ANTIBODIES TO TISSUE-TYPE PLASMINOGEN ACTIVATOR (tPA) IN PATIENTS WITH ANTIPHOSPHOLIPID SYNDROME. EVIDENCE OF INTERACTION BETWEEN THE ANTIBODIES AND THE CATALYTIC DOMAIN OF tPA IN TWO PATIENTS

Anti-tPA antibodies in antiphospholipid syndrome

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ABSTRACT

The causes of thrombosis and pregnancy loss in antiphospholipid syndrome (APS) are still unknown, although several hypotheses have been proposed and hypofibrinolysis has been implicated. Anti-tissue-type plasminogen activator (tPA) antibodies may induce fibrinolytic defects and preliminary data indicate an association with thrombosis in APS. We measured plasma anti-tPA antibody levels in 91 consecutive APS patients, 91 normal controls, 40 patients with antiphospholipid antibodies without APS symptoms and 23 patients with systemic lupus erythematosus (SLE) without antiphospholipid antibodies and APS symptoms. APS patients had anti-tPA antibody levels higher than controls (p=0.0001), SLE patients (p=0.0001) and asymptomatic antiphospholipid patients (p=0.05). A subgroup of 53 patients had plasma levels of tPA antigen higher (p=0.0001) and tPA activity lower (p=0.05) than controls, with an inverse correlation (r=-0.454, p=0.003) between anti-tPA antibody levels and tPA activity and no correlation with tPA antigen. The two patients with the highest antibody levels had tPA activity below the normal range. Their antibodies were respectively IgG1 and IgG3; both recognized human tPA, recombinant tPA and the catalytic domain of tPA, but not β2-glycoprotein-I, prothrombin, or plasminogen. Our data indicate that anti-tPA antibodies specifically interacting with the catalytic domain of tPA can be found in patients with APS, representing a possible cause of hypofibrinolysis.

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INTRODUCTION

The antiphospholipid syndrome (APS) is characterized by the occurrence of events such as arterial, venous thrombosis and/or recurrent pregnancy loss, associated with positivity for lupus anticoagulant (LA) or anticardiolipin antibodies (aCL), or both. The syndrome may be primary, or secondary to autoimmune or other diseases. The etiopathogenesis of thrombosis in APS is still unknown, but a number of hypotheses have been proposed to explain the mechanisms by means of which antiphospholipid antibodies may promote thrombotic events. These include the activation of endothelial cells, oxidant-mediated vascular endothelium lesions, interference with or modulation of the phospholipid binding proteins regulating hemostasis, and mechanisms similar to heparin-induced thrombocytopenia. Reduced fibrinolytic activity has also been described in patients with APS, and may be responsible for thrombotic events, and antibodies directed against tissue-type plasminogen activator (tPA) might lead to a hypofibrinolytic state. Our previous studies have shown that an association between anti-tPA antibodies and thrombosis seems to be a peculiarity of APS because normal plasma anti-tPA antibody levels were found in 100 subjects with a history of deep vein thrombosis (DVT) without APS, but high levels in 3/39 patients with primary APS who had a history of thrombosis. Furthermore, the patients who had experienced a previous stroke had higher plasma IgG anti-tPA levels than the patients without a history of thrombosis.

The goal of this study was to measure the plasma levels of anti-tPA antibodies in 91 patients with primary or secondary APS who have developed a thrombotic event or recurrent pregnancy loss, in 91 normal controls, in 40 patients with antiphospholipid antibodies without history of APS symptoms and in 23 patients with systemic lupus erythematosus (SLE) without antiphospholipid antibodies and history of APS symptoms. We also evaluated the correlations between the presence of anti-tPA antibodies and the patients’ clinical and laboratory data, particularly the type and site of thrombosis and tPA activity and antigen. Finally, in two patients with high anti-tPA antibodies, we isolated the immunoglobulin G fraction and studied antibody interactions with the different types of tPA and its catalytic domain obtained by recombinant DNA technologies.
PATIENTS AND METHODS

