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Development and clinical application of a new ELISA assay to determine plasmin–α_2-antiplasmin complexes in plasma

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Summary. Plasmin–α_2-antiplasmin complexes (PAP) are considered good markers of fibrinolytic activation *in vivo*. The presence of neoantigens in these complexes offers the possibility to develop specific immunoassays to determine PAP levels. We have developed a sensitive PAP purification method *in vitro* by adding urokinase to fresh plasma followed by affinity chromatography to lysine-sepharose and elution with ε-aminocaproic acid. This material, characterized by SDS-PAGE and Western blotting, was used to raise monoclonal antibodies (MoAbs). We describe a new enzyme-linked immunosorbent assay (ELISA) to quantify PAP complexes in plasma. The assay follows the sandwich principle and is based on two MoAbs, CPL12 and CPL15, that bind to the modified α_2-antiplasmin moiety and the plasmin moiety of the complex respectively. The calibration curve was constructed with definite concentrations of purified PAP. The lower limit of the assay is 75 ng/ml and the variation coefficients are 3·5% (intra-assay) and 10·6% (interassay). A mean value of $573{\cdot}5 \pm 131{\cdot}4$ ng/ml was obtained from PAP concentration in a healthy population ($n = 30$). Significantly higher PAP levels were observed under diverse clinical conditions in which fibrinolysis is activated: clinical sepsis, acute myocardial infarction (AMI), malignancy, diabetes, pregnancy, elderly people and thrombolytic therapy. From our results we conclude that this ELISA is suitable to measure *in vivo* plasma PAP levels.

Keywords: plasmin–antiplasmin, monoclonal antibodies, ELISA, fibrinolysis.

Activation of the fibrinolytic system leads to plasmin formation. Plasmin is a serine protease that cleaves fibrin into soluble fragments. This enzyme is rapidly inactivated by α_2-antiplasmin in a two-step reaction. The first reaction, a reversible interaction that involves the lysine binding sites (LBS) of kringle 1 in the heavy chain of plasmin and the carboxyl-terminal end of α_2-antiplasmin, is followed by covalent bond formation between the active site of the enzyme and the reactive region of the inhibitor. A stable, inactive complex with a half-life of 12 h is thus formed (Wiman & Collen, 1979; Collen & Wiman, 1979). Therefore quantification of plasmin–antiplasmin complexes (PAP) in plasma reflects plasmin generation and could be a useful tool to study the behaviour of the fibrinolytic system in pathological situations.

Attempts have been made to quantitate PAP in plasma using polyclonal antibodies (Collen & De Cock, 1975; Collen *et al*, 1977; Harpel, 1981; Wiman *et al*, 1983; Holvoet *et al*, 1986) but this approach may create cross-reaction problems. The emergence of neoantigens in the PAP (Collen & De Cock, 1974) offers the possibility of obtaining monoclonal antibodies directed specifically against these structures, thus facilitating the design of immunoassays to measure plasma levels of PAP avoiding interferences from plasmin and α_2-antiplasmin.

We purified PAP from fresh plasma and obtained monoclonal antibodies (MoAbs) directed against PAP using spleen cells from mice immunized with the purified complexes. We subsequently developed an enzyme-linked immunosorbent assay (ELISA) to determine plasma levels of PAP in healthy subjects, in patients with different clinical conditions characterized by an increased risk of thrombotic complications and after thrombolytic therapy.

MATERIAL AND METHODS

Purification of PAP complexes. 300 ml fresh human plasma was supplemented with 500 IU/ml urokinase (Roger S.A. Lab., Spain) and incubated at 37°C for 30 min. After addition of 100 kIU/ml aprotinin (Trasylol, Bayer AG, Leverkusen, Germany) to prevent further proteolysis by an excess of free plasmin, affinity chromatography on lysine-sepharose (Deutsch & Mertz, 1970) at 4°C and at a flow rate of 20 ml/h was performed. The protein peak obtained after

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elution with 0·05 mol/l ϵ-aminocaproic acid (EACA) (Fluka, Switzerland) was concentrated (Minicon B, Amicon, U.S.A.) and fractionated in three aliquots prior to gel filtration on Sephadex G-200 SF (Pharmacia, Sweden) at 4°C and a flow rate of 3 ml/h. The fractions obtained were analysed by SDS-PAGE (10%, under non-reducing conditions) and immunoanalysis subsequent to Western blotting. Concentration of purified PAP was determined by the method of Bradford (1976) using equimolar solutions of plasminogen and α_2-antiplasmin (Biopool, Sweden) as reference.

