Autoantibodies to Receptor Induced Neoepitopes of Fibrinolytic Proteins in Rheumatic and Vascular Diseases

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ABSTRACT. ***Objective.*** Abnormal plasminogen activation has been implicated in vascular and rheumatic diseases. The development of an autoimmune response to neoepitopes of plasminogen and its activator (tissue-type plasminogen activator, t-PA) was explored in sera from patients with rheumatoid arthritis (RA, n = 30), Behçet's disease (n = 20), primary antiphospholipid syndrome (APS, n = 23), and idiopathic arterial (n = 33) or venous thrombosis (n = 16).

Methods. Sera diluted 1/50 were incubated with either plasminogen or t-PA bound to their natural receptors (immobilized fibrin or monocytic cells), and bound immunoglobulins were detected using a sheep peroxidase labeled anti-human Fab IgG. Controls included plates coated with fibrin or cells alone or plasminogen passively adsorbed to the plastic. Sera were considered positive when the absorbance at 405/490 nm was above the mean + 2 SD of normal sera.

Results. Reactivity of sera against plasminogen bound to cells (28%) or to fibrin (22%) was a predominant feature in patients with RA compared with other patient groups and controls. However, some patients with primary APS had reactivity against cell and fibrin bound plasminogen (9 and 13%, respectively). Autoantibodies against fibrin bound t-PA were detected in only 8% of patients with arterial or venous thrombosis.

Conclusion. Conformational changes induced by molecular assembly of plasminogen on cell or fibrin surfaces result in the expression of neoepitopes recognized by autoantibodies. These autoantibodies could be markers of the proteolytic events associated with plasminogen activation in autoimmune diseases. (J Rheumatol 2001;28:302–8)

Key Indexing Terms:
AUTOANTIBODIES   TISSUE-TYPE PLASMINOGEN ACTIVATOR
PLASMINOGEN   FIBRIN BOUND t-PA

Plasminogen activation is involved in the dissolution of fibrin clots and the removal of fibrin deposits in the vascular wall and in extravascular tissues such as the synovial membrane[1]. This system is also responsible for the activation of growth factors and metalloproteinases, thereby facilitating extracellular matrix remodeling or degradation and cell migration[2].

Plasminogen activation is triggered by the assembly of plasminogen and either tissue- or urokinase-type plasminogen activator (u-PA) on specific surface structures of fibrin and cell membranes, respectively. For instance, plasminogen interacts with carboxy-terminal lysine residues exposed on fibrin[3] and cell membrane proteins, such as annexin II and α-enolase, considered candidate plasminogen acceptors[4]. Tissue-type plasminogen activator (t-PA) binds to fibrin through interaction between its finger domain and a sequence in the fibrin D region of fibrinogen[5], whereas binding to endothelial cells requires a specific receptor[6]. Urokinase has no fibrin binding domain but binds to cell membranes through a glycosyl-phosphatidylinositol anchored specific receptor[7].

These ligand binding interactions induce conformational changes that are necessary for optimal activator/plasminogen recognition and plasmin formation[8]. It has been shown[9,10] that the compact spiral coiled structure of circu-

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lating plasminogen changes to an open form upon binding to C-terminal lysines of fibrin that facilitates its activation to plasmin[11]. These conformational changes of a molecule after binding to its acceptor or receptor could lead to exposure of new epitopes. Ligand and receptor induced neoepitopes have thus been recognized with the use of monoclonal antibodies[12]. Such neoepitopes may also evoke an autoimmune response in patients with immunological disorders[13-15].

We explored this possibility in patients with rheumatoid arthritis (RA), primary antiphospholipid syndrome (APS), and Behçet's disease (BD), and patients with no history of autoimmune disease who had recurrent thrombotic events (arterial thrombosis or deep venous thrombosis) in the absence of any congenital or acquired hemostatic defect. The monocytic cell line THP-1, to which plasminogen is known to bind[16], and a well characterized solid phase fibrin surface[3,17] were used to investigate in the sera of these patients the presence of antibodies to epitopes induced by binding of plasminogen or its activator to these surfaces.