Patients

We tested the plasma of 91 consecutive APS patients regularly attending three different Italian centers (Department of Hematology of Bergamo, Istituto Auxologico Italiano and Hemophilia and Thrombosis Center of Milano). Twenty-six were men and 65 women with a mean age of 47 years (range 19-79); 68 had primary disease according to the Sapporo criteria, and 23 secondary disease associated with an autoimmune disease (18 cases had systemic lupus erythematosus according to the American Rheumatism Association [ARA] criteria; two lupus-like syndrome; one rheumatoid arthritis; one Sjögren’s syndrome and one polymyalgia rheumatica). All the patients had a history of episodes of thrombosis or pregnancy loss according to the Sapporo criteria. Twenty-four patients had had previous arterial thrombosis, 48 venous thrombosis and 8 both. Deep venous thrombosis and ischemic stroke were respectively diagnosed by Doppler ultrasonography and computed tomography; the other cases by angiography. Eight of the patients with a history of thrombosis had also experienced a previous episode of pregnancy loss. The remaining 11 patients had never suffered from thrombosis but had a history of pregnancy loss (one or more unexplained deaths of a morphologically normal fetus beyond the 10th week of gestation or three or more unexplained consecutive spontaneous abortions before the 10th week of gestation). The time since the last episode of thrombosis ranged between 6 months and 5 years. The control group consisted of 91 age- and gender-matched healthy subjects (26 men and 65 women with a median age of 46 years; range 19-79 years). We also studied 40 patients with persistently elevated antiphospholipid antibody levels without history of thrombosis or pregnancy loss (11 men and 29 women; age range 21-72 years) and 23 patients with systemic lupus erythematosus (SLE) without antiphospholipid antibodies, history of thrombosis or pregnancy loss (all women, age range 20-65 years).

Blood samples were collected in the morning by clean puncture of an antecubital vein with minimal stasis using sodium citrate as anticoagulant. The anti-tPA antibody levels were measured in plasma obtained after centrifuging the blood samples at 2500 g for 20 minutes, and the plasma samples were
frozen in small aliquots and stored at -80°C until testing. Two patients with high antibody levels were further studied in order to characterize the antibodies: a 37-year-old woman with lupus-like syndrome and a previous history of three spontaneous pregnancy losses, and a 46-year-old woman with a previous ischemic stroke. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki, and all of the subjects gave their informed consent before participation.

**Diagnosis of lupus anticoagulants**

Lupus anticoagulants were diagnosed according to the revised criteria proposed by the Subcommittee for the Standardization of Lupus Anticoagulants using a battery of assays consisting of activated partial thromboplastin time (aPTT; Thrombofax, Ortho, Raritan, NJ, USA; or automated aPTT reagent; Organon Teknika, Durham, NC, USA), kaolin clotting time according to Exner et al., dilute Russel's viper venom time according to Thiagarajan et al., and colloidal silica clotting time at low and high bovine phospholipid concentrations (Ortho) according to Chantarangkul et al. In the case of patients on oral anticoagulants, the Textarin/Ecarin test was also used.

**Measurement of anticardiolipin antibodies**

IgG and IgM anticardiolipin antibodies were measured using ELISA "in house" techniques as described by Loizou et al. The results were expressed as GPL or MPL units according to Harris et al., with one unit being equivalent to 1 µg of affinity purified anticardiolipin antibodies/ml of plasma. Values of more than 21 GPL or MPL units were considered elevated.

**Assays for anti-tPA antibodies**

In order to detect anti-tPA antibodies, rtPA (Actilyse; Boehringer Ingelheim, Ingelheim, Germany) (10 µg/ml in phosphate buffered saline, PBS, pH 7.4) was coated overnight onto ELISA microtitration plates (Maxisorp; Nunc, Roskilde, Denmark). After three washes with 250 µl of 0.1% PBS Tween 20, the wells were coated with 250 µl of β2-GPI-free bovine albumin (Sigma Chemical, St Louis, Mo., USA, product number A3803) (10 mg/ml in PBS Tween 20) and then incubated for two hours at 37°C to avoid aspecific binding. After repeated washes, the plasma samples (starting from a 1:20 dilution in PBS containing 0.1% Tween 20) were placed into the coated wells and incubated for 45 minutes at
room temperature. The 1:20 dilution was chosen to avoid false positive and non-specific interference due to total immunoglobulin level. After repeated washes, the rtPA-bound immunoglobulins were identified by class-specific mouse monoclonal antibodies (50 µl diluted 1:1000 in washing buffer) (Sigma Chemical), which were in turn detected by means of peroxidase-conjugated anti-mouse Ig antibodies (50 µl diluted 1:2000 in washing buffer) (Sigma Chemical). The bound peroxidase was revealed by ortho-phenylene-diamine (OPD, 0.5 mg/ml in citrated buffer containing H₂O₂), and absorbance (OD) was read at 492 nm. The results were expressed as U/ml, referred to an internal standard (plasma collected from a patient with a high anti-tPA antibody titre) arbitrarily fixed at 100 U/ml. This reference plasma was stored in aliquots at -80°C. The standard curve was linear until 0.78 U/ml (r=0.999) and the same linearity was obtained in plasma samples from patients and controls. Within-assay and between-assay coefficients of variation were lower than 15%.