Preparation of monoclonal antibodies. Female BALB/c mice were immunized with PAP as follows: three subcutaneous and intraperitoneal doses (300 μg antigen in Freund's adjuvant) every 2 weeks, and two boosts (300 μg antigen in saline) 4 and 2 d before fusion.

Fusion was performed according to Galfrè & Milstein (1981). Splenocytes were incubated with the myeloma cell line P3X63-Ag8-6.5.3 in the presence of polyethylene glycol 1500 (Merck, Germany). Emerging hybridomas were tested by the screening method described below. Selected lines were subcloned by limiting dilution and injected into the peritoneal cavity of pristane-primed female BALB/c mice. Antibodies from ascitic fluid were purified by protein A affinity chromatography (Bio-Rad, U.S.A.).

Screening to select specific monoclonal anti-PAP antibodies. Supernatants of emerging hybridoma clones were analysed by using ELISAs employing equimolar concentrations of PAP, plasminogen and α_2-antiplasmin as antigens. An antigen concentration of $0{\cdot}5 \times 10^{-9}$ mol/l was employed in a preselection step. Higher concentrations were used for the experiments.

Characterization of monoclonal antibodies. The affinity of MoAbs for PAP, plasminogen and α_2-antiplasmin was determined as follows. Antigens were passively adsorbed to microtitre plates by incubating 0·75 mg/ml of protein in phosphate buffer (0·05 mol/l phosphate pH 7·4–0·08 mol/l NaCl) overnight at 4°C. Free binding sites on the surface were saturated with a solution of 10 mg/ml bovine serum albumin (BSA, Sigma, U.S.A.) in the same phosphate buffer. Serial dilutions of the antibodies (7×10^{-6} to 0·11 nmol/l) were then incubated with solid-phase antigens overnight at 4°C. A peroxidase conjugated anti-mouse Ig rabbit polyclonal antibody (Amersham, U.K.) was used to detect bound antibodies and the peroxidase reaction was performed by adding 0·4 mg/ml O-phenylenediamine (Sigma, U.S.A.) and 0·006% H_2O_2 in a 0·15 mol/l citrate–0·065 mol/l disodium hydrogen phosphate buffer, pH 5·0. After 30 min at room temperature the reaction was arrested with 2 N H_2SO_4. The absorbances were read at 492 nm using a multiscan spectrophotometer (Organon Teknika, The Netherlands). Untransformed data were analysed by non-linear regression methods. The equilibrium dissociation constant (K_d) was derived using the simple Langmuir equation for adsorption at interfaces (Adamson, 1990).

Competition experiments were performed using increasing concentrations of antigens (0·5–50 nmol/l) and both CPL12 and CPL15 ($5{\cdot}5 \times 10^{-3}$ mmol/l). The comparative binding of these antibodies was further assessed in a ELISA with a fixed amount of PAP (0·15 mg/ml).

ELISA to measure plasma concentration of PAP. MoAb CPL12 was used as coating antibody at 5 mg/ml in phosphate buffer (0·05 mol/l phosphate pH 7·4–0·08 mol/l NaCl). After 16 h at 4°C, 20 mg/ml BSA in the same buffer were incubated for 1 h at room temperature to saturate free binding sites. Plasma samples were diluted (1:50 or more if higher values expected) in phosphate buffer containing 20 mg/ml BSA, 0·01% Tween 20 (Merck, Germany), and 0·1 mol/l EACA and incubated with the solid-phase antibody for 1 h at 37°C. The plate was then extensively washed with 0·05% Tween 20 in phosphate buffer. The MoAb CPL15, previously conjugated to horseradish peroxidase (Boehringer Mannheim, Germany) by the periodate method (Tijssen & Kurstak, 1984), was added as tag antibody at $3{\cdot}4 \times 10^{-2}$ mmol/l in sample buffer. The peroxidase reaction was performed as described above. Standard curves were obtained by adding different quantities of purified PAP in the same buffer used to dilute the samples.