MATERIALS AND METHODS

Patients. Serum samples obtained from 30 unselected patients with RA (age 51 ± 17 yrs) followed at the Department of Immunology and Rheumatology of the Hospital General de Occidente were studied. In addition, serum samples from patients with a history of repeated thrombotic events (BD, n = 20, age 42 ± 12 yrs; idiopathic arterial thrombosis, n = 33, age 49 ± 14 yrs; deep venous thrombosis, n = 16, age 44 ± 6 yrs) or with primary APS (n = 23, age 47 ± 6 yrs) selected from patients attending the Department of Internal Medicine of the Groupe Hospitalier Pitié-Salpêtrière were also studied. Control serum samples were obtained from 20 healthy adult volunteers. Antiphospholipid antibodies (aPL) were assessed in all subjects except controls using an ELISA (Phospholisa, Biomedical Diagnostics, Marne-la-Vallée, France) as described[18]. Detection of anticardiolipin antibodies (IgG, IgM) was performed with an ELISA (Reaads Medical Products, Westminster, CO, USA) and lupus anticoagulant using a sensitive APTT reagent (PTT LA, Diagnostica Stago, Asnières, France). All patients with arterial or venous thrombosis had normal coagulation and fibrinolytic studies, and possible causes of inherited/acquired thrombophilia including antigens of the protein C-S system, antithrombin III, activated protein C resistance, aPL, and hyperhomocysteinemia were eliminated.

Buffers. Buffer A was 0.05 mol/l sodium phosphate, pH 7.4, containing 0.08 mol/l NaCl, 0.01% Tween 20, and 0.01% thymerosal; assay buffer was buffer A containing 2 mg/ml of bovine serum albumin; binding buffer was buffer A containing 4% albumin. Phosphate buffered saline (PBS) was Na_2HPO_4 0.015 mol/l, KH_2PO_4 0.06 mol/l, pH 7.4, containing NaCl 0.14 mol/l.

Proteins. Native human plasminogen and fibrinogen were purified from fresh-frozen human plasma as described[19,20] with minor modifications[3]. The IgG fraction from normal sera was purified by protein A affinity chromatography. Human t-PA (> 95% single-chain) purified from Bowes melanoma cell conditioned medium on immobilized anti-t-PA monoclonal antibody was from Biopool (Upsala, Sweden); it had a specific activity of 680,000 IU/mg. Protein concentration was determined photometrically at a wavelength of 280 nm using E (1%, 1 cm) = 16.8 for plasminogen, 15.1 for fibrinogen, and 14.1 for IgG.

Preparation of fibrin surfaces. Fibrin surfaces were prepared as described[3]. Briefly, fibrinogen was covalently bound to polyvinyl chloride bound stable polyglutaraldehyde derivatives, transformed into a fibrin surface by treatment with thrombin, and treated with plasmin to expose carboxy-terminal lysine residues.

Cell culture and immobilization of THP-1 cells. The human monocytic cell line THP-1[21] was obtained from the American Type Culture Collection (Rockville, MD, USA) and grown in tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal calf serum, 4 mmol/l glutamine, 0.5 mmol/l sodium pyruvate, 0.5% nonessential amino acids, and 1% antibiotics (penicillin, streptomycin). Cells were collected, washed with PBS containing 1 mg/ml BSA, acid treated for 3 min with 50 mmol/l glycine-HCl, pH 3.0, containing 100 mmol/l NaCl, to elute surface bound endogenous u-PA, and immediately neutralized by addition of an equal volume of 50 mmol/l Hepes, pH 7.4, containing 100 mmol/l NaCl. The cells were then immobilized on microtitration plates as described[22]. Briefly, flexible polyvinyl chloride plates were first treated with 2.5% polyglutaraldehyde for 2 h at 22°C, followed by washing with distilled water and addition of 80,000 cells per well. After centrifugation at 230 g for 10 min the plates were incubated 1 h at 22°C, followed by an incubation of 10 min in a bath of 0.25% polyglutaraldehyde, washing with PBS, and addition of 100 µl per well of PBS containing 4 mg/ml BSA and 0.01% thymerosal. The plates were sealed and stored at 4°C until further use.