Characterization of anti-tPA antibodies

The immunoassay was identical to that described above except for the fact that the first antibody was an immunoglobulin anti-human immunoglobulin with subclass specificity for IgG1, IgG2, IgG3 or IgG4 (Sigma Chemical Company St. Louis, Mo., USA).

IgG fraction isolation

The plasma of each patient (2 ml) was added to protein G column (Immunopure Immobilized Protein G, Pierce, Rockford, IL, USA) capable of binding immunoglobulin of the IgG class and equilibrated in PBS, pH 7.4. After washing with 40 ml of PBS, the immunoglobulin fractions specifically bound to protein G were eluted in glycine buffer 0.1 M at pH 2.5, and 66 µl of Tris buffer (pH 9.5) was added to every eluted milliliter. After repeated washing of the column, sodium azide was added to avoid the growth of moulds. The eluted fractions were collected, concentrated in Minicon B 15 (Amicon, Beverly, MA, USA), and stored at -80°C until testing. Protein recovery was evaluated by measuring absorbance at 280 nm. The recovery of anti-tPA antibodies was evaluated by ELISA and ranged from 50% to 75%. The same method was applied to the plasma of a healthy individual taken as control.
Production of the catalytic domain of tPA.

The recombinant molecule consisting of the catalytic domain of the enzyme was obtained by inserting the cDNA coding for the catalytic domain and the first three N-terminal residuals of human tPA, tPA del (Val 14-Cys 261), in the vector pET11c, which was then transfected into E. coli cells. It has been demonstrated that the molecule expressed in this way has the same activity as whole tPA in a dog model of coronary thrombosis.\textsuperscript{17}

Evaluation of purified IgG binding to different tPA forms immobilized on microplates.

The microplates (Maxisorp, Nunc; Roskilde, Denmark) were coated overnight (4°C) with 50 µl of rtPA (rtPA, Actilyse Boehringer Ingelheim Gmbh, Ingelheim, Germany), or tPA obtained from human melanoma cells (mtPA, single chain tPA, Biopool Sweden), or the recombinant molecule constituted by the catalytic domain of tPA at a 10 µg/ml concentration in PBS, pH 7.4. After three washes with 250 µl of 0.1% PBS Tween 20, the wells were coated with 250 µl of bovine albumin (10 mg/ml in PBS Tween 20) and then incubated for two hours at 37°C. After repeated washes with PBS Tween 20, increasing amounts of IgG purified from the plasma of the patients and the normal control were placed in the wells starting from a concentration of 16 µg/ml to 250 µg/ml and then incubated for 45 minutes at room temperature. The immunoglobulins specifically bound to the tPA forms were detected by means of ELISA. The absorbance (OD) was read at 492 nm.

Interactions between the anti-tPA antibodies and the different tPA forms in fluid phase.

After the patients’ plasma had been incubated for one hour at 37°C with increasing concentrations of rtPA, human tPA or the catalytic domain of tPA (0-0.5 mg/ml), we evaluated the free anti-tPA antibodies still capable of binding to the microplate-immobilised rtPA (ELISA).

Interactions between the anti-tPA antibodies and beta2-glycoprotein I (β2-GP I), prothrombin or plasminogen in fluid phase.

After the patients’ plasma had been incubated for one hour at 37°C with increasing concentrations of β2-GP I (0-9 mg/ml; a kind gift of Dr EM Bevers, Cardiovascular Research Institute Maastricht, The Netherlands), prothrombin (0-4.5 mg/ml; Diagnostica Stago, Asnieres, France) or plasminogen (0-5
mg/ml, Hyphen BioMed, Andresy, France), we evaluated the free anti-tPA antibodies still capable of binding to the microplate-immobilised rtPA (ELISA).

