6], and tissue-type plasminogen activator [7] in plasma of such patients possible.

Plasminogen is a key component of fibrinolysis. Based on the structural similarities between some domains in serum proteins and plasminogen the latter is considered to be an autoantigen in patients with connective tissue diseases [8]. For this reason we isolated anti-plasminogen autoantibodies from sera of patients with SLE and APS to investigate of their binding specificity. This report presents the results of the study.

Abbreviations: ELISA) enzyme-linked immunosorbent assay; PMSF) phenylmethylsulfonyl fluoride; SLE) systemic lupus erythematosus; APS) anti-phospholipid antibody syndrome.

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MATERIALS AND METHODS

Reagents. The following reagents were used in this work: Tris, glycine, PMSF, EDTA, BSA, ammonium persulfate, acrylamide, sodium citrate and phosphates, and ε-aminocaproic acid from Sigma (USA); CNBr-Sepharose 4B, lysine-Sepharose 4B, Sephacryl S300, and protein G-Sepharose 4B from Amersham (USA). Other reagents were domestic products of chemical purity grade.

Patients. Eleven patients (females) with SLE seen in the Institute of Rheumatology were selected for this study based on the presence of APS. Diagnosis of SLE was made according to the revised American Rheumatism Association criteria [9]. Their median age was 41 years (range, 32 to 50 years). All patients had histories of vascular occlusions, including deep venous thrombosis (n = 7) and stroke (n = 4). This was evaluated as features of APS, defined as secondary APS according to Wilson et al. [10].

Purification of plasminogen. Blood samples of seven healthy donors were collected in plastic tubes containing 12 mM sodium citrate, 2 mM EDTA, 1 mM PMSF in 0.1 M sodium phosphate buffer, pH 7.4. The blood samples for the preparation of plasma were centrifuged at 3000g for 10 min at 4°C. Plasma of healthy donors (120 ml) was used as the source of plasminogen used in our experiments. Plasminogen was purified from human plasma by affinity chromatography on lysine-Sepharose 4B (Amersham, USA) as described by Deutsch and Mertz [11]. The eluted fractions were concentrated to 2.1 mg/ml on an Amicon (USA) cell equipped with a Diaflo YM-10 membrane and dialyzed against 10 mM sodium phosphate, pH 7.4.

To remove impurities the plasminogen preparation was then chromatographed on a column of rabbit antibodies to human serum albumin coupled to Sepharose 4B (Amersham) and further purified with protein G-Sepharose 4B (Amersham) according to a described procedure [12]. Protein concentrations were determined spectrophotometrically at 280 nm using a published absorption coefficient for plasminogen $A_{1\ cm}^{0.1\%} = 1.68$ [13].

Analysis of purity of plasminogen included SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5% resolving slab gel as described by Laemmli [14] and immunoelectrophoresis in 1% agarose gel as described by Grabar and Williams [15] with multispecific antiserum to human plasma proteins (Behringwerke AG, Germany) and specific rabbit anti-human albumin and IgG antibodies (Sigma). No contamination with fibrinogen, IgA, IgM, and C-reactive protein was detected in the plasminogen preparation by sandwich ELISA using specific rabbit anti-human antibodies (Sigma) to these proteins [16]. The purity of plasminogen estimated by the SDS-PAGE and by immunoelectrophoresis was at least 95%.

The presence of Lys- and Glu-plasminogen isoforms in plasminogen preparations was shown by immunoblotting with specific goat anti-human plasminogen antibodies (ICN, USA) [14, 17].

Plasminogen (10 mg) was coupled to CNBr-activated Sepharose 4B (2 g) according to the manufacturer's instruction. The effectiveness of plasminogen immobilization and protein nativity was examined by microimmunoadsorbent assay using specific goat anti-human plasminogen antibodies (ICN).

