JCI The Journal of Clinical Investigation

Alpha2-plasmin inhibitor and alpha2-macroglobulin-plasmin complexes in plasma. Quantitation by an enzyme-linked differential antibody immunosorbent assay.

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J Clin Invest. 1981;68(1):46-55. https://doi.org/10.1172/JCI110253.

Research Article

An enzyme-linked differential antibody immunosorbent assay has been developed for the quantification of alpha2-plasmin inhibitor-plasmin and alpha2-macroglobulin-plasmin complexes. In this method the inhibitor-plasmin complex is bound to a surface by an inhibitor-specific antibody, and the plasmin bound to the inhibitor is quantified by a second antibody, rabbit antiplasminogen F(ab')2, labeled with alkaline phosphatase. The hydrolysis of p-nitrophenyl phosphate by the alkaline phosphatase is expressed in femtomoles of plasminogen per milliliter, by reference to a standard plasminogen curve. Inhibitor-enzyme complexes were generated in plasma by the addition of plasmin or of urokinase. The concentration of plasmin added was well below the plasma concentration of alpha2-plasmin inhibitor (1 microM) or of alpha2-macroglobulin (3.5 microM), so that neither inhibitor would be fully saturated with enzyme. Under these conditions increasing amounts of plasmin generated an increase in both alpha2-plasmin inhibitor-plasmin and alpha2-macroglobulin-plasmin complexes. Varying amounts of plasmin were incubated with each of the purified inhibitors in the concentration found in plasma, and the complexes. Varying amounts of plasmin were incubated with each of the purified inhibitors in the concentration found in plasma, and the complexes that formed were quantified by immunoassay. These studies made it possible to quantify the distribution of plasmin between the two inhibitors in plasmin or urokinase-treated plasma. In plasmin-treated plasma, 10% or less of the plasmin bound to both inhibitors […]

α_2-Plasmin Inhibitor and α_2-Macroglobulin-Plasmin Complexes in Plasma

QUANTITATION BY AN ENZYME-LINKED DIFFERENTIAL ANTIBODY IMMUNOSORBENT ASSAY

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ABSTRACT An enzyme-linked differential antibody immunosorbent assay has been developed for the quantification of α_2-plasmin inhibitor-plasmin and α_2-macroglobulin-plasmin complexes. In this method the inhibitor-plasmin complex is bound to a surface by an inhibitor-specific antibody, and the plasmin bound to the inhibitor is quantified by a second antibody, rabbit antiplasminogen F(ab')$_2$, labeled with alkaline phosphatase. The hydrolysis of *p*-nitrophenyl phosphate by the alkaline phosphatase is expressed in femtomoles of plasminogen per milliliter, by reference to a standard plasminogen curve. Inhibitor-enzyme complexes were generated in plasma by the addition of plasmin or of urokinase. The concentration of plasmin added was well below the plasma concentration of α_2-plasmin inhibitor (1 μM) or of α_2-macroglobulin (3.5 μM), so that neither inhibitor would be fully saturated with enzyme. Under these conditions increasing amounts of plasmin generated an increase in both α_2-plasmin inhibitor-plasmin and α_2-macroglobulin-plasmin complexes. Varying amounts of plasmin were incubated with each of the purified inhibitors in the concentration found in plasma, and the complexes that formed were quantified by immunoassay. These studies made it possible to quantify the distribution of plasmin between the two inhibitors in plasmin or urokinase-treated plasma. In plasmin-treated plasma, 10% or less of the plasmin bound to both inhibitors was in complex with α_2-macroglobulin. In contrast, between 19 and 51% of the plasmin generated in urokinase-activated plasma was bound to α_2-macroglobulin. Thus, major changes in the distribution of plasmin were observed, according to whether plasmin was added to plasma or whether plasminogen was activated endogenously. The pattern of inhibitor-plasmin complexes generated in vivo by the therapeutic infusion of urokinase was similar to that found for urokinase-activated plasma. 23 normal individuals had low levels of α_2-plasmin inhibitor-plasmin complexes, whereas six patients with laboratory evidence for disseminated intravascular coagulation demonstrated a 16- to 35-fold increase in the concentration of these complexes. These data indicate that a useful new probe for the study of the fibrinolytic enzyme system has been developed.

Received for publication 16 January 1981 and in revised form 2 March 1981.

INTRODUCTION

Activation of the blood coagulation and fibrinolytic enzyme systems leads to the production of a variety of protein-cleaving enzymes. The direct measurement of these enzymes in plasma has proven to be difficult because the enzymes involved may rapidly bind to cell surfaces, the developing fibrin network, or to circulating plasma proteinase inhibitors. Shuman and Majerus (1) have succeeded in developing a radioimmunoassay sufficiently sensitive to detect the evolution of thrombin in clotting blood. Measuring free plasmin directly in plasma is probably not feasible since the enzyme is rapidly bound to circulating proteins that inhibit its activity. Another approach, used in the present study, is to follow enzyme activation by measuring the generation of inhibitor-enzyme complexes in plasma. Previously, Collen et al. (2) have produced antisera directed against the α_2-plasmin inhibitor-plasmin complex that detected these complexes in urokinase- and streptokinase-activated plasma and in patients with intravascular coagulation using a semiquantitative latex agglutination test. The

J. Clin. Invest. © *The American Society for Clinical Investigation, Inc.* *0021-9738/81/07/0046/10 $1.00*
Volume 68 July 1981 46–55

possibility, however, that other inhibitor-enzyme complexes might also induce agglutination of the latex particles was not excluded. Plow et al. (3) failed to show an absolute antigenic difference between the plasmin inhibitor-plasmin complex and its individual constituents, and demonstrated that discrimination in the latex system was due to the more rapid binding of the complex.