Several quantities of PAP (100–1600 ng/ml) were added to two plasma samples that were subsequently diluted 1:20 and 1:50 and assayed in the ELISA.The detection rate for the added complexes was then calculated.

Three plasma samples supplemented with α_2-antiplasmin to reach a 30% higher than normal final concentration were assayed in the ELISA at three different dilutions (1:10, 1:20 and 1:50) before and after the addition of α_2-antiplasmin.

Coefficients of variation (CV) were calculated for intra-series ($n = 5$, 10 simultaneous assays) and inter-series ($n = 5$, 10 consecutive assays).

Influence of different anticoagulant conditions. In order to evaluate the influence of anticoagulants on the emergence *in vitro* of complexes after venipuncture, healthy donor samples ($n = 10$) were taken under the following conditions: 0·012 mmol/l sodium citrate (1:10, v/v), sodium citrate with 100 kIU/ml aprotinin and sodium citrate with 1 mg/ml PPACK (Sigma, U.S.A.). The ELISA was performed as indicated above.

Plasma samples. After informed consent was obtained, blood samples were collected by venipuncture in tubes containing 0·012 mmol/l sodium citrate (1:10, v/v) and centrifuged for 15 min at 2200 *g* at 4°C. Plasma aliquots were assayed immediately or stored frozen at −20°C.

Plasma samples were obtained from the following groups of patients: (i) Sepsis ($n = 20$): patients with clinical evidence of infection and positive blood cultures (mean age 61·15 ± 15·17; M/F: 14/6). (ii) Acute myocardial infarction (AMI) ($n = 20$): patients were studied during the first 7 d from the onset of symptoms (mean age 58·43 ± 8·75; M/F: 14/6). (iii) Malignancy ($n = 20$): patients with cancer tumours in the active phase of the disease (mean age 49·53 ± 20·42; M/F: 13/7). (iv) Diabetes ($n = 20$): both insulin and non-insulin metabolically controlled diabetic patients (mean age 51·38 ± 17·42; M/F: 11/9). (v) Pregnancy ($n = 20$): women during the third trimester of gestation (mean age 30·56 ± 4·12). (vi) Elderly ($n = 20$): healthy subjects over 65 years in which other well-established risk factors for atherosclerosis were excluded (mean age 71·92 ± 5·81; M/F: 13/7). (vii) Patients undergoing thrombolytic therapy for AMI ($n = 5$).

These samples were assayed with the ELISA and results were compared with those obtained in a healthy population ($n = 30$) whose mean age was 36 ± 12 years (M/F: 17/13).

Comparison with a commercially available PAP kit. Samples from healthy donors ($n = 30$) were used to compare the new ELISA with a commercial kit (EnzygnostR PAP micro, Behringwerke AG, Germany) to assess whether a correlation between the methods was present.

Statistical analysis. Results are expressed as mean $\pm$ SD. Differences among groups were tested for significance by the Dunn test when comparing anticoagulant conditions and by the Mann-Whitney test for the comparison between controls and subjects with different clinical conditions.

RESULTS

Purification of PAP

Material bound to lysine–Sepharose and subjected to SDS-PAGE was further submitted to gel filtration chromatography, rendering three protein peaks of distinct molecular masses (Fig 1A) corresponding to the bands observed in the SDS-PAGE pattern (Fig 1A, insert). Further characterization of fraction 2 by SDS-PAGE and subsequent Western-blot analysis with anti-plasminogen and anti-α_2-antiplasmin antisera showed a single band ($M_r = 140$ kD) strongly stained by both antibodies (Fig 1B). Protein quantification revealed that about 1·5 mg of PAP complexes could be obtained from 100 ml of fresh human plasma.