Isolation of anti-plasminogen autoantibodies from patients' blood plasma. Human IgG was isolated from blood plasma of SLE patients with APS (n = 11) by affinity chromatography on protein G-Sepharose 4B as described elsewhere [12]. The IgG preparation was concentrated to 6 mg/ml on an Amicon cell equipped with a Diaflo YM-10 membrane, then was extensively dialyzed versus PBS (0.15 M NaCl, 10 mM sodium phosphate, pH 7.4). The material obtained was subjected to gel filtration on the Sephacryl S300 column in a buffer listed above. The eluted fraction containing monomeric IgG was used for further analysis.

Protein concentrations were determined spectrophotometrically at 280 nm using the known absorption coefficient for IgG, $A_{1\ cm}^{0.1\%} = 1.47$.

Purity of isolated IgG was assessed by SDS-PAGE in 11% slab gel [14], immunoelectrophoresis with multispecific antiserum to human serum proteins (Behringwerke AG) [15], and ELISA using horseradish peroxidase conjugated to monoclonal anti-human IgG, IgA, or IgM antibodies (Sigma) [16].

Specific anti-plasminogen IgG autoantibodies were purified by immunoadsorption to plasminogen-Sepharose 4B resin [18], 5 ml of which was used to pack a chromatographic column.

To isolate anti-plasminogen autoantibodies, 100 mg IgG obtained before from plasma of SLE patients with APS were dissolved in 50 ml PBS, pH 7.4, containing 2 mM EDTA and 1 mM PMSF. The immunoadsorbent was first equilibrated with the same buffer and the resulting IgG solution was passed through at 4°C during 2 h. The immunoadsorbent was then extensively washed with PBS. When the absorbance of the effluent at 280 nm was 0.05 or less the proteins adsorbed were eluted at 4°C with 0.2 M glycine-HCl buffer, pH 2.7, containing 0.15 M NaCl. The protein concentration in the effluent was measured by the Bradford method [19].

The eluted protein was subjected to chromatography on a protein G-Sepharose 4B as the final purification step [12]. Isolated by this procedure anti-plasminogen autoantibodies were dialyzed and lyophilized.

The possibility that autoantibody IgG preparation was contaminated with plasminogen was tested in immunoelectrophoresis with specific goat anti-human plasminogen antibodies (ICN) [15].

Thus obtained IgG autoantibodies from eleven SLE patients with APS were pooled and subjected to further analysis.

Enzyme-linked immunosorbent assay. Binding specificity of anti-plasminogen IgG autoantibodies isolated from patients' blood plasma was evaluated by ELISA as described previously [5].

The wells of polystyrene plates (Titertek, Finland) were coated overnight at 4°C with 100 μl of 10 μg/ml solution of human plasminogen in 40 mM sodium carbonate, pH 8.3. After coating the plates were washed three times with PBS, pH 7.4, containing 0.05% Tween 20 (PBS-Tween) to remove unbound protein. Nonspecific sites were blocked by incubation with PBS-Tween containing 1% bovine serum albumin (Sigma) for 1 h at room temperature. For assays increasing concentrations of antibody preparations ($(0.05\text{-}6.7)\cdot10^{-7}$ M) in PBS-Tween or plasma samples dilutions (from 1 : 2 to 1 : 64) in PBS-Tween were added in duplicate in a 100 μl final volume and incubated for 2 h at 23°C. The plates were then washed three times with PBS-Tween and incubated with horseradish peroxidase conjugated to monoclonal anti-human IgG antibodies (0.5 μg/ml) (Sigma) for 1 h. The plates were then washed with PBS-Tween and 100 μl of substrate added to each well. The substrate was 2.5 mM *o*-phenylenediamine in 0.15 M phosphate/citrate buffer, pH 5.0. The enzyme reaction was terminated by addition of 50 μl of 2 M sulfuric acid to each well. The color development at 492 nm was followed in a Titertek Multiscan photometer (Flow Laboratories, Multiscan, Finland). Each sample was tested in duplicate.