Recently we have developed a new method for the quantification of α_2-macroglobulin-enzyme complexes in biological fluids, making use of the fact that the enzyme bound to α_2-macroglobulin retains its activity in hydrolyzing low molecular weight substrates (4). Our approach consists of binding α_2-macroglobulin-proteinase complexes to the IgG fraction of rabbit antihuman α_2-macroglobulin antisera that is covalently coupled to an agarose gel. After this immunocapture step, the hydrolytic activity of the enzyme bound to α_2-macroglobulin on the solid-phase antibody is assayed with a suitable chromogenic substrate. Using bovine β-trypsin as a model enzyme system, we have determined that the Michaelis-Menten kinetics for the hydrolysis of a low molecular weight substrate by the α_2-macroglobulin-trypsin complex in the fluid phase is the same as for the complex on the immobilized antibody. It also has been demonstrated that the α_2-macroglobulin-trypsin complex can be recovered quantitatively from plasma to which trypsin has been added (4). These studies have been extended to human thrombin, where similar results have been obtained, and the generation of α_2-macroglobulin-thrombin complexes have been quantified in clotting blood.[1]

In contrast to α_2-macroglobulin, when other plasma proteinase inhibitors bind with their enzymes, the catalytic capacity of the proteinase is entirely inhibited. Thus, the method detailed for the quantitation of α_2-macroglobulin-enzyme complexes would not be possible. In the present study we have developed a novel approach to the quantification of plasma plasmin inhibitor-enzyme complexes by extending our previous observations. In the new method the inhibitor-enzyme complex is bound to a surface by an inhibitor-specific antibody; subsequently, the enzyme bound to the inhibitor is detected and quantified by a second antibody that is immunospecific for the proteinase. To develop this technique we modified the enzyme-linked immunosorbent technique developed by Engvall and Perlmann (5), using microtitration plates as detailed by Voller et al. (6). We have selected the two principle plasmin inhibitors of plasma, α_2-plasmin inhibitor and α_2-macroglobulin, for analysis (7). The complex formed between plasmin and each of these inhibitors fulfills a critical requirement for the immunocapture assay described in this study; that is, that antigenic determinants on plasmin remain exposed in the inhibitor-enzyme complex so as to allow for antibody recognition. Thus, Müllertz and Clemmensen (8) have demonstrated that plasminogen antisera form an immunoprecipitate with the α_2-plasmin inhibitor-plasmin complex. In a similar manner, plasminogen antisera precipitate the α_2-macroglobulin-plasmin complex (9, 10).

The present study describes the development of an enzyme-linked differential antibody immunosorbent assay that measures both α_2-plasmin inhibitor-plasmin and α_2-macroglobulin-plasmin complexes either in purified systems or formed in vitro when plasmin or urokinase are added to plasma. This assay also measures plasmin inhibitor-plasmin complexes that are either generated in vivo after urokinase infusion or occur in the plasma of patients with disseminated intravascular coagulation.

METHODS

Blood samples. Venous blood was obtained in plastic syringes from normal volunteers and added to 3.8% sodium citrate (9:1, vol/vol). Alternatively, portions of blood samples sent to the Blood Coagulation Laboratory of the New York Hospital for coagulation studies were tested for plasmin inhibitor-plasmin complexes. The plasma, harvested following centrifugation at 2,500 rpm for 20 min, was frozen in plastic tubes at −70°C.

Purification of human plasma proteins. α_2-Plasmin inhibitor was isolated from outdated blood bank plasma as described by Moroi and Aoki (11). The final preparation contained no detectable α_2-macroglobulin, $C\bar{1}$ inactivator, α_1-antitrypsin, antithrombin III, inter-α-trypsin inhibitor, or chymotrypsin inhibitor, as analyzed by double diffusion in agarose gels with specific antibodies obtained from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. A reference standard of α_2-plasmin inhibitor was also kindly provided by Dr. Désiré Collen. α_2-Macroglobulin was isolated from fresh human plasma as has been previously described (12, 13). Plasminogen was isolated from plasma by lysine affinity chromatography (14), which was followed by gel filtration chromatography (Bio-Gel A-0.5 m, Bio-Rad Laboratories, Richmond, Calif.). The concentration of each protein was determined by their extinction coefficient (α_2-plasmin inhibitor = 7.03 [11]; α_2-macroglobulin = 8.93 [15]; plasminogen = 17.0 [16]).

Plasminogen was activated with insolubilized urokinase in 0.1 M barbital buffer, pH 8.3, containing 25% glycerol. The urokinase (Abbott Diagnostics, Diagnostic Products, North Chicago, Ill.) was coupled to Bio-Gel A 5.0 m (Bio-Rad Laboratories) by the cyanogen bromide method (17). Each gram of activated gel was coupled with 125,000 U of urokinase. In a typical activation mixture, plasminogen (6.6 mg) was incubated with insolubilized urokinase (0.3 ml packed gel) in a total volume of 1.0 ml at room temperature for 16 h. The activity of the plasmin was established by active site titration (18). The plasmin was stored at −70°C in 50% glycerol, at a concentration of 2.0 mg/ml active plasmin.

Preparation of antisera and immunoglobulin fractions. Rabbits were immunized intradermally with α_2-plasmin inhibitor, α_2-macroglobulin, or plasminogen in equal volumes

[1] Harpel, P. Unpublished observations.

of complete Freund's adjuvant. The IgG of the antisera was isolated, after dialysis against 0.02 M K_2HPO_4 buffer, pH 8.0, by chromatography on DE-52 cellulose (H. Reeve Angel & Co., Inc., Clifton, N. J.) to which cibacron-blue F 3GA (Polysciences, Inc., Warrington, Pa.) was coupled (19). The rabbit anti-α_2-plasmin inhibitor or anti-α_2-macroglobulin IgG fraction was absorbed with insolubilized human plasminogen to remove any plasminogen-reactive antibody.