**Plasma levels of tPA antigen** were measured by means of a commercial immunoenzymatic method (Imulyse tPA kit, Biopool, Umea, Sweden).

**Plasma activity of tPA** was measured using a chromogenic method (Spectrolyse (fibrin) tPA assay kit, American Diagnostica, New York, NY).

**Plasma PAI-1 activity** was measured using a commercial immunoassay (Chromolize PAI-1, Biopool, Umea, Sweden).

**Statistical analysis**

As the tPA and anti-tPA data were skewed, they were log-transformed before analysis. The results are reported as antilog values of means with SD. The data were statistically analyzed using Student's test for unpaired values. The significance level was set at P<0.05. Spearman’s correlation coefficient was calculated to assess relationships between the variables. The associations between abnormally high anti-tPA antibody levels and APS, thrombosis or pregnancy loss and primary or secondary syndrome were evaluated by logistic regression. Odds ratios and 95 percent confidence intervals were reported.
RESULTS

The anti-tPA antibody levels of the APS patients (geometric mean values ± SD 6.7±2.1) were significantly higher (p=0.0001) than those of normal controls (3.5±2.5 U/ml) and those of patients with SLE without APS symptoms (4.1±1.7 U/ml). There was no significant difference between normal controls and patients with SLE without APS symptoms and between patients with primary (6.9±1.9 U/ml) or secondary syndrome (5.8±2.6 U/ml). Anti-tPA levels in asymptomatic patients with antiphospholipid antibodies (5.24±1.84 U/ml) were slightly lower than in symptomatic APS patients (p=0.05) and higher than in normal controls (p=0.01). Three of them were above the 95th percentile of the controls. Fourteen of the APS patients had anti-tPA antibody levels above the 95th percentile of the controls (Figure 1). In patients with APS, the association with abnormally high anti-tPA antibodies was higher than in normal controls (odds ratio 3.955 [95% C.I. 1.249-12.523]) (p=0.01) and tended to be higher than in asymptomatic patients with antiphospholipid antibodies (odds ratio 2.242 [95% CI 0.607-8.287]) (p=0.171).

Of the 91 patients, eight had anticardiolipin antibodies alone, 24 lupus anticoagulant alone, and 59 both. There was no relationship between anti-tPA antibody levels and patient age or gender, anticardiolipin antibody levels or the presence of lupus anticoagulants. No significant differences in anti-tPA levels were observed among patients with venous thrombosis (6.38±1.99), arterial thrombosis (7.87±1.97) or a history of pregnancy loss (6.25±3.41) but all the groups had levels significantly higher than normal controls. Patients with arterial thrombosis and patients with venous thrombosis were not significantly different in the association with abnormally high anti-tPA antibodies (odds ratio 1.171 [95% C.I. 0.307-4.473]) and no difference was found between patients with thrombosis and patients with pregnancy loss (odds ratio 0.794 [95% C.I. 0.152-4.137]), primary and secondary syndrome (odds ratio 0.777 [95% C.I. 0.197-3.073]) or male and female patients (odds ratio 1.562 [95% C.I. 0.398-6.126]).

Due to limited sample supply we could measure tPA antigen and activity only in a subgroup of 53 APS patients (10 men and 43 women; age range 19-78 years; 40 primary and 13 secondary). In these patients
we found plasma levels of tPA antigen (9.8±1.6 ng/ml) significantly higher than in 53 of the 91 normal subjects, age and gender matched with the patients (6.4±1.3 ng/ml) (p=0.0001). Plasma levels of tPA activity were lower in patients (0.51±1.71 IU/ml) than in normal controls (0.66±1.40 IU/ml) (p=0.05) and were even lower in the nine patients who had anti-tPA levels above the 95 percentile of normal subjects (0.42±2.00 IU/ml) (p=0.01). The two female patients with the highest anti-tPA antibody levels (130 U/ml and 100 U/ml) had plasma levels of tPA activity below the normal range (0.12 and 0.10 IU/ml respectively; normal range 0.20-2.00 IU/ml). An inverse correlation was found between anti tPA plasma levels and tPA activity (r=-0.454, p=0.003). No significant correlation was found between plasma levels of tPA antigen and anti tPA antibodies or tPA activity.