The measured absorbance value is proportional to the concentration of plasminogen–autoantibody IgG–conjugate triple complex. Binding curves representing the optical density as a function of the concentration of IgG autoantibodies were obtained. Positive control was obtained by adding goat anti-human plasminogen antibodies (ICN). Negative control was obtained by incubation with monoclonal antibodies to γ-interferon (Sigma).

The inhibition of anti-plasminogen IgG binding to plasminogen by ε-aminocaproic acid or streptokinase was tested by adding isolated anti-plasminogen IgG autoantibodies ($(0.05\text{-}6.7)\cdot10^{-7}$ M) in PBS-Tween containing ε-aminocaproic acid (10 mM final concentration) to the wells of microtitration plates coated with immobilized plasminogen. Streptokinase (2 μM; Sigma) was added in buffer containing anti-plasminogen IgG in the presence of 1 mM PMSF and processed as above.

Cross-reactivity of the anti-plasminogen IgG autoantibodies with human thrombin (Boehringer Mannheim, Germany), fibrinogen (Boehringer Mannheim), C-reactive protein (Calbiochem, USA), serum albumin (Calbiochem) was tested using sandwich ELISA. Plates were coated with 100 μl solutions containing 10 μg/ml above-listed proteins in 40 mM sodium carbonate, pH 9.6, and incubated overnight at 4°C. Increasing concentrations of isolated anti-plasminogen IgG autoantibodies were added to the wells and processed as above.

SDS-PAGE and Western blot analysis. Anti-plasminogen IgG was separated in 11% polyacrylamide gel under reducing conditions using the discontinuous buffer system of Laemmli [14] and transferred to nitrocellulose (Bio-Rad Co., USA) as described previously by Towbin [17]. The nitrocellulose blot was reacted with anti-plasminogen autoantibodies (0.7 mg/ml) in PBS-Tween overnight at 4°C and reacted with peroxidase-labeled monoclonal anti-human IgG (0.1 mg/ml) (Sigma) in PBS-Tween.

Control of reaction with monospecific goat anti-human plasminogen antibody (ICN) reacted with peroxidase-labeled rabbit anti-goat (ICN) diluted 1 : 1000 was included.

RESULTS

Isolation of anti-plasminogen autoantibodies. Anti-plasminogen autoantibodies isolation and purification consists of two steps: isolation and immobilization of antigen (plasminogen) and anti-plasminogen autoantibodies purification from IgG obtained from plasma of SLE patients with APS.

Affinity purification of anti-plasminogen autoantibodies imposes requirements on immobilized antigen purity by reason of high cross-reactivity of the immunoglobulins from SLE patients' serum [20]. Affinity purification of plasminogen on Lys-Sepharose cannot rule out plasminogen contamination with other serum proteins. Additional use of immunoadsorbents to remove human serum albumin and IgG increases the plasminogen purity substantially.

By SDS-PAGE and immunoblotting it has been showed that plasminogen exhibited two closely spaced bands of the estimated mass 91 kD Glu-plasminogen and 89 kD Lys-plasminogen (Fig 1). Purity of the plasminogen preparation was confirmed by immunoelectrophoresis (Fig. 2).

The native form of immobilized plasminogen used as immunoadsorbent for autoantibody isolation is an important factor influencing its binding specificity. The use of polyclonal goat anti-human plasminogen antibodies in a microimmunoadsorbent assay provides a ready means of investigating binding capacity of immunoadsorbent. We observed that plasminogen-Sepharose 4B can bind about 1.8 mg goat anti-human plasminogen per ml of affinity resin.

Affinity chromatography on plasminogen-Sepharose 4B allows isolation of anti-plasminogen IgG autoantibodies from plasma of patients and healthy subjects. SLE patients' anti-plasminogen IgG levels were defined by affinity chromatography. The levels of the anti-plasminogen IgG autoantibodies in SLE patients with APS and healthy donors were found to be 1.06 ± 0.13 and 0.82 ± 0.12 mg per 100 mg serum IgG, respectively ($p < 0.05$).