To minimize the occurrence of spurious high values in the immunoassay caused by naturally occurring human antibodies directed against the Fc portion of immunoglobulin molecules (Rheumatoid factor) (20), $F(ab')_2$ fragments were prepared from the rabbit antiplasminogen IgG and subsequently labeled with alkaline phosphatase. The $F(ab')_2$ fragments were prepared by digestion of rabbit antihuman plasminogen IgG with 0.2 mg pepsin/100 mg IgG (pepsin, 2,700 U/mg, Worthington Biochemical Corp., Freehold, N. J.) in 0.1 M sodium acetate buffer, pH 4.0, for 6 h at 37°C (21). After adjusting the pH to 7.4 with 1 M Tris base, the digest was passed through two columns in series, the first containing 6.0 ml Protein A-Sepharose CL-4B (Pharmacia Diagnostics, Div. of Pharmacia Inc., Piscataway, N. J.) to bind undigested IgG and pFc fragments, and the second containing immobilized human plasminogen (5 mg/ml Sepharose CL-4B, coupled by the method of March [17]). After washing of the insoluble plasminogen antigen column with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.25 M NaCl and 0.5% Tween 80, the immunospecific $F(ab')_2$ fragments were eluted with 0.1 M glycine-HCl, pH 2.5, into tubes containing 2 M K_2CO_3. The yield of $F(ab')_2$ from 50.0 ml of rabbit antisera was 22 mg.

Enzyme-linked differential antibody immunosorbent assay. The performance of the assay was essentially as detailed by Voller et al. (6). Microtitration plates (Linbro, E.I.A. Microtitration Plate, Flow Laboratories, Inc., Hamden, Conn.) were coated with the IgG fraction of normal rabbit serum, or of the antisera against α_2-plasmin inhibitor or α_2-macroglobulin. Thus, 0.2-ml portions of the IgG in the bicarbonate coating buffer (6) were incubated in a humid chamber overnight at 4°C. Preliminary studies using IgG concentrations ranging from 1.25 to 20 μg/ml demonstrated that optimal binding of either α_2-plasmin inhibitor-plasmin, or α_2-macroglobulin-plasmin complexes in plasma to which either plasmin or urokinase had been added occurred at IgG concentrations of 2.5 μg/ml or greater. A concentration of 5.0 μg IgG/ml in coating buffer, therefore, was used in this study. Contents of the microtitration plates were removed, and the wells washed three times, for 3 min each in 0.15 M phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS-Tween)[2] (6). The solutions containing the inhibitor-plasmin complexes were diluted in PBS-Tween buffer containing 0.05 M epsilon amino caproic acid (EACA) to prevent plasminogen activation after dilution. These mixtures (0.2 ml) were added in duplicate to coated wells, and the plates incubated for 2 h at 37°C, and then for an additional 2 h at 4°C in a humid chamber. The washing procedure was repeated and the alkaline phosphatase labeled antiplasminogen $F(ab')_2$, prepared as detailed below, was added for an 18-h incubation in a humid chamber at 4°C. The wells were then emptied, the wash step repeated, and the substrate (0.2 ml) *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) (1 mg/ml in 10% diethanolamine buffer, pH 9.8) added. The color development was followed at 10-min intervals by repeated readings at 405 nm in a Titertek Multiscan photometer (Flow Laboratories, Inc., Rockville, Md.). The change in absorbance per minute was calculated by subtracting the 10-min value from the 60-min reading and dividing by 50. When the reading was >2.0, a shorter time interval was used. Plots of the color development with time demonstrated a linear relationship.

Alkaline phosphatase labeling of antihuman plasminogen $F(ab')_2$. This was performed, as described by Voller et al. (6), with Sigma type VII, calf mucosa alkaline phosphatase. The activity of the labeled antibody preparations was assessed by coating the microtitration plates with plasminogen (1 μg/ml in carbonate-coating buffer) for 18 h in a humid chamber at 4°C. After washing three times for 3 min each in PBS-Tween, dilutions of the stock alkaline phosphatase-labeled immunoglobulin preparation (1:200–1:1,600) were then added. After an overnight incubation at 4°C and washing, 0.2 ml of the substrate *p*-nitrophenyl phosphate was added, and the absorbance followed with time at 405 nm. The concentration producing the highest reading (1:400 dilution) was selected for the present studies.

Plasminogen standard curve. A standard curve was generated with each experiment. Purified human plasminogen, diluted from 1.25 to 25 ng/ml in carbonate-coating buffer in glass tubes, was passively adsorbed in duplicate (0.2-ml portions) to the wells of microtitration plates, 18 h at 4°C in a humid chamber. The wells were washed three times with PBS-Tween, and the alkaline phosphatase-labeled antiplasminogen $F(ab')_2$ antibody added for an additional 18 h at 4°C. The washing step was repeated, the *p*-nitrophenyl phosphate substrate added, and the change in absorbance monitored. The best fit curve of the change in absorbance (405 nm), plotted against plasminogen concentration, was calculated by linear regression analysis with a statistical package supplied by Hewlett-Packard and a Hewlett-Packard model 9815A calculator with a model 9862A X-Y plotter (Hewlett-Packard Co., Palo Alto, Calif.). The concentration of plasminogen was converted to femtomoles per milliliter (plasminogen M_r = 92,000). For the standard plasminogen curve illustrated (Fig. 1), the ΔA_{405} nm/min per fmol per ml is 1.81×10^{-4}.

Generation of α_2-plasmin inhibitor-plasmin or α_2-macroglobulin-plasmin complexes by the addition of plasmin or urokinase to plasma. Plasmin, diluted in glass tubes in 0.1 M barbital buffer, pH 8.3, containing 25% glycerol, was added to plasma in concentrations of 0.5 μg to 24 μg/ml plasma for 10 min at 37°C. Alternately, urokinase (Abbott Laboratories), also diluted in barbital-glycerol, was added to plasma in concentrations from 31.25 to 500 U/ml plasma and incubated 30 min at 37°C. These incubation mixtures were diluted variously (1:10, 1:20, 1:40, 1:80, 1:100, 1:120) in PBS-Tween buffer containing 0.05 M EACA and added to microtitration plate wells previously coated with normal rabbit IgG, rabbit anti-α_2-plasmin inhibitor, or anti-α_2-macroglobulin IgG. The assay was performed as described above. The results of the 1:10–1:80 dilutions of plasma were similar, whereas the values obtained at dilutions >1:80 progressively decreased. Thus, 1:40 dilution of plasma was selected to study normal and patient plasma samples.