The two patients with the highest anti-tPA antibody levels underwent further studies: their main clinical and laboratory data are shown in Table 1. The IgG fractions were isolated from their plasma. The anti-tPA antibodies were IgG1 in one and IgG3 in the other (Figure 2), whereas the antiprothrombin antibodies and anti-β2-GPI antibodies were IgG2 in both, as demonstrated by the IgG binding to immobilized β2-GPI and prothrombin. The IgGs of the two patients bound to recombinant tPA, the catalytic domain of the tPA molecule, and to human tPA immobilized on microplates in a concentration-dependent manner. The immunoglobulins isolated from a normal subject did not bind to rtPA, the tPA catalytic domain or human tPA (Figure 3). When added to the patients’ plasma at concentrations of up to 0.5 mg/ml, both rtPA and the catalytic domain of tPA inhibited the binding of anti-tPA antibodies to the recombinant tPA immobilized on microplates in a concentration-dependent manner, but the addition of prothrombin (at concentrations of up to 50 times higher than physiological level), β2-GPI (at concentrations of up to 45 times higher than physiological levels and 50 times higher than those used in vitro to obtain an inhibition of anti-β2-GPI activity in fluid phase18) or plasminogen (at concentrations of up to 25 times higher than physiological levels) did not lead to any such inhibition (Figure 4). The isolated immunoglobulins from the two patients were tested for in vitro inhibition of tPA activity by adding increasing amount of immunoglobulins to a normal human plasma and measuring tPA activity.
after 1 hour incubation at 37°C (Figure 5). We observed a slight inhibition of tPA activity with both preparations. Immunoglobulins purified from a normal subject served as negative control and goat anti-tPA antibodies (American Diagnostica, Greenwich, CT, USA) served as positive control.
DISCUSSION

Previous studies have demonstrated that the plasma of patients with antiphospholipid antibodies also contain antibodies directed against different plasma proteins involved in hemostasis, such as prothrombin, protein C, protein S, factor XII and annexin V. Antibodies interfering with the "plasminogen/tissue-type plasminogen activator" system have also been described in patients with APS. In a previous study of 39 patients with antiphospholipid antibodies, we found that three had antibodies against tPA and four had antibodies against fibrin-bound tPA; all the patients with antibodies against tPA had a history of thrombosis. In this study, we analyzed a larger group of patients with APS who had had thrombotic events or recurrent pregnancy loss: their plasma levels of anti-tPA antibody were significantly higher than those of normal controls that showed a range of antibody levels. This is not surprising because it has been demonstrated that naturally occurring autoantibodies can also be found in normal subjects, thus tPA can act as described for other autoantigens. APS patients had plasma levels of anti-tPA antibody significantly higher than those of patients with antiphospholipid antibodies without history of APS symptoms and SLE patients without antiphospholipid antibodies or history of APS symptoms. No difference was found between primary and secondary syndrome which indicates that anti-tPA antibodies can be found in APS patients regardless of any associated autoimmune disease. This is further supported by the observation of normal levels of anti-tPA antibodies in SLE without APS. The presence of anti-tPA antibodies in patients with SLE is associated with a higher frequency of severe Raynaud phenomenon and thrombotic events and, among patients with primary APS, the highest antibody levels are found in those with a history of ischemic stroke. In this study, anti-tPA antibody levels were similarly high in the patients with a history of vein or arterial thrombosis or recurrent pregnancy loss, although we have no information on their antibody levels at the time of the clinical event or soon afterwards. No age-related difference in anti-tPA antibody levels was found, although it is known that anticardiolipin antibody levels increase with age. No differences were observed between men and women.
Antiphospholipid antibodies are heterogeneous in relation to their antigen specificity, and previous studies showed a strict correlation between anticardiolipin antibodies and anti-β2-GPI antibodies, and cross reactivity between anti prothrombin and anti plasminogen antibodies. Thus we looked for a possible cross-reactivity between anti-tPA and antiphospholipid antibodies. In our group of patients no correlation was found between the plasma levels of anticardiolipin and anti-tPA antibodies, suggesting that they are not the same. In the two patients with the highest anti-tPA antibody levels, the antibody subclasses were IgG1 and IgG3, whereas we found that their antiphospholipid antibodies (anti-β2-GPI and anti-prothrombin) were IgG2. Studies of patients with SLE and primary APS have also confirmed that the majority of anti-β2-GPI antibodies belong to the IgG2 subclass. These data show that anti-tPA antibodies are different from antiphospholipid antibodies antibodies and, being IgG1 and IgG3, are probably T lymphocyte-dependent and activate the classical complement pathway. Recent studies support an association between the inflammatory state and the clinical manifestations of APS with mechanisms involving complement and oxidative processes related to inflammation. Complement activation is also important in the fetal loss caused by antiphospholipid antibodies, as shown in an animal model. The absence of inhibition of anti-tPA binding to tPA immobilized on microplates after the addition of prothrombin and β2-GPI in fluid phase confirms the absence of cross-reactivity between anti-tPA and antiphospholipid antibodies. However, when a rtPA solution is added under the same conditions, the binding of anti-tPA antibodies to the tPA molecule is remarkably inhibited, thus demonstrating that anti-tPA antibodies bind to the tPA molecule that is not modified by contact with the well (i.e. a condition more similar to the in vivo situation). Patients’ immunoglobulins can also bind the tPA obtained from human melanoma cells and immobilized on microplates. The recombinant tPA molecule produced from hamster ovarian cells, starting with cDNA derived from the mRNA of human melanoma, has a glycosylation pattern similar but not identical to that of endogenous tPA. In contrast, melanoma tPA is identical to the endogenous molecule, and so the anti-tPA antibodies can also react with human tPA. Both tPA and prothrombin molecules have two "kringle" domains in their
secondary structure. The fact that anti-tPA antibodies do not interact with prothrombin suggests that they are not directed against the kringle domains, the only parts that these two molecules share.