Binding of immunoglobulins to Fc receptors of THP-1 cells. Receptors for the Fc domain of immunoglobulins (Fcγ RI and RII) are constitutively expressed by THP-1 cells[23]; to exclude binding of IgG from patients' sera to this receptor, the following experiments were performed. The interaction of IgG with the Fc receptor was first determined by incubating increasing concentrations of purified human IgG in mass buffer with the immobilized THP-1 cells for 2 h at 37°C; bound IgG was detected with a sheep peroxidase labeled IgG directed against human Fab IgG. ABTS (Boehringer Mannheim, Mannheim, Germany) was used as a substrate for color development and the absorbance at a double wavelength (405/490 nm) ratio was determined. The variables of the binding, dissociation constant (K_d), and maximum bound (B_{max} = the concentration of IgG that saturates the binding sites) were calculated from the binding isotherm by fitting raw data to the Langmuir equation[24]. The concentration of protein A able to inhibit the binding of IgG was determined as follows. A fixed concentration of IgG was incubated with varying amounts of purified protein A (Sigma, St. Louis, MO, USA) in mass buffer for 1 h at 22°C. A volume of 50 µl of these solutions was then incubated on the immobilized THP-1 cells and the amount of bound IgG determined as above.

Detection of receptor induced neoepitopes. This assay was performed using immobilized THP-1 cells and fibrin surfaces. For plasminogen antibody detection, a 1 µM solution of plasminogen in mass buffer was incubated with these surfaces for 1 h at 37°C. This concentration of plasminogen saturates ~50% of lysine binding sites, as established from binding isotherms on fibrin (K_d = 1.1 ± 0.2 µM)[3] and cell surfaces (K_d = 0.9 ± 0.3 µM)[25]. Unbound plasminogen was discarded and the surfaces were washed with assay buffer. Sera dilutions (1:10, 1:50) in binding buffer containing 0.95 µmol/l of protein A were then added in triplicate. After 2 h incubation at 37°C, the plates were washed twice with assay buffer and bound antibodies were detected using a sheep peroxidase labeled antibody to human Fab IgG (Valbiotech, Paris, France) and ABTS as above. Parallel experiments were performed using fibrin and cell surfaces without adsorbed plasminogen. Serum samples were considered positive when the values obtained on fibrin bound and cell surface bound plasminogen were above the mean + 2 SD value obtained with sera from 20 healthy controls.

Detection of anti-t-PA antibodies was as described[15] with minor modifications. In this system, t-PA is first bound to fibrin by incubating a constant amount of the activator (5.25 nmol/l) for 1 h at 37°C. Unbound protein is removed by washing and the assay started by adding triplicated 50 µl/well of sera diluted 1:50 in mass buffer. After 2 h at 37°C, the plate is washed 3 times with assay buffer and bound immunoglobulins detected as above. Sera were considered positive when the absorbance measured on fibrin bound t-PA was 2 SD above the mean value obtained on solid phase fibrin alone.

RESULTS

Determination of antigen concentration for autoantibody detection. The specific binding of plasminogen to fibrin[3] and THP-1 cells[25], and of t-PA to fibrin[17], has been characterized. Variables of the binding constants B_{max} (the maximal amount bound) and K_d (the dissociation constant) were calculated applying the Langmuir equation to raw data and are summarized in Table 1. The concentrations of plasminogen and t-PA immobilized on fibrin and cell surfaces that are able to capture autoantibodies upon incubation of these surfaces with sera were determined from the respec-

Table 1. Binding variables and definition of antigen concentrations.

Antigen	Biological Surface	Dissociation Constant K_d, moles/l	Concentration Used for Antibody Detection
Plasminogen	THP-1 cells	1.3×10^{-6}	1 µmol/l
Plasminogen	Fibrin	0.9×10^{-6}	1 umol/l
t-PA	Fibrin	1.4×10^{-9}	5.25 nmol/l

tive binding isotherms. A concentration of plasminogen (1 µM) that saturates ~50% of lysine binding sites, i.e., similar to the K_d, provided a significant signal in the antibody assay, whereas for t-PA a concentration that approached saturation (5.25 nmol/l) was established.