Assay of purified α_2-plasmin inhibitor-plasmin and α_2-macroglobulin-plasmin complexes. Plasmin, in concentrations ranging from 128 ng to 2.048 μg, diluted in glass tubes in barbital buffer containing either 25% glycerol or buffer alone was added to 6 μg purified α_2-plasmin inhibitor or to 200 μg α_2-macroglobulin in a total volume of 0.1 ml and incubated 10 min at 37°C. The amidolytic activity of plasmin in hydrolyzing the substrate H-D-Val-Leu-Lys-pNA (Kabi, Stockholm, Sweden), measured as previously described (7), was completely inhibited in the mixtures of α_2-plasmin inhibitor and plasmin.

[2] *Abbreviations used in this paper*: PBS-Tween, phosphate-buffered saline containing Tween; EACA, epsilon amino caproic acid.

In addition, soybean trypsin inhibitor (Worthington Biochemical Corp.) failed to inhibit the amidolytic activity of the mixtures of plasmin and α_2-macroglobulin mixtures. These studies indicate that both inhibitors had formed complexes with plasmin (7, 10). PBS-Tween buffer containing 0.05 M EACA was added to the inhibitor-plasmin mixtures to a total volume of 4.0 ml. Each final inhibitor concentration was therefore equivalent to the concentration of a 1:40 dilution of normal plasma. 0.2-ml portions were added in duplicate to microtitration plate wells coated with rabbit anti-α_2-plasmin inhibitor IgG, anti-α_2-macroglobulin IgG, or with normal rabbit IgG (5 μg/ml in carbonate buffer). After an incubation period (2 h, 37°C; 2 h, 4°C) and a washing step, the antiplasminogen F(ab')$_2$ labeled with alkaline phosphatase was added. After 18 h at 4°C and further washing, *p*-nitrophenyl phosphate was added and the absorbance recorded. The change in absorbance per minute obtained with normal rabbit IgG was subtracted from those values obtained with the specific antisera. Results were converted to plasminogen equivalents using a standard plasminogen curve.

Quantification of circulating plasmin inhibitor-plasmin complexes by the enzyme-linked differential antibody immunosorbent assay. Various plasma samples from normal individuals or from hospitalized patients studied in the clinical coagulation laboratory were diluted 1:40 in PBS-Tween buffer containing 0.05 M EACA. 0.2-ml portions in duplicate were added to microtitration plate wells coated with normal rabbit IgG, anti-α_2-plasmin inhibitor, or anti-α_2-macroglobulin IgG (5.0 mg/ml). After an incubation of 2 h at 37°C and 2 h at 4°C in a humid chamber, and a washing step, rabbit antiplasminogen F(ab')$_2$ labeled with alkaline phosphatase was added (0.2 ml) and incubated 18 h at 4°C. After a washing step the substrate, *p*-nitrophenyl phosphate, was added, and the absorbance recorded. The change in absorbance per minute of the plasma sample added to the well coated with normal rabbit IgG was subtracted from the value that resulted when the sample was added to the wells coated with antibody directed against either α_2-plasmin inhibitor or α_2-macroglobulin. This A_{405} nm was then divided by the change in absorbance per minute per femtomole of plasminogen, derived from a plasminogen curve run simultaneously, as detailed above. The results are expressed in plasminogen equivalents, in femtomoles of plasminogen per milliliter. With the aid of a standard curve the results are also expressed as the concentration of α_2-plasmin inhibitor-plasmin complexes by conversion of plasminogen equivalents to micromoles per liter of complex composed of purified α_2-plasmin inhibitor-plasmin complexes as detailed above.

RESULTS

Plasminogen standard curve. Plasminogen, in increasing concentrations, was passively adsorbed to the wells of the plastic microtitration plate, and rabbit antiplasminogen F(ab')$_2$ labeled with alkaline phosphatase was added. The resulting hydrolysis of the alkaline phosphatase substrate *p*-nitrophenyl phosphate was linear with the amount of plasminogen added in the concentration range of 1.25 to 15 ng/ml (Fig. 1). Thus, hydrolysis of substrate was proportional to plasminogen concentration. This plasminogen curve was repeated with each experiment detailed in this report, and hydrolysis of substrate is expressed by conversion from the standard curve to plasminogen equivalents in femtomoles per milliliter.

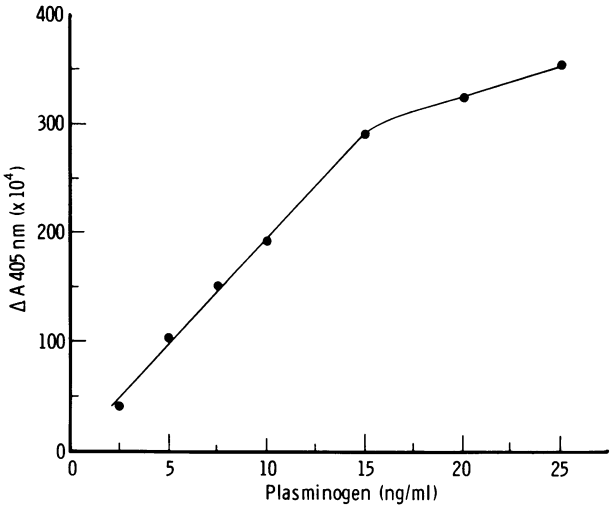

FIGURE 1 Plasminogen standard curve. Human plasminogen diluted from 1.25 to 25 ng/ml in carbonate buffer was applied to the plastic wells for 18 h at 4°C. After washing, the enzyme-labeled immunospecific rabbit F(ab')$_2$ antiplasminogen was added. After an 18-h incubation and washing, the substrate *p*-nitrophenyl phosphate was added and color development followed in a Titertek Multiscan photometer.

Detection and quantification of α_2-plasmin inhibitor and α_2-macroglobulin-plasmin complexes in plasma. Increasing concentrations of plasmin, activated by insoluble urokinase, were added to plasma to generate plasmin inhibitor-plasmin complexes. As illustrated in Fig. 2, α_2-plasmin inhibitor-plasmin complexes were first identified at added plasmin concentrations of 6.7 nM (0.5 μg/ml plasma), and α_2-macroglobulin-plasmin complexes at 160 nM (12.0 μg/ml). The concentration of both complexes increased with increasing concentration of exogenously added plasmin, but much less activity appeared to be associated with the α_2-macroglobulin-plasmin complexes than with the α_2-plasmin inhibitor-plasmin complexes.