The catalytic domain of the tPA molecule is completely different from the kringle domains and is located on the C-terminal side: for this reason, we evaluated the interactions between anti-tPA antibodies and the active site of the enzyme using a recombinant molecule obtained by inserting a cDNA coding for the catalytic domain and the first three N-terminal residues of human tPA in a vector. The anti-tPA IgGs of the two patients investigated interacted with the microplate-immobilised tPA catalytic domain in a dose-dependent manner. We excluded that IgG immunoglobulins bind specifically to the catalytic domain by observing that normal IgG did not interact with this domain.

When the tPA catalytic domain molecule was added to a solution containing the plasma of the two patients, we observed a dose-dependent inhibition of binding between anti-tPA antibodies and microplate-immobilised tPA, thereby showing that the antibodies are mainly directed against the catalytic domain. This result is relevant because it suggests that anti-tPA antibodies may potentially affect enzyme function. Recent studies have demonstrated that IgG purified from the plasma of APS patients can inhibit the degradation of fibrin clots. In our two patients, the plasma levels of tPA enzymatic activity were lower than in normal controls, with normal or slightly increased antigen levels and normal levels of the main tPA inhibitor (PAI-1). The other patients with abnormally high plasma levels of anti-tPA antibodies had lower levels of tPA activity than normal controls. Since a defect of tPA has been associated with thrombosis, APS patients with abnormally high levels of anti-tPA antibodies may have an additional risk for thrombosis. Thus hypofibrinolysis due to anti-t-PA antibodies could be added to the list of possible pathological processes responsible for clinical symptoms in APS. The increased levels of tPA antigen observed in our APS patients have been already found in a previous study and could be related to an activation of endothelial cells which represent a target for antiphospholipid antibodies. The slight inhibition of tPA activity by anti-tPA antibodies we observed in vitro, along with the fact that tPA activity is low in plasma of APS patients and inversely
correlates with anti-tPA levels, supports the hypothesis that anti-tPA antibodies may act in vivo as inhibitors of tPA function.

In conclusion, it is possible to find antibodies specifically recognising the tPA catalytic domain in the plasma of patients with APS. These antibodies may participate in the hypofibrinolytic state described in APS.
REFERENCES


TABLE 1
Main clinical and laboratory data of the two patients with the highest anti-tPA antibody levels.

<table>
<thead>
<tr>
<th></th>
<th>PATIENT V.A.</th>
<th>PATIENT L.P.</th>
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<tr>
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Normal ranges for anticardiolipin antibodies: GPL<21, MPL<21; anti-tPA antibodies: <11 U/ml; tPA antigen: 1.7-12.3 ng/ml; tPA activity: 0.20-2.00 IU/ml, PAI activity levels: 0.1-18.4 IU/ml. N.A.: not available.
FIGURE LEGENDS

Figure 1. Plasma levels of tissue-type plasminogen activator (tPA) antibodies in patients with antiphospholipid syndrome (APS) and controls.
Anti-tPA antibody levels are expressed in U/ml; the horizontal lines represent geometric means. The results show that 91 APS patients had anti-tPA antibody levels higher than 91 normal controls (p=0.0001), 23 patients with systemic lupus erythematosus (SLE) without antiphospholipid antibodies and APS symptoms (p=0.0001), and 40 patients with antiphospholipid antibodies without APS symptoms (p=0.05). Fourteen APS patients and three patients with antiphospholipid antibodies without APS symptoms had anti-tPA antibody levels higher than the 95th percentile of normal controls (indicated by the dashed line).