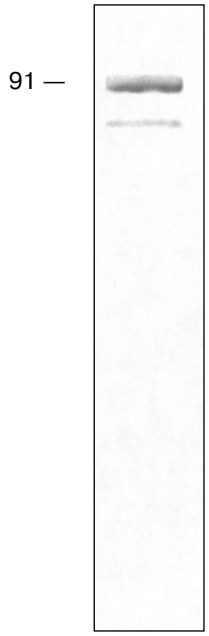

Fig. 1. Western blot analysis of Glu-, Lys-plasminogen preparation. Plasminogen (0.5 μg) was resolved by electrophoresis in 7.5% SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane. The blot was reacted with goat polyclonal antibody to human plasminogen followed by reaction with peroxidase-labeled rabbit anti-goat antibody. Molecular mass of protein is indicated in kD.

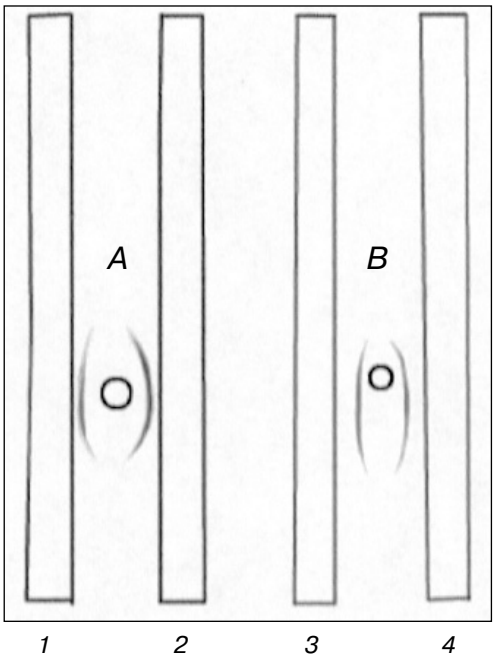

Fig. 2. Immunoelectrophoresis of plasminogen and anti-plasminogen IgG autoantibodies. The protein samples plasminogen (10 μg) (well A) and anti-plasminogen autoantibodies IgG (7 μg) (well B) were resolved in 1% agarose gel, pH 8.0. Multispecific antiserum to human plasma proteins (trough *1*) and goat anti-plasminogen antiserum (*2*) were reacted with plasminogen (well A) and antiserum to human immunoglobulins (*3*) and multispecific antiserum to human plasma proteins (*4*) were reacted with anti-plasminogen IgG autoantibodies (well B).

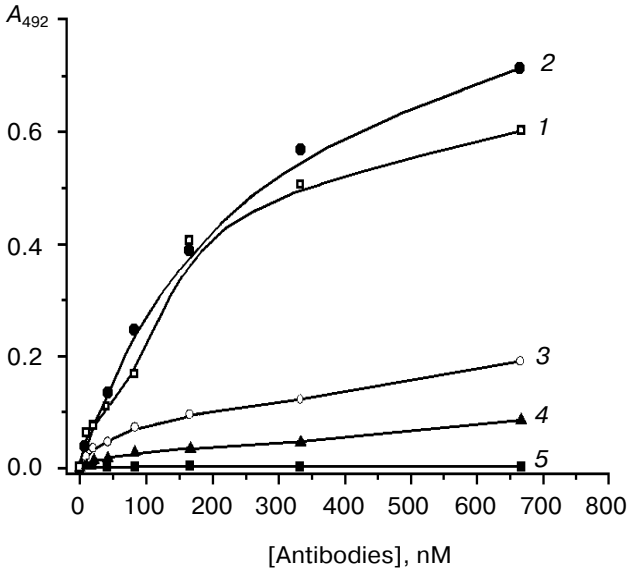

Fig. 3. ELISA of autoantibody IgG binding to plasminogen: *1*) anti-plasminogen IgG autoantibodies from SLE patients' plasma; *2*) monospecific goat anti-human plasminogen antibodies; *3*) IgG pool from SLE patients' plasma depleted of anti-plasminogen autoantibodies by passage over plasminogen-Sepharose 4B; *4*) monoclonal antibodies to human IFN-γ; *5*) monoclonal antibodies to human IgG.