Inhibitor-plasmin complexes were also detected in plasma to which increasing concentrations of urokinase, the plasminogen activator, were added (Fig. 3). Both α_2-plasmin inhibitor-plasmin and α_2-macroglobulin-plasmin complexes were detected at 31.25 U urokinase/ml plasma following a 30-min incubation. As with the addition of plasmin, increasing concentrations of urokinase produced increasing amounts of both complexes. In contrast, however, to the complexes generated with plasmin, there appeared to be proportionally more α_2-macroglobulin-plasmin complexes formed in relationship to the α_2-plasmin inhibitor-plasmin complexes in urokinase-activated plasma.

Quantification of purified α_2-plasmin inhibitor or α_2-macroglobulin-plasmin complexes. To define further the distribution of plasmin between α_2-plasmin inhibitor and α_2-macroglobulin, the immunoreactivity

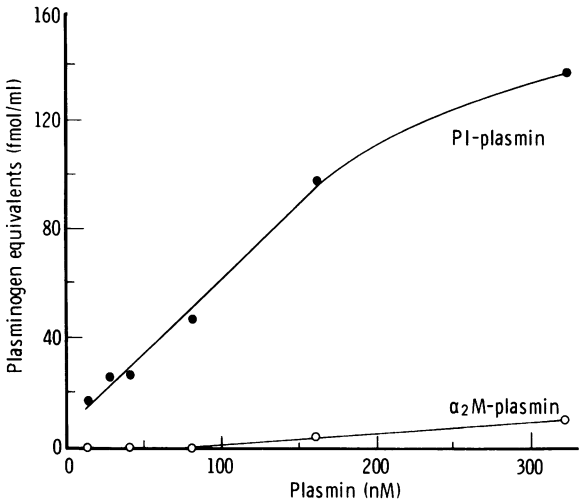

FIGURE 2 Generation of α_2-plasmin inhibitor(PI)-plasmin and α_2-macroglobulin(α_2M)-plasmin complexes by the addition of plasmin to plasma, as measured by the enzyme-linked differential antibody immunosorbent assay. Increasing concentrations of plasmin (0.5 to 24 μg/ml plasma) were added to plasma. After incubation for 10 min at 37°C, the plasma was diluted 40-fold with PBS-Tween buffer containing 0.05 M EACA, and samples were added to microtitration plates coated with rabbit anti-α_2-plasmin inhibitor, anti-α_2-macroglobulin, or normal rabbit IgG, and assayed as detailed in Methods. The results are expressed as plasminogen equivalents by converting the readings to femtomoles plasminogen per milliliter by means of a standard plasminogen curve (see Fig. 1). The curves are fitted by linear regression analysis. For the α_2-plasmin inhibitor-plasmin complex curve (first five data points), $y = 0.547x + 7.89$; and for the α_2-macroglobulin-plasmin complex curve, $y = 0.043x - 3.15$.

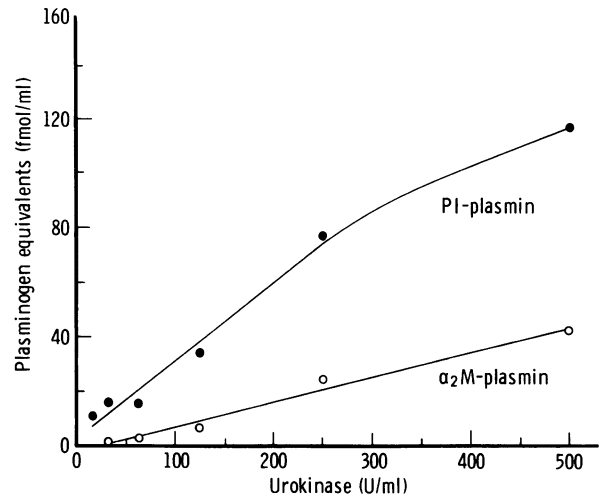

FIGURE 3 Generation of α_2-plasmin inhibitor(PI)-plasmin and α_2-macroglobulin(α_2M)-plasmin complexes by the addition of urokinase to plasma, as measured by the enzyme-linked differential antibody immunosorbent assay. Increased concentrations of urokinase (31.25–500 U/ml plasma) were added to plasma and incubated 30 min at 37°C. The plasma was diluted 40-fold in PBS-Tween-EACA buffer and assayed for α_2-plasmin inhibitor-plasmin and α_2-macroglobulin-plasmin complexes as described in Methods. For α_2-plasmin inhibitor-plasmin complex curve (first five data points), $y = 0.285x + 2.85$; and for α_2-macroglobulin-plasmin complex curve, $y = 0.091x - 2.55$.

of plasmin bound to each purified inhibitor was examined (Fig. 4). The concentration of each inhibitor used was similar to that found for the normal plasma that was diluted 1:40 for the assay. Thus, plasmin was added to 1.5 μg/ml α_2-plasmin inhibitor or to 50 μg/ml α_2-macroglobulin, so that the molar ratios of the enzyme to α_2-plasmin inhibitor were 0.018 to 0.29 and to α_2-macroglobulin were 0.006 to 0.096. Studies of the amidolytic activity of the plasmin in these incubation mixtures indicated that the added enzyme was in complex with its inhibitor. Each of the purified complexes was added to microtitration plate wells coated with the inhibitor antibody. The IgG fraction of rabbit α_2-plasmin inhibitor antisera did not bind α_2-macroglobulin-plasmin complexes as compared with the control rabbit IgG nor did the rabbit α_2-macroglobulin antisera bind α_2-plasmin inhibitor-plasmin complexes. These experiments demonstrate the lack of cross-reactivity in the enzyme-linked assay, and further confirm its specificity.