Interaction of IgG with the Fcγ receptor and inhibition by protein A. Binding of human IgG to the Fcγ receptors expressed by THP-1 cells was analyzed. As shown in Figure 1, the binding of IgG was dose dependent and saturating. Fitting of raw data to the Langmuir equation provided a K_d value (0.08 µmol/l) within the same order of magnitude as reported[23]. The inset in Figure 1 shows that blocking of the Fc region of IgG with protein A inhibits its binding to the Fcγ receptor. Inhibition of 50% of the binding of a concentration of IgG close to the B_{max} (0.666 µmol/l) was obtained with 0.13 µmol/l of protein A. A concentration of 0.95 µmol/l of protein A inhibited most of the binding and was subsequently used in all experiments to eliminate reactivity through Fcγ receptor-IgG interaction in the autoantibody detection assay.

Detection of receptor induced epitopes. We investigated the appearance of new epitopes induced by binding of plas-
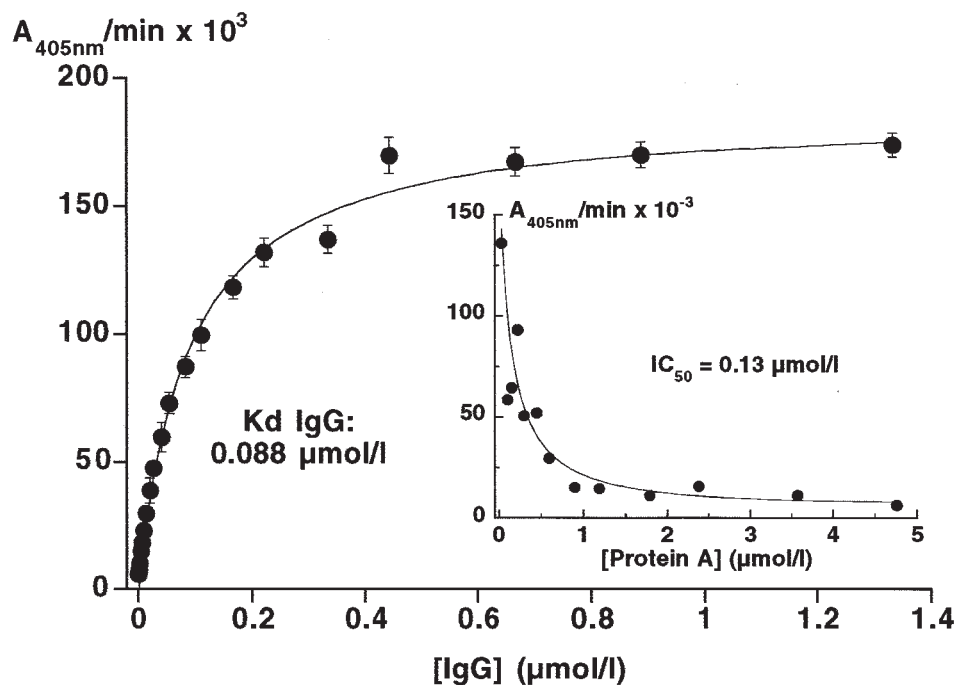

Figure 1. Isotherm of the binding of human IgG to THP-1 cells. Serial dilutions of purified human IgG were incubated with THP-1 cells and the amount bound was detected with a rabbit peroxidase labeled anti-Fab IgG fragment. The molar concentration of IgG is plotted against the amount bound, expressed in absorbance 405 nm/min; the dissociation constant (K_d) = 0.08 µmol/l was calculated by fitting raw data to the Langmuir equation. Data represent 3 separate experiments performed in triplicate. The inset represents inhibition by protein A of the binding of a concentration of IgG that approaches saturation (B_{max}). Serial dilutions of purified protein A (0 to 5 µmol/l) and 0.666 µmol/l of IgG were incubated with THP-1 cells for 2 h at 37°C and bound IgG was detected as described in Materials and Methods. A 0.13 µmol/l concentration of protein A decreased the amount of bound IgG by 50% (IC_{50}).

minogen to fibrin and membrane acceptor proteins of THP-1 cells. For this purpose, the reactivity of sera against immobilized cells or fibrin was evaluated in the absence and in the presence of bound plasminogen as described in Materials and Methods. Results obtained in the different groups of patients are summarized in Table 2. Results obtained with the solid phase fibrin–t-PA system are shown in Figure 2.