Characterization of selected monoclonal antibodies (MoAbs)

MoAbs CPL12 and CPL15 were selected from the pool obtained after fusion. As shown in Fig 2 (top), CPL12 exhibited a higher affinity for PAP than for α_2-antiplasmin. It did not recognize plasminogen at all. At low antigen concentrations (range 0·5–3 nM), this antibody recognized PAP complexes exclusively (K_d PAP $= 5{\cdot}22 \times 10^{-11}$ M). Competition experiments showed that antibody response dropped to about 10% of normal when pre-incubated with 1 nM PAP complexes. In contrast, a similar concentration of α_2-antiplasmin hardly neutralized 40% of CPL12 activity.

CPL15 strongly recognized both PAP and plasminogen but had no affinity for α_2-antiplasmin (Fig 2, bottom). This was confirmed by competition experiments (data not shown). The K_d for PAP was $3{\cdot}98 \times 10^{-11}$ M.

Characteristics of the ELISA to determine PAP

Results obtained by assaying serial dilutions of purified PAP

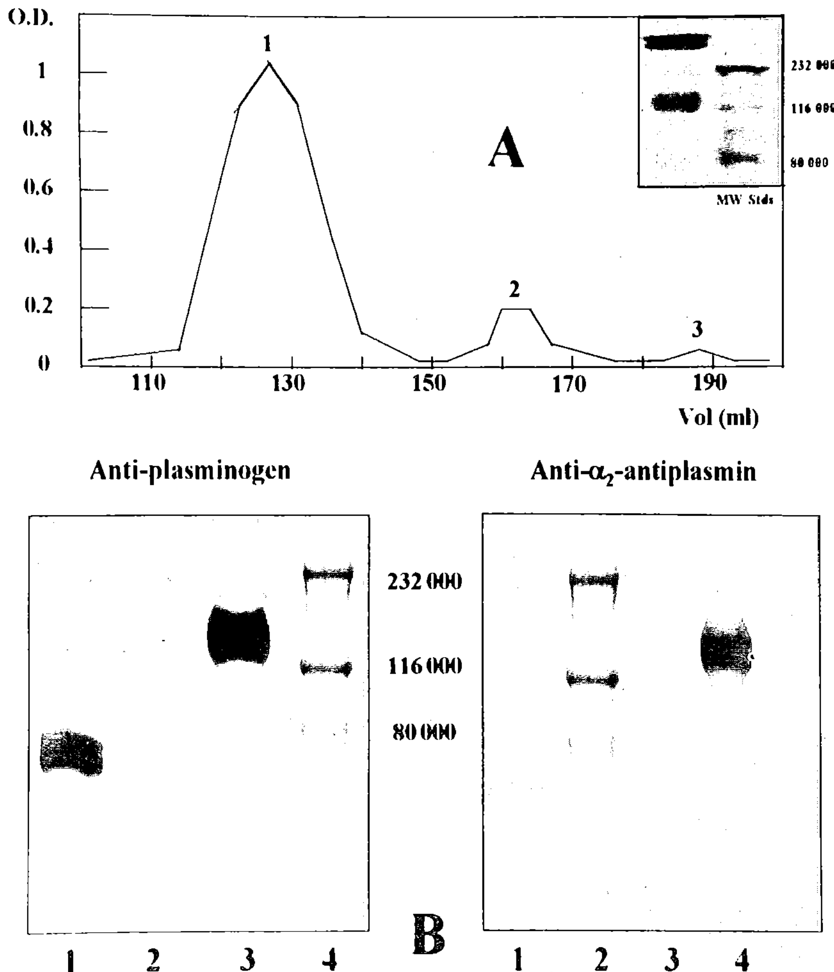

Fig 1. (A) Profile of the EACA-eluted material subjected to Sephadex G200 SF chromatography. The insert shows the SDS-PAGE pattern prior to column injection.
(B) Characterization of fraction 2 from Sephadex G200 SF by Western blotting with anti-plasminogen polyclonal antiserum in the left panel (lane 1: plasminogen; lane 2: α_2-antiplasmin; lane 3: fraction 2 from Sephadex G200 SF.; lane 4: MW standards) and anti-α_2-antiplasmin polyclonal antiserum in the right panel (lane 1: α_2-antiplasmin; lane 2: MW standards; lane 3: plasminogen; lane 4: fraction 2).