Increasing concentrations of plasmin added to each inhibitor yielded a linear increase in reactivity with the enzyme-linked plasminogen antibody when the inhibitor-enzyme complex was bound to the microtitration plate wells by the specific anti-inhibitor antibody (Fig. 4). The sensitivity of the assay is greater for the α_2-plasmin inhibitor-plasmin complex than for the α_2-macroglobulin-plasmin complex. The ratio of the slopes of the two curves in Fig. 4 is 3.6. Since plasmin forms a 1:1 molar complex with α_2-plasmin inhibitor and α_2-macroglobulin (10, 11), the abscissa of Fig. 4 indicates both the concentration of plasmin added and the concentration of inhibitor-plasmin complex formed. These standard curves thus permit direct comparison of the binding of plasmin to each inhibitor when either plasmin or urokinase is added to plasma as well as the quantification of inhibitor-plasmin complexes that may occur in plasma in vivo. The activity of the α_2-plasmin inhibitor-plasmin or α_2-macroglobulin-plasmin complexes at each concentration of plasmin (Fig. 2) or urokinase (Fig. 3) added to plasma, was converted to nanomolars of plasmin bound to each inhibitor by referring to the purified inhibitor curves (Fig. 4). The percentage of plasmin bound to α_2-macroglobulin compared with the total bound to both inhibitors in treated plasma was then plotted against the activity of the α_2-plasmin inhibitor-plasmin complex in each plasma sample expressed in plasminogen equivalents (Fig. 5).

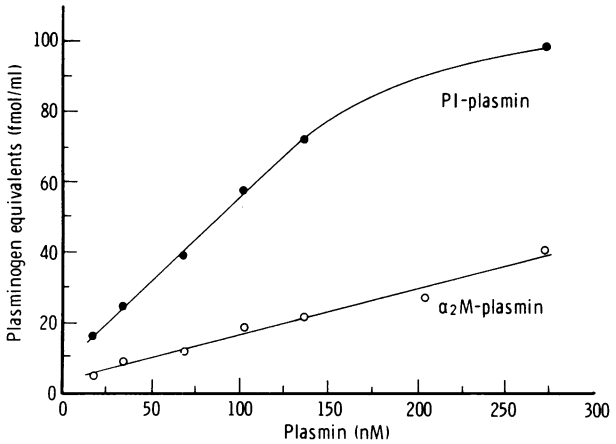

FIGURE 4 Quantification of purified α_2-plasmin inhibitor-(PI)-plasmin, and α_2-macroglobulin(α_2M)-plasmin complexes by enzyme-linked differential antibody immunosorbent assay. Varying concentrations of plasmin (0.128–2.048 μg) were added to either 6.0 μg α_2-plasmin inhibitor or 200 μg α_2-macroglobulin and the volume adjusted to 4.0 ml with PBS-Tween buffer containing 0.05 M EACA. The concentration of inhibitor used represents a 1:40 dilution of that amount found in normal plasma. Samples were applied to microtitration plates coated with either rabbit anti-α_2-plasmin inhibitor or anti-α_2-macroglobulin IgG, and processed as detailed in Methods. The plasmin concentration added to the inhibitor was converted to nanomoles per liter after multiplication by 40. For the α_2-plasmin inhibitor-plasmin complex curve (first five data points), $y = 0.475x + 7.9$; and for the α_2-macroglobulin-plasmin complex curve, $y = 0.131x + 3.2$.

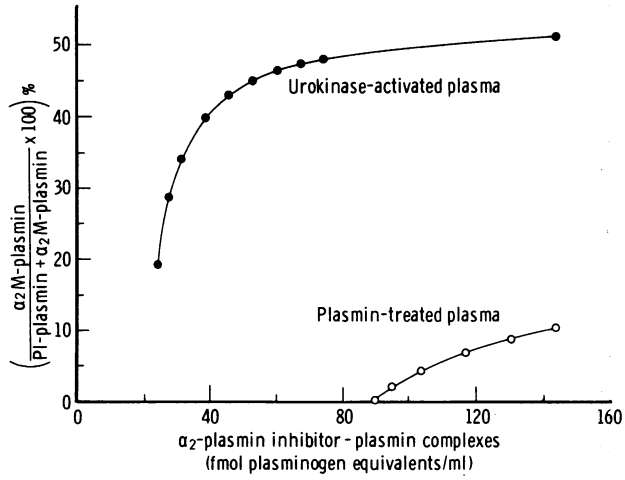

FIGURE 5 Percentage of α_2-macroglobulin-plasmin complex generated in plasmin or urokinase-treated plasma as assessed by the enzyme-linked differential antibody immunosorbent assay. The relative amount of plasmin bound to α_2-macroglobulin (α_2M) or to α_2-plasmin inhibitor (PI) in plasmin or urokinase-treated plasma in Figs. 2 and 3 was calculated from the purified inhibitor-plasmin curves of Fig. 4. The percentage of activity of the α_2-macroglobulin-plasmin complex in either the plasmin or urokinase-plasma compared with the total activity of both complexes is plotted against the activity of the α_2-plasmin inhibitor-plasmin complex in each treated plasma.

In urokinase-activated plasma, the α_2-macroglobulin-plasmin complex increased from 19 to 51% as the concentration of activator increased from 75 to 500 U/ml plasma. Thus, for example, when 125 U of urokinase/ml plasma is added, the activity of the α_2-plasmin inhibitor-plasmin and the α_2-macroglobulin-plasmin complexes are 38.5 and 8.83 fmol plasminogen equivalents/ml, respectively (Fig. 3). This amount of activity in the purified system is equivalent to the addition of 64.4 nM plasmin to α_2-plasmin inhibitor and 43.0 nM plasmin to α_2-macroglobulin (Fig. 4). The ratio of plasmin bound to α_2-macroglobulin compared with the total bound when 125 U of urokinase are added to plasma is therefore 43.0/107.4 or 40% (Fig. 5). Since the concentration of α_2-plasmin inhibitor in plasma is 1.0 μM and of α_2-macroglobulin, 3.5 μM, it is apparent that in urokinase-activated plasma, the plasmin that is generated binds to both inhibitors before stoichiometric saturation of α_2-plasmin inhibitor occurs.