Among the 30 RA sera tested, 5 displayed a reactivity against the cell membrane (16.6%) and 3 others against fibrin (10%), suggesting interaction with an unidentified membrane antigen and fibrin, respectively. The 25 remaining sera negative against the immobilized cells were analyzed for reactivity against cell bound plasminogen; a positive result was detected in 7 (28%) of these sera (Figure

Table 2. Autoantibodies against proteins of the plasminogen activation system.

	Rheumatoid Arthritis, n = 30	Arterial Thrombosis, n = 33	Venous Thrombosis, n = 16	Behçet's Disease, n = 20	PAPS, n = 23
Fn	3	1	—	—	1
Fn-t-PA*	1	2	2	—	—
Fn-Pg*	6	—	1	—	3
THP-1	5	—	—	—	1
THP-1-Pg*	7	1	1	1	2

*Sera negative on fibrin, THP-1 cells, or plasminogen bound to plastic. Fn-t-PA: fibrin-bound t-PA, Fn-Pg: fibrin-bound plasminogen, THP-1: monocytic cell line, THP-1-Pg: cell-bound plasminogen, PAPS: primary antiphospholipid syndrome.
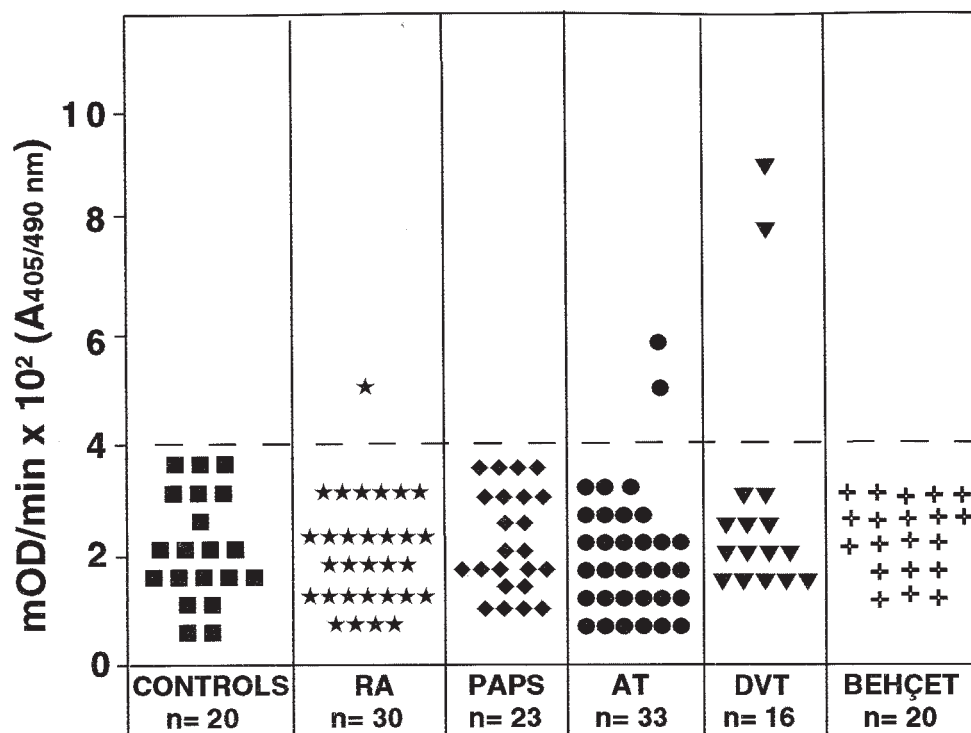