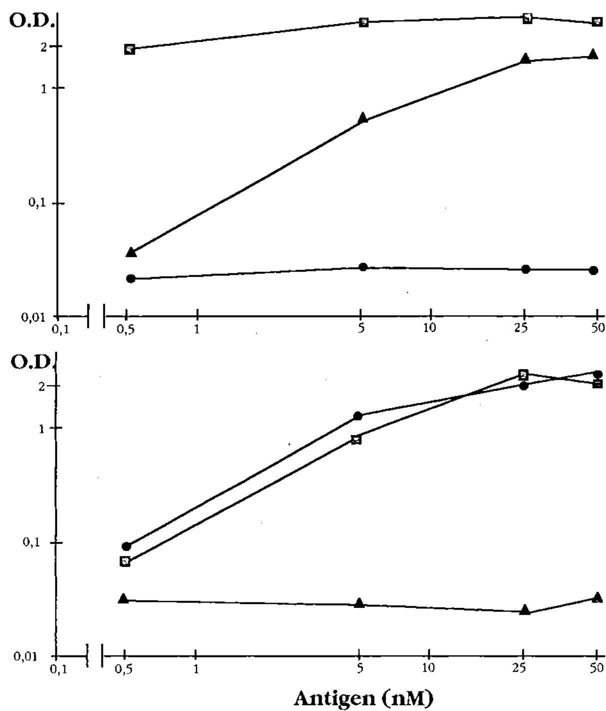

Fig 2. Binding of monoclonal antibodies CPL12 (top) and CPL15 (bottom) to PAP (■), plasminogen (●) and α_2-antiplasmin (▲) (0·5–50 mM). The antibody concentration was 0·8 mg/ml.

(0·75–200 ng/ml) in the ELISA exhibited a linear correlation between 1·5 and 100 ng/ml (Fig 3). We selected this range to construct the standard curve of the assay. Within this range, the optimal dilution of plasma was 1:50 and the lower detection limit of the assay was 75 ng/ml. Under these conditions, recovery of PAP complexes (100, 400 and 1600 ng/ml) added to normal plasma ranged from 80% to 100%. Addition of α_2-antiplasmin (30% over the basal value) did not impair detection of PAP complexes. Intra-assay and inter-assay variation coefficients were 3·5% and 10·6% respectively for a PAP concentration within the normal control range (350–790 ng/ml). No such coefficients were calculated for values beyond that normal range.

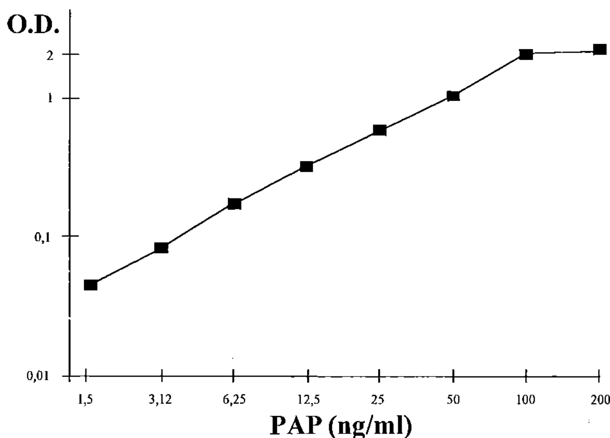

Fig 3. Reference standard curve for the PAP ELISA. Single points representing the average of three experiments are shown.

To determine the effect of plasminogen–α_2-antiplasmin complexes on the ELISA, experiments were performed using EACA in the dilution buffer as this fibrinolysis inhibitor is able to dissociate such reversible complexes. When the inhibitor is absent or in low concentration, PAP measurements were not reliable because plasminogen–α_2-antiplasmin complexes are also detected giving an overestimation of PAP values, as seen in Fig 4.