Binding specificity of anti-plasminogen autoantibodies. The anti-plasminogen autoantibodies were characterized for binding specificity by ELISA. A curve representing binding of plasminogen to anti-plasminogen autoantibodies from SLE/APS patients' serum is shown (Fig. 3). A similar curve was obtained with polyclonal antibody to plasminogen. A significant reduction in binding (more than 80%) was obtained in the presence of starting IgG pool from patients' serum depleted of anti-plasminogen autoantibodies by passage over plasminogen-Sepharose 4B (Fig. 3). When the anti-plasminogen autoantibody preparation was replaced by monoclonal antibodies to IFN-γ, binding was not concentration dependent and had negligible absorbance values (less 9% the same amount of anti-plasminogen IgG).

On immunoelectrophoresis of plasminogen with Coomassie blue staining no precipitin bands with anti-Pg autoantibodies were observed. No reactivity was detected with denatured and reduced plasminogen and with plasmin heavy and light chains as determined by Western blot analysis (Fig. 4).

Effect of specific inhibitors on the binding of autoantibodies to plasminogen. The influence of 10 mM ε-aminocaproic acid on the binding of anti-plasminogen IgG to plasminogen is shown (Fig. 5). The ε-

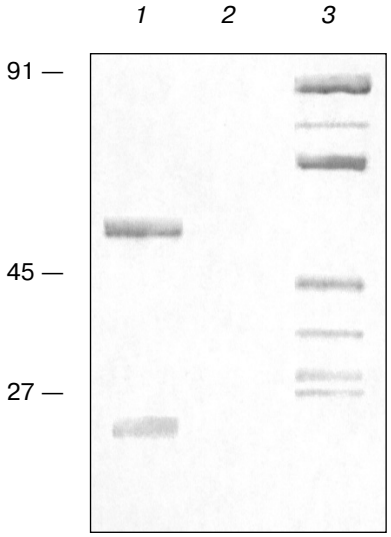

Fig. 4. Western blot analysis of the interaction of human plasminogen and anti-plasminogen autoantibody IgG. Autoantibodies IgG (0.6 μg) (*1*) and plasminogen (0.5 μg) (*2*, *3*) were resolved by electrophoresis in 8% SDS-PAGE under reducing conditions and electroblotted to nitrocellulose paper. Lanes: *1*) nitrocellulose paper incubated with peroxidase labeled anti-human IgG monoclonal antibody; *2*) nitrocellulose paper incubated with anti-plasminogen IgG autoantibodies followed by reaction with peroxidase labeled anti-human IgG monoclonal antibody; *3*) nitrocellulose paper incubated with goat anti-human plasminogen antiserum followed by reaction with peroxidase labeled rabbit anti-goat antibody. Molecular mass of proteins is indicated in kD.