Figure 2. SOFIA t-PA analysis for different categories of human sera. An amount of 250 IU/ml of t-PA was incubated with the fibrin coated surface for 1 h at 37°C; unbound protein was discarded by washing, and sera diluted 1:50 were incubated for 2 h at 37°C. After 3 washes with assay buffer, bound immunoglobulins were detected as described in Materials and Methods. All sera were tested in duplicate in 3 different experiments. The broken line indicates the cutoff point, calculated as mean + 2 SD of values obtained with sera from 20 healthy subjects. AT: arterial thrombosis, DVT: deep vein thrombosis, PAPS: primary APS.
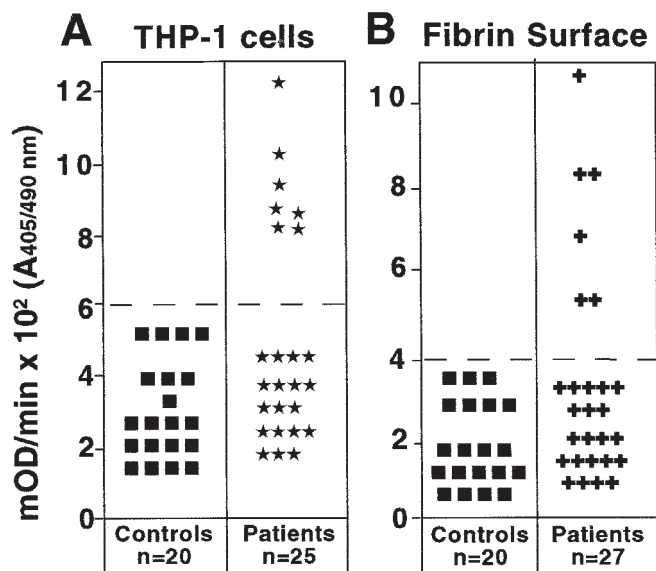
A THP-1 cells
B Fibrin Surface

Figure 3. Detection of receptor induced neoepitopes on cell and fibrin bound plasminogen in RA sera. Serum samples diluted (1:50) in mass buffer with 0.95 µmol/l protein A were incubated on cell and fibrin bound plasminogen for 2 h at 37°C. After washing 3 times with assay buffer, autoantibodies were detected with a sheep peroxidase labeled antibody against human Fab IgG fragment. The milli-OD/min values at 405/490 nm were determined after adding ABTS substrate. Broken line represents the cutoff point: mean + SD of 20 healthy controls. A. Cell bound plasminogen. B. Fibrin bound plasminogen.

Table 3. Clinical and serological features of RA patients with antibodies against fibrin and THP-1 cell bound plasminogen.

Features	Anti-THP-1-Pg (%)	Anti-Fn-Pg (%)
Patients	7/25 (28)	6/27 (22)
Mean age, yrs	56	49
Sex, F/M	7/0	5/1
Arthritis	7/7 (100)	6/6 (100)
Vasculitis	0/7	0/6
Rheumatoid nodule	2/7 (28.5)	1/6 (17)
Keratoconjunctivitis sicca	1/7 (14)	1/6 (17)
Raynaud's phenomenon	0/7	0/6
Thrombotic events	0/7	0/6
Rheumatoid factor	7/7 (100)	5/6 (83)
Antinuclear antibodies	0/7	0/6

Fn-Pg: fibrin bound plasminogen, THP-1-Pg: cell bound plasminogen.

3A). Among the 27 sera negative against fibrin, only one displayed reactivity against fibrin bound t-PA (3.7%), whereas 6 (22%) were positive against fibrin bound plasminogen (Figure 3B). Interestingly, the sera positive against fibrin or cell bound plasminogen did not show cross reactivity. Clinical and laboratory features in positive patients are summarized in Table 3. There was no significant correlation with antibody reactivity.

All sera from patients with arterial thrombosis (n = 33) except 2 were negative against cells or fibrin either in the presence or in the absence of plasminogen. Of the positive sera, one reacted against plasminogen bound to cells and another against fibrin. Two other sera were positive against fibrin bound t-PA.

Among the sera from patients with deep venous thrombosis (n = 16), one reacted against plasminogen bound to fibrin, 2 against fibrin bound t-PA, and one against plasminogen bound to cells, but none reacted against the cells or fibrin alone.

Sera from patients with BD (n = 20) were negative against fibrin in the presence and in the absence of plasminogen or t-PA; only one serum showed reactivity against cell bound plasminogen.

One of the sera from patients with the primary APS (n = 23) reacted against fibrin and 3 were positive against fibrin bound plasminogen, but none reacted against t-PA bound to fibrin. Reactivity against the cell membrane was detected in only one case, whereas 2 sera reacted against cell bound plasminogen.