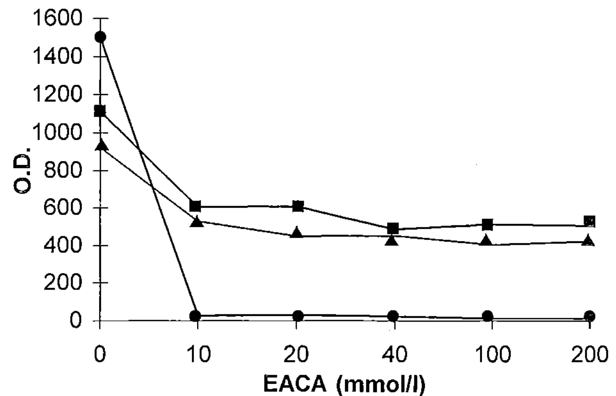

Fig 4. Influence of the addition of EACA to the sample dilution buffer. Plasma samples from two healthy subjects diluted 1:50 (■, ▲), and a mixture of plasminogen and α_2-antiplasmin at a concentration 50-fold lower than physiological (●) were subjected to the PAP ELISA in the presence of increasing concentrations of EACA in the dilution buffer.

Effect of different anticoagulant conditions

No differences in the levels of PAP complexes were observed between citrated samples without inhibitor (585 ± 126 ng/ml, $n = 10$) and samples supplemented with either aprotinin (529 ± 126 ng/ml, $n = 10$) or PPACK (516 ± 86 ng/ml, $n = 10$).

PAP concentration in plasma samples

PAP concentration in healthy subjects ($n = 30$, mean age 36 ± 12 years) was $573{\cdot}5 \pm 131{\cdot}4$ ng/ml (range 350–790 ng/ml).

Results obtained in samples from different groups of patients are shown in Fig 5. Higher PAP concentrations ($P < 0{\cdot}0001$), as compared to controls, were observed in sepsis (1739 ± 849 ng/ml), AMI (2489 ± 1232 ng/ml), malignancy (1804 ± 589 ng/ml), diabetes (1022 ± 414 ng/ml) and elderly subjects (1485 ± 546 ng/ml), indicating that the fibrinolytic system is activated in these situations. No differences were observed between elderly subjects and patients with sepsis, malignancy and diabetes. No differences were found between pregnancy and the control group. As expected, a marked increase in PAP was detected in samples from patients undergoing thrombolytic therapy with values between 2×10^4 and 2×10^5 ng/ml, clearly indicating extensive plasmin formation.

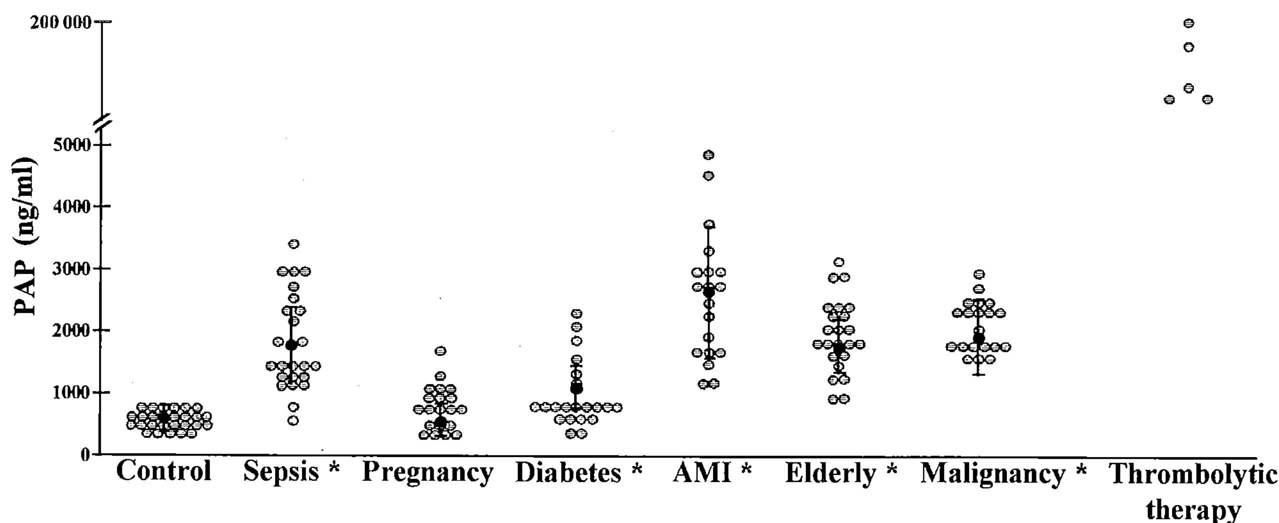

Fig 5. Individual distribution of plasma PAP levels in healthy donors and in several clinical conditions. The mean ±SD value is shown. $^*P < 0{\cdot}0001$.