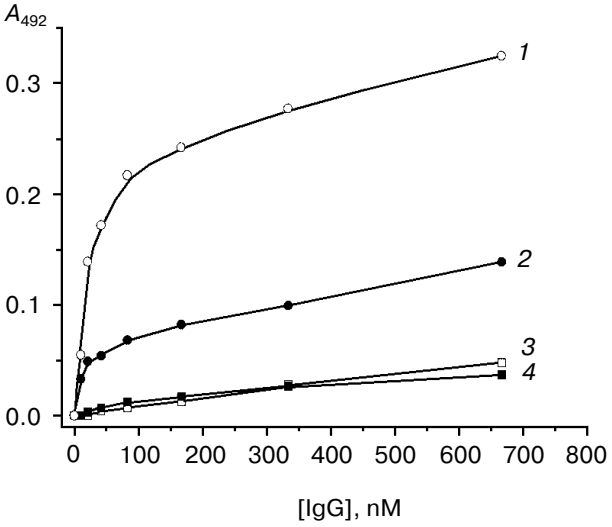

Fig. 5. Binding of anti-plasminogen IgG autoantibodies to plasminogen in the presence of 10 mM ε-aminocaproic acid: *1*) anti-plasminogen IgG autoantibodies from SLE patients' plasma; *2*) anti-plasminogen IgG autoantibodies from SLE patients' plasma in the presence of 10 mM ε-aminocaproic acid; *3*) IgG pool from SLE patients' plasma depleted of anti-plasminogen autoantibodies by passage over plasminogen-Sepharose; *4*) IgG pool from SLE patients' plasma depleted of anti-plasminogen autoantibodies by passage over plasminogen-Sepharose in the presence of 10 mM ε-aminocaproic acid.

aminocaproic acid reduced the binding by 67%, but it did not cause complete inhibition. With starting IgG pool from SLE patients' sera depleted of anti-plasminogen autoantibodies, ε-aminocaproic acid had no effect on antibody binding.

When streptokinase at final concentration 2 μM was incubated in the assay the reactivity of anti-plasminogen IgG with immobilized plasminogen was not inhibited (Fig. 6).

Cross-reactivity of anti-plasminogen IgG autoantibodies with other human serum proteins. The binding curves of anti-plasminogen autoantibodies to several other human serum proteins are shown (Fig. 7). When compared to plasminogen, only human fibrinogen and to a lesser extent human thrombin showed capacity to bind to these antibodies.

DISCUSSION

Stefanescu et al. were the first to investigate sera of patients with one of the connective tissue diseases, rheumatoid arthritis, for the presence of anti-plasminogen autoantibodies [6]. Sera from 47.3% of patients with rheumatoid arthritis and 4% of healthy donors were found to contain anti-plasminogen autoantibodies.

Binding of anti-plasminogen autoantibodies to plasminogen was demonstrated by Gonzalez-Gronow et al. [5]. IgG and IgA anti-plasminogen autoantibod-

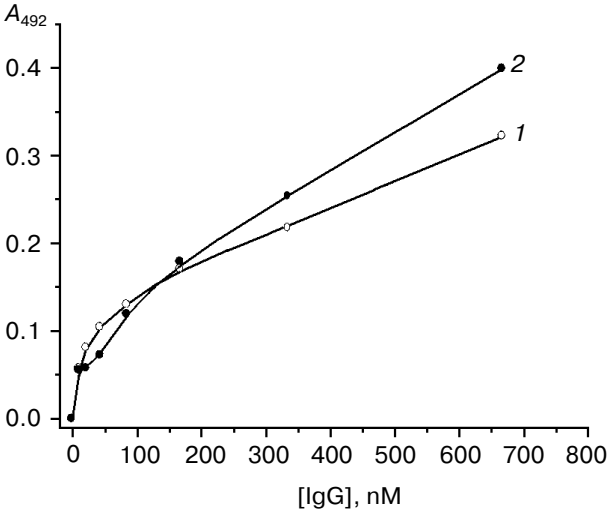

Fig. 6. Binding of plasminogen to anti-plasminogen IgG autoantibodies from SLE patients' plasma in the absence (*1*) and presence of 2 mM streptokinase (*2*).

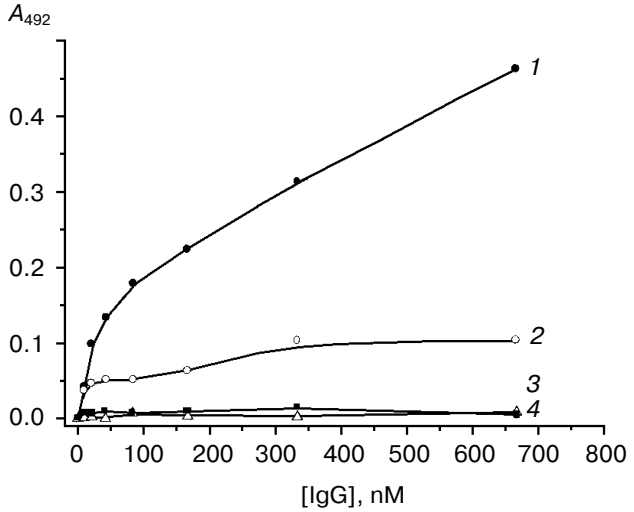

Fig. 7. Binding of anti-plasminogen IgG autoantibodies to human serum proteins: *1*) human fibrinogen; *2*) human thrombin; *3*) human serum albumin; *4*) human C-reactive protein.