In contrast to the findings in urokinase-activated plasma, much less plasmin binds to α_2-macroglobulin and proportionally more to α_2-plasmin inhibitor when the active enzyme plasmin is added to plasma (Fig. 5). With increasing concentrations of plasmin, the percentage bound to α_2-macroglobulin increases progressively to 10.5% at an added plasmin concentration of 250 nM. It is also apparent that in urokinase-activated plasma, α_2-macroglobulin-plasmin complexes become detectable at lower levels of α_2-plasmin inhibitor-plasmin complex activity. α_2-Macroglobulin-plasmin complexes are detected in urokinase-activated plasma when the concentration of the α_2-plasmin inhibitor-plasmin complex is ~34 nM. In plasmin-treated plasma, however, the α_2-macroglobulin-plasmin complex is not detected until an α_2-plasmin inhibitor-plasmin complex concentration of 141 nM is reached. These results demonstrate that the distribution of plasmin between the two inhibitors studied differs markedly, according to whether plasmin is activated endogenously in plasma or is added directly to plasma.

These in vitro results with urokinase were extended to the in vivo situation in which α_2-plasmin inhibitor-plasmin and α_2-macroglobulin-plasmin complexes were quantified in a patient receiving continuous intravenous infusion of urokinase (300,000 U initially followed by 300,000 U/h for 12 h), for a massive pulmonary embolus (Table I). After 2 h of fibrinolytic therapy, the fibrinogen concentration fell by 50%, and the reptilase clotting time was significantly elevated. There was a 24-fold increase in the level of α_2-plasmin inhibitor-plasmin complex and a 173-fold increase in α_2-macroglobulin-plasmin complex as compared with the preinfusion value. 2 h after cessation of urokinase infusion, the fibrinogen concentration and reptilase

TABLE I

Effect of Urokinase on Circulating Plasmin Inhibitor-Plasmin Complexes In Vivo

Patient	Fibrinogen	Reptilase time	Plasminogen equivalents: α_2-plasmin inhibitor-plasmin	Plasminogen equivalents: α_2-macroglobulin-plasmin
	mg/dl	*s*	*fmol/ml*	
Normal range, n = 23	180–400	18–21	4.1±3.5	0.6±0.8
Preurokinase	550	28.7	6.4	0.5
On urokinase	280	48.7	150.4	86.5
Posturokinase	470	39.0	177.6	107.1

time had returned toward the pretreatment level, but the inhibitor-plasmin complexes remained elevated.

Measurement of α_2-plasmin inhibitor-plasmin or α_2-macroglobulin-plasmin complexes in the plasma of patients with disseminated intravascular coagulation. The enzyme-linked differential antibody immunosorbent assay was used to measure α_2-plasmin inhibitor-plasmin or α_2-macroglobulin-plasmin complexes in the plasma of healthy individuals, or in the plasma of six patients with laboratory evidence for disseminated intravascular coagulation (Table II). The mean and SD of α_2-plasmin inhibitor-plasmin complex levels of 23 normal individuals were 4.1±3.5 with a range of 0 to 11.1 fmol plasminogen equivalents/ml. The patients studied had depressed fibrinogen levels and platelet counts, prolonged thrombin times, and elevated fibrin degradation products. α_2-Plasmin inhibitor-plasmin complex levels in the group with overt defibrination varied between 65 and 142 fmol plasminogen equivalents/ml. These data were converted to the plasma concentration of α_2-plasmin inhibitor-plasmin complexes in micromoles per liter with the aid of the curve generated by the assay of purified complexes (Fig. 4). The patients demonstrated levels ranging from 0.12 to >0.28 μM of α_2-plasmin inhibitor-plasmin complexes. Values >0.28 μM were not quantified, as these were beyond the range of the standard curve shown in Fig. 4. In contrast to the results with α_2-plasmin inhibitor-plasmin complexes, quantification of α_2-macroglobulin-plasmin complexes failed to detect significant differences in the patients as compared with the control group.

DISCUSSION

Human plasma contains five well-characterized proteinase inhibitors that possess the capacity to inhibit the fibrinolytic enzyme plasmin in vitro (22, 23). These inhibitors include the α_2-plasmin inhibitor (8, 11, 24), α_2-macroglobulin (10, 25), $C\bar{1}$ inactivator (26, 27),

TABLE II

α_2-Plasmin Inhibitor-Plasmin Complexes in the Blood of Patients with Disseminated Intravascular Coagulation

Patient	Diagnosis	Fibrinogen	Thrombin clotting time	Fibrinogen degradation products	Platelets	α_2-Plasmin inhibitor-plasmin complexes: Plasminogen equivalents	α_2-Plasmin inhibitor-plasmin complexes: Plasma concentration
		mg/dl	*s*	*μg/ml*	*per ml*	*fmol plasminogen/ml*	*μM*
	Normal range, n = 23	180–400	18–21	<10	180,000–400,000	4.1±3.5	—
1	Adenocarcinoma liver	75	36	>40	101,000	79.3	0.15
2	Hemorrhagic shock	148	—	>40	79,000	141.8	>0.28
3	Acute promyelocytic leukemia	119	33	>10 < 40	75,000	141.1	>0.28
4	Epidermoid carcinoma of lung	130	48	>10 < 40	Decreased on peripheral smear	65.9	0.12
5	Sepsis; hepatic failure	65	48	>10 < 40	48,000	67.2	0.13
6	Sepsis	105	>120	>40	49,000	93.5	0.24

antithrombin III (28), and α_1-antitrypsin (29, 30). α_2-Plasmin inhibitor, a protein of M_r = 67,000 whose plasma concentration is 1.0 μM, is an extremely effective inhibitor of plasmin. The dissociation constant of the reaction ($K = 1.9 \times 10^{-10}$) is low in comparison with other protease inhibitor reactions (31, 32). Inherited deficiency of this protein is associated with a life-long hemorrhagic diathesis characterized by rapid dissolution of fibrin thrombi (33, 34). In purified systems, when the distribution of plasmin is compared between α_2-macroglobulin, $C\bar{1}$ inactivator, and α_1-antitrypsin, the α_2-macroglobulin binds the majority of the enzyme (30). Studies from our laboratory examining the binding of plasmin in mixtures of purified α_2-plasmin inhibitor and α_2-macroglobulin found that the plasmin inhibitor bound over 90% of the added plasmin (7). These and other studies suggest that α_2-plasmin inhibitor is the physiologically most important inhibitor of plasmin-mediated fibrinolysis (35, 36).