Comparison with a commercially available method
The correlation degree between the new ELISA and the commercial kit was highly significant in the range of normal values (Fig 6).

DISCUSSION

In recent years considerable effort has been made to develop new methods to detect prethrombotic states. The latter are defined as situations in which haemostasis is activated but intravascular fibrin deposits are not detected. The study of the fibrinolytic system should contribute to a better knowledge of such states, because fibrin is an important structural component of the thrombus.

A variety of assays to determine plasminogen or α_2-antiplasmin have been used, but they lack sensitivity and specificity. The measurement of molecules that may be considered as markers of fibrinolytic activation in circulating blood is a more attractive proposal. Among them, those generated from plasmin action on fibrinogen or fibrin (fibrinogen degradation products, D dimer, Bβ 15-42 fragment or X oligomers) are of particular interest (Gaffney *et al*, 1988).

There have been few attempts to measure circulating plasmin because of its short half life (10 min) and its antigenic similarity to proenzyme plasminogen. In contrast, the amount of complexes formed between plasmin and its main inhibitor α_2-antiplasmin seems to be a good indicator of the plasma concentration of the enzyme. The emergence of neo-epitopes upon formation of these complexes (Collen & De Cock, 1974) makes it possible to design specific immunoassays. In this work we describe a specific ELISA assay to detect plasma PAP complexes. For this purpose we have purified PAP in enough quantities to immunize mice

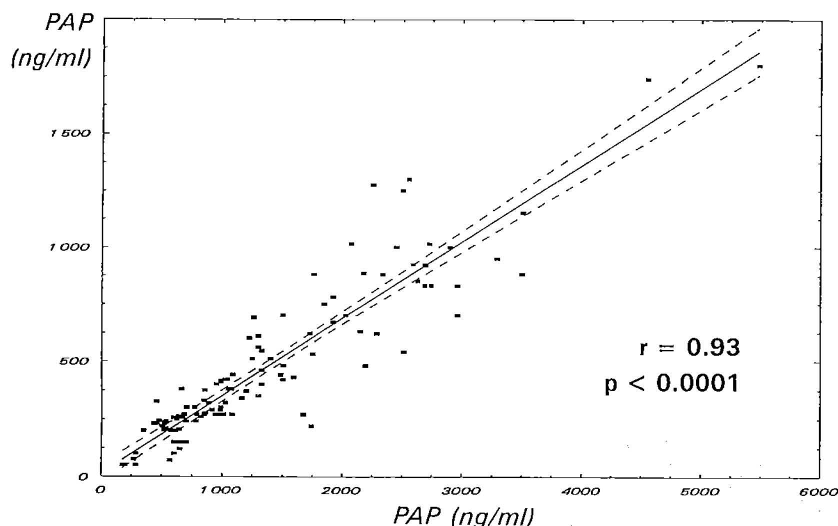

Fig 6. Correlation between the new ELISA (*X* axis) and a commercial kit (*Y* axis), assessed by the Spearman rank test.

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and detect specific monoclonal antibodies. So far the available literature in this field proposed cumbersome and time-consuming protocols which include either plasma manipulations or the use of prepurified zymogen or inhibitor (Wiman & Collen, 1979; Wiman, 1982). We describe an easy and suitable PAP purification method. Plasma does not have to be previously handled and there is no need to add purified proteins. The addition of 500 IU/ml urokinase to fresh plasma is enough to activate plasminogen to such an extent that the complexes formed can be effectively isolated by sequential lysine-sepharose and Sephadex G-200 SF chromatography. The amount of complexes obtained, 1·5 mg from 100 ml fresh plasma, covers our needs.