Figure 2. Subclass characterisation of anti-tPA immunoglobulins.
The immunoglobulins bound to immobilised tPA were revealed using mouse subclass-specific monoclonal antibodies against human immunoglobulins in an ELISA system. The results are expressed as absorbance at 492 nm (mean values ± SE of 3 experiments), and indicate that the anti-tPA antibodies of patient V.A. are IgG1 whereas those of patient L.P. are IgG3. No significant signal was obtained from the immunoglobulins of a normal control.

Figure 3. Binding of immunoglobulins purified from the plasma of two patients and a normal control to different tPA forms immobilised on microplates.
The binding of purified IgG to human tPA (top panel), recombinant tPA (middle panel) and the catalytic domain of tPA (bottom panel) was evaluated in patient V.A. (diamonds), patient L.P. (squares), and a normal control (triangles). The horizontal axis indicates the concentrations of purified immunoglobulins. Our results indicate that both patients have immunoglobulins directed against the different tPA forms; no significant binding was observed in the normal control.
Figure 4. Inhibition of IgG binding to insolubilised rtPA by soluble tPA forms, prothrombin, β2-glycoprotein I and plasminogen.

Plasma from patients V.A. and L.P. was incubated for one hour with rtPA or the catalytic domain of tPA at concentrations of 0.500 mg/ml, 0.250 mg/ml, 0.125 mg/ml, 0 mg/ml, with prothrombin up to a concentration of 4.500 mg/ml, with β2-glycoprotein I (Beta-2-GP-I) up to a concentration of 9.000 mg/ml or plasminogen up to a concentration of 5.000 mg/ml, and then tested using the solid phase immunoassay described in Methods.

The results are expressed as the percentage binding recorded in the absence of added soluble proteins (buffer). When added to the patients’ immunoglobulins, both rtPA and the catalytic domain of tPA inhibited the binding of anti-tPA antibodies to the recombinant tPA immobilised on microplates, but prothrombin, β2-glycoprotein I and plasminogen did not inhibit the binding.

Figure 5. Inhibition of plasma tPA activity by immunoglobulins purified from the plasma of two patients.

The tPA activity was measured in a normal human plasma after one hour incubation at 37°C with increasing amounts of immunoglobulins purified from the plasma of patients V.A. and L.P. Immunoglobulins purified from a normal subject served as negative control and goat anti-tPA antibodies served as positive control. Results are expressed as IU/ml of tPA activity.
Figure 1

Anti-tPA antibodies (U/mL)

APS patients  Normal controls  SLE without APS  Asymptomatic patients with antiphospholipid antibodies
Figure 3

OD

Anti-human tPA Ab

V.A.
L.P.
Normal

µg/ml

15.6 31.3 62.5 125 250

OD

Anti-rtPA Ab

V.A.
L.P.
Normal

µg/ml

15.6 31.3 62.5 125 250

OD

Anti-catalytic domain Ab

V.A.
L.P.
Normal

µg/ml

15.6 31.3 62.5 125 250
Figure 4

IgG binding to immobilised tPA
% of control

Buffer
- 0.125
- 0.250
- 0.500

rtPA
- 0.125
- 0.250
- 0.500

Cat domain
- 0.125
- 0.250
- 0.500

Prothrombin 4.500

Beta-2-GP-I 9.000

Plasminogen 5.000

% of control

Figure 4
Figure 5

Graph showing the effect of Immunoglobulins (mg/ml) on Normal plasma tPA activity (I.U./ml). The graph compares different samples:
- V.A.
- L.P.
- Normal
- Goat anti-tPA
Antibodies to tissue-type plasminogen activator (tPA) in patients with antiphospholipid syndrome: evidence of interaction between the antibodies and the catalytic domain of tPA in two patients

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