ies from rheumatoid arthritis, SLE, and systemic sclerosis patients were investigated in that study. The presence of anti-plasminogen autoantibodies in sera from SLE and rheumatoid arthritis patients and some healthy donors was demonstrated by ELISA assay using plasminogen-coated plates and by Western blot analysis. Analyses of the specificity of these autoantibodies demonstrated that the anti-plasminogen IgG autoantibodies reacted with both whole plasminogen and plasmin light chains, located in the C-terminal region corresponding to the catalytic domain of plasmin [5]. Using Western blot analysis it was found that anti-plasminogen IgA autoantibodies were directed against continuous epitopes of plasminogen, plasmin heavy and light chains.

The titers of serum anti-plasminogen autoantibodies quantified by ELISA were elevated in 28% of rheumatoid arthritis patients and in 13% of primary APS patients compared with healthy donors [21].

We found that anti-plasminogen IgG autoantibodies were present in sera of all SLE patients with APS (11 of 11). Anti-plasminogen autoantibody levels in SLE with APS quantified by column affinity chromatography were greater than the background seen in normal.

A considerable reduction in binding of anti-plasminogen autoantibodies to plasminogen was shown in the presence of 10 mM ε-aminocaproic acid. These results are similar to the previous work which demonstrated that ε-aminocaproic acid reduced the binding of anti-plasminogen autoantibodies to plasminogen by 79% [22]. It was shown that the L-lysine analog, ε-aminocaproic acid, binds to plasminogen [23] and competitively inhibits the interaction of plasminogen with polyclonal antibodies or monoclonal anti-plasminogen antibodies, which bind to conformational epitopes located in the lysine-binding domain of plasminogen [24, 25].

The results obtained suggest that at least some of the isolated anti-plasminogen autoantibodies react with an epitope located in the lysine-binding domain of plasminogen. It should be noted that 10 mM ε-aminocaproic acid did not completely displace anti-plasminogen autoantibodies from plasminogen. These findings suggest that there are other autoantibodies in the population of anti-plasminogen IgG that react with epitopes located elsewhere on plasminogen.

Our findings that streptokinase poorly inhibits the binding of anti-plasminogen IgG autoantibodies to plasminogen are in accordance with the results of previous study [5]. This is evidence that streptokinase binding sites on the C-terminal region of plasminogen corresponding to the catalytic domain of the molecule do not overlap with those of anti-plasminogen autoantibodies.

It was previously reported that anti-plasminogen autoantibodies cross-react with both lactate dehydrogenase isoenzyme M [5] and human prothrombin [26]. We found that anti-plasminogen IgG autoantibodies from SLE patient sera reacted also with human fibrinogen (Fig. 5). The structural basis of such cross-reactivity is poorly understood.

We could not detect binding of anti-plasminogen IgG autoantibodies to reduced plasminogen by Western blot analysis. Autoantibody recognition of prothrombin, tissue-type plasminogen activator, and factor XII also requires the presence of native epitopes of these molecules [7, 27, 28]. Binding specificity of the autoantibodies with the just listed proteins was examined using ELISA.

The exact mechanism of the development of anti-plasminogen autoantibodies is not apparent. A couple of possibilities have been considered. One possibility is that under uncertain circumstances the conformation of plasminogen alters and that makes it immunogenic [8]. In connective tissue diseases it could lead to autoantibody formation to plasminogen. The second possibility is that it involves common epitope recognition of prothrombin and plasminogen. These autoantibodies react with prothrombin (anti-prothrombin antibodies) and cross-react with plasminogen [26].

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