The performance of the hybridoma technology rendered a pool of anti-PAP MoAbs, one of which, CPL12, exhibited a low degree of cross-reaction with α_2-antiplasmin, without any interference from plasminogen. Another of the obtained antibodies, CPL15, recognized PAP and plasminogen in a very similar way but did not react with α_2-antiplasmin at all. These features made CPL15 and CPL12 suitable MoAbs for the development of a specific immunoassay for the detection of PAP complexes.

The reference curve obtained in the PAP ELISA, between 1·5 and 100 ng/ml, covers a range of concentrations broad enough to detect subnormal, normal and increased PAP values. The influence of EACA in the sample buffer is remarkable. This molecule dissociates the plasminogen–α_2-antiplasmin complexes, which are not fibrinolysis activation markers. The detection of these complexes would introduce a source of error that is avoided by adding this inhibitor. On the other hand, the recovery rate as well as the experiments performed in excess of α_2-antiplasmin helped us to validate the definitive conditions of the assay. Despite the high specificity for PAP exhibited by CPL12, plasma samples had to be diluted by a factor of 50.

The addition of different protease inhibitors to citrated tubes showed no effect on the PAP levels in normal plasma. Similar data were obtained by Pelzer *et al* (1993) using a different immunoassay, indicating that *in vitro* formation of PAP is negligible or cannot be prevented under the conditions assayed. Whether the inhibitors are needed for PAP determination in clinical situations with increased fibrinolysis (e.g. thrombolytic therapy) requires further evaluation.

The mean PAP value in a healthy population was $573 \cdot 5 \pm 131 \cdot 4$ ng/ml (range 350–790 ng/ml). Other authors using a variety of assays have found different results. Whilst Pelzer *et al* (1993) found a mean value of 210 ± 88 ng/ml (Enzygnost^R PAP, Behringwerke), Hattey *et al* (1987) established normality under 150 ng/ml (PAP ELISA, Technoclone). Meijer *et al* (1994) recently obtained a mean concentration of 758 ng/ml by using the PIC test from Teijin Diagnostics and a mean value of 900 ± 270 ng/ml was reported by Wiman & Haegerstrand-Björkman (1993) using their own method. This heterogeneity could be attributed to the different antibodies and calibration systems used in each case, because anticoagulation conditions do not seem to make any difference as shown by our own data and by Pelzer *et al* (1993).

It is worth mentioning that the real test for evaluating the usefulness of an assay lies in its capacity to detect subnormal as well as higher than normal concentrations of the desired molecule. In a small series of cirrhotic patients ($n = 9$) we were able to detect a PAP concentration as low as 215 ng/ml in four of them (unpublished observations). Although we did not include these data because of the small sample size, it is worth emphasizing that it would be possible to detect subnormal levels in selected groups of patients. By using our method we are able to measure a wide range of PAP values. We have also shown a good correlation between this assay and other commercially available ELISA already proven to be useful to assess plasma PAP. Despite the different absolute values obtained, our previous experiments to avoid cross-reaction and the good linearity observed when using our purified PAP as standard indicates that the calibration method was correctly performed.

In order to evaluate the capacity of this ELISA in monitoring fibrinolysis, we tested samples from different groups of patients where fibrinolysis activation takes place. We observed significantly higher PAP concentrations in sepsis, malignancy, diabetes, AMI and elderly groups as compared to the healthy control population, with no differences in the pregnancy group, in agreement with previous reports (Lorente *et al*, 1993; Kario *et al*, 1992; Vaziri *et al*, 1992; Takahashi *et al*, 1989, 1990). A dramatic PAP increase was also seen in patients under thrombolytic therapy, indicating extensive plasmin formation as previously reported (Pelzer *et al*, 1993). Since no differences were found between the elderly population and the patients with sepsis, malignancy and diabetes, a possible influence of age in the PAP concentration in these groups cannot be ruled out.

In view of these results, we consider that the described method is useful in evaluating the activation rate of the fibrinolytic system. Its reduced time factor makes it suitable to be introduced in routine laboratories. More work will be required to better establish the relevance of PAP levels in relation to different clinical conditions.

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