The present study details a new method for detection and quantification of α_2-plasmin inhibitor-plasmin and α_2-macroglobulin-plasmin complexes that has made it possible to follow the generation of α_2-plasmin inhibitor-plasmin and α_2-macroglobulin-plasmin complexes in plasma to which plasmin is added. The α_2-plasmin inhibitor-plasmin complex was detected when as little as 6.7 nM plasmin was added, the equivalent of a 0.3% conversion of plasma plasminogen to plasmin. α_2-Macroglobulin-plasmin complexes were detected when 160 nM plasmin was added to plasma. Since plasma contains ~1 μM of α_2-plasmin inhibitor, it is apparent that α_2-macroglobulin binds plasmin in plasma well below the saturation level of α_2-plasmin inhibitor. These data indicate that recent conclusions that plasmin forms a complex with α_2-macroglobulin only after saturation of α_2-plasmin inhibitor must be revised (8, 37).

The ability of the new assay described in this study to measure both α_2-plasmin inhibitor-plasmin and α_2-macroglobulin-plasmin complexes in plasma has produced an unanticipated result. There appears to be a major difference in the distribution of plasmin between α_2-plasmin inhibitor or α_2-macroglobulin according to whether preformed plasmin is added to the plasma or the plasminogen in plasma is activated endogenously with urokinase. When plasmin is added to plasma at concentrations well below the molar binding activity of α_2-plasmin inhibitor, >90% of the plasmin associated with the two inhibitors forms a complex with α_2-plasmin inhibitor. In urokinase-activated plasma, however, when the α_2-plasmin inhibitor-plasmin complex activity is equivalent to that in plasmin-treated plasma and where α_2-macroglobulin-plasmin complexes are still undetectable, >40% of the plasmin bound to both inhibitors forms a complex with α_2-macroglobulin.

Although our data do not explain why plasmin is distributed differently between the two inhibitors, according to whether plasmin is activated endogenously or added to the plasma, recent observations by Lijnen et al. (38) may provide an explanation. These authors demonstrated that the plasma protein histidine-rich glycoprotein, first isolated by Haupt and Heimburger (39, 40), interacts with the high affinity lysine-binding site in plasmin to reduce markedly the reaction rate between plasmin and α_2-antiplasmin. They postulated that about one-half of the plasminogen in blood forms a reversible complex with the histidine-rich glycoprotein. Thus, a plausible explanation for the results of the present study is that when preformed plasmin, free of histidine-rich glycoprotein, is added to plasma, it rapidly binds to α_2-plasmin inhibitor. A portion of the plasminogen in plasma, however, when activated by urokinase, is in complex with the histidine-rich glycoprotein and thereby loses its α_2-plasmin inhibitor high affinity binding site. Presumably, a portion of this form of plasmin binds to α_2-macroglobulin. Studies have been initiated to confirm the possibility that the histidine-rich protein may modulate the distribution of plasmin between its circulating inhibitors.

To explore the potential usefulness of this new assay, the plasma of a patient undergoing urokinase infusion or the plasma of patients with laboratory evidence of overt disseminated coagulation were studied. High levels of both α_2-plasmin inhibitor-plasmin and α_2-macroglobulin-plasmin complexes were generated during urokinase therapy. The distribution of plasmin between the two inhibitors was similar to that observed when urokinase was added to plasma in vitro. These results are compatible with those of Aoki et al. (35), who identified α_2-plasmin inhibitor-plasmin complexes in the plasma of patients receiving urokinase treatment by antigen-antibody crossed electrophoresis. In contrast to our findings, they did not detect α_2-macroglobulin-plasmin complexes. The elevated levels of α_2-macroglobulin-plasmin complexes as quantified by our immunoassay indicates the greater sensitivity of the present method. The immunoassay demonstrated a 16- to 35-fold increase in levels of α_2-plasmin inhibitor-plasmin complexes in six patients with disseminated intravascular coagulation. Quantification of the concentration of α_2-plasmin inhibitor-plasmin complexes in these patients by reference to a standard curve of the purified complex indicates that 0.12 to >0.28 μM of the inhibitor, whose normal plasma concentration is 1 μM (11), circulates as a complex. Study of α_2-macroglobulin-plasmin complexes in patients' plasma demonstrated minimal elevations. These results are compatible with the lower sensitivity of the immunoassay for α_2-macroglobulin-plasmin complexes as compared with the α_2-plasmin inhibitor-plasmin complexes. Differential clearance rates of the two-plasmin

complexes may also explain our findings. In the experimental animal, α_2-macroglobulin-enzyme complexes are cleared in <60 min (41), faster than the $t_{1/2}$ of 0.5 d reported for the α_2-plasmin inhibitor-plasmin complex (42). Alternatively, in disseminated intravascular coagulation, plasminogen activation may occur only on the surface of fibrin, where the plasmin formed is inhibited by α_2-plasmin inhibitor, but not by α_2-macroglobulin (34, 36).

Our results indicate that the enzyme-linked differential antibody assay as detailed in this report serves as sensitive probe for quantifying activation of the fibrinolytic enzyme system in purified systems, in plasma in vitro, and in the circulation. Using this assay, we have found that the distribution of plasmin between α_2-plasmin or α_2-macroglobulin differs according to whether plasmin is added to the plasma or endogenous plasma plasminogen is activated with urokinase. The immunocapture assay has established that circulating inhibitor-plasmin complexes are elevated in patients with disseminated intravascular coagulation. Thus, a new approach for the study of the fibrinolytic enzyme system has been developed. The methods described may also prove valuable in the detection and quantification of other biologically important protein complexes.

ACKNOWLEDGMENTS

This study could not have been accomplished without the dedicated technical assistance of Mr. T. S. Chang and Mrs. Maureen Kraics and excellent editorial assistance of Ms. Pauline Santos.

This study was supported by U. S. Public Health Service grant HL-18828 (Specialized Center of Research in Thrombosis).

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