DISCUSSION

We describe detection of autoantibodies directed against neoepitopes expressed by t-PA and plasminogen bound to fibrin and cells. Two assay methods were specifically designed for this. The solid phase fibrin immunoassay (SOFIA) is a well characterized method for the detection of Mab directed against t-PA[26]. With this method, antibodies directed against conformational epitopes exposed on t-PA after binding to fibrin were described in 3 groups of patients with autoimmune disorders[13-15].

We used the THP-1 monocytic cell line to detect autoantibodies directed against plasminogen bound to cell membrane proteins. This cell line was isolated from a boy with monocytic leukemia. It resembles the human monocyte by numerous criteria, including morphology and expression of membrane antigens and molecules. Among these, Fcγ receptors and plasminogen acceptors[16,23], as well as secretory products such as the urokinase-type plasminogen activator[27], are relevant to this study. To rule out the possibility of any interference in the antibody binding assay through binding of IgG to the Fcγ receptor, sera were diluted in binding buffer containing a concentration of protein A (0.95 µmol/l) determined experimentally. Binding of IgG to its Fcγ receptors (Figure 1) was thus inhibited and antibody in complex with cell immobilized plasminogen could be detected with a secondary antibody directed against human Fab IgG. This antibody specifically recognizes the antigen binding fragment of IgG and was used in all assays to avoid cross reactivity with rheumatoid factors. Screening of sera with this method showed reactivity of cells with 5 out of 30 RA sera. Reactivity against fibrin alone was also detected in 5 cases. A likely explanation is that autoantibodies in the sera of some patients recognize cell membrane or fibrin epitopes. It is possible that circulating autoantibodies

against fibrin derivatives described in inflammatory conditions[28] may react against immobilized fibrin. On the other hand, circulating immune complexes containing complement may bind to monocyte complement receptors that can be detected in the cell binding assay. The absence of cross reactivity between cell bound and fibrin bound plasminogen among positive sera (28% and 22% of cases, respectively) suggests the presence of autoantibodies that may react with distinct conformational changes of plasminogen induced by its binding to fibrin or THP-1 cells. Some sera from patients with primary APS also showed reactivity against both cell and fibrin bound plasminogen (9% and 13%, respectively). In contrast, the development of antibodies against plasminogen in venous and arterial thrombosis and BD did not show significant differences from controls. The presence of autoantibodies directed against plasminogen bound to cells or fibrin appears therefore to be a predominant feature in RA. Plasminogen passively adsorbed to plastic may also react with sera from patients with RA[29]. In this study, however, sera positive for plasminogen bound to cells or fibrin had no cross reactivity with plasminogen passively adsorbed to plastic (data not shown).

Plasminogen autoantibodies may interfere with plasmin activity, which might result in inadequate fibrinolysis or pericellular proteolysis[30]. Supporting this hypothesis, Puurunen, et al[31] described recently a group of patients with plasminogen autoantibodies who later developed myocardial infarction.

Reactivity against fibrin bound t-PA in the different groups of patients was not significant (Figure 2). It was found in only 8% of the patients with arterial or venous thrombosis. The other 2 groups of patients (primary APS and BD) were negative. The reactivity of an RA serum positive with both t-PA and plasminogen most probably represented cross reactivity with the latter. These results indicate that anti-t-PA antibodies are not prevalent in the inflammatory process of RA and vascular occlusive diseases. In contrast, anti-t-PA antibodies appear to be frequent in systemic lupus erythematosus[14].

These results may have implications in 2 different areas. First, the different reactivity of some sera against fibrin and cell bound plasminogen suggests that in spite of a similar interaction between plasminogen kringles 1 and 4 with carboxy-terminal lysine residues in fibrin and membrane proteins[3,4], the conformation of human plasminogen may not be the same. These differences in reactivity stress the importance of antigen presentation in the detection of autoantibodies. In other words, methods that mimic as closely as possible the conformational changes induced by binding of soluble proteins to specific receptors or acceptors must ideally be used in the detection of autoantibodies. Second, the antiplasminogen reactivity of RA sera suggests, in agreement with recent reports[32,33], that assembly and activation of plasminogen on cells and fibrin may be an important component of extracellular matrix remodeling in rheumatoid arthritis.

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