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Fibrinolysis Inhibition after a Major Standardized Trauma

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ABSTRACT

The present investigation on 20 patients after total hip replacement surgery has confirmed that the posttraumatic increase of the fibrinolysis inhibition activity (FIA) in serum and plasminogen-depleted serum is due to the primary fibrinolysis inhibitor (PFI,α_2-antiplasmin). This protein exists in at least two forms and it was indicated that PFIα with affinity to immobilized plasminogen, is mainly responsible for the posttraumatic variations of the FIA in plasminogen-depleted serum. PFIβ, the major part of the PFI-related antigen, which has none or low such affinity, displayed weak FIA and relatively small increase after the surgical trauma.

It was established that the posttraumatic increase of the FIA was not derived from the low molecular weight fraction in serum of those patients.

INTRODUCTION

Inhibition of the fibrinolytic system during intravascular coagulation resulting in a delayed elimination of fibrin from the lungs, is probably a major factor in the pathogenesis of the delayed microembolism syndrome (DMS) (12,13).

There are several conceivable mechanisms which might contribute to the impaired fibrinolysis, for example decreased serum levels of plasminogen and plasminogen activators and increased levels of different fibrinolysis inhibitors. However, we have previously found that during intravascular coagulation an artificially induced increase of the fibrinolysis inhibition activity (FIA) in serum alone can cause an impairment of fibrin elimination from the rat lung similar to that seen after trauma in the same animal (1).

The posttraumatic increase in FIA seems to be due to a raised blood concen-

tration of the primary fibrinolysis inhibitor (PFI) (2). This inhibitor is also called α_2-plasmin inhibitor (10) and α_2-antiplasmin (15).

Two forms of PFI and α_2-antiplasmin have been demonstrated in serum and plasma (2,5), in the former investigation by two-step affinity chromatography on Lysine-Sepharose® 4B and immobilized plasminogen. The form absorbed to the latter matrix (PFIα) was well correlated to FIA in plasminogen-depleted serum and showed the larger increase posttraumatically (2). The unbound form (PFIβ) displayed weak FIA.

In the present investigation the fibrinolytic system was studied during the seven days immediately following total hip replacement surgery in 20 patients in order to further elucidate the post-traumatic fibrinolysis inhibition and the role of PFI in this inhibition.

MATERIAL

Patients: Twenty patients (12 women and 8 men, aged 60-74 years) suffering from severe osteoarthritis of the hip joint undergoing total hip replacement by low friction arthroplasty (4) were studied. The patients had no history of heart or lung disease, diabetes, previous thromboembolism, varicose veins or leg ulcers. The operations were performed either under lumbar analgesia or under intravenous balanced anaesthesia with controlled ventilation. Care was taken to replace blood loss by packed red cells, balanced electrolyte solutions and whole blood intra- and postoperatively. No dextran or other colloidal solution was given either during or after the operation.

Chemicals: Human fibrinogen (Grade L), human plasminogen, human plasmin, ε-aminocaproic acid (EACA) and trans-4-aminomethyl-cyclohexanoic acid (AMCA) (AB Kabi, Sweden); Chromogenic substrate for plasmin (S-2251, Kabi Diagnostica, Sweden); Sephadex® G-25 Medium in PD-10 columns, CNBr-activated Sepharose®4B and Lysine Sepharose® 4B (Pharmacia Fine Chemicals, Sweden); Urokinase (Leo, Denmark); Thrombin (Topostasine®, Hoffman-La-Roche, Switzerland); Rabbit antibodies against human α_2-macroglobulin, α_1-antitrypsin and plasminogen (DAKO-PATTS Denmark); Rabbit antibodies against PFI (own product or "RaHu/Apl", Nordic Immunological Laboratories, The Netherlands); Immobilized plasminogen: plasminogen purified by the method of Deutsch and Mertz (7) was coupled to CNBr-activated Sepharose® 4B at a concentration of 10 mg/g gel.

Affinity chromatography columns: Empty PD-10 columns (cross-sectional area= 1.5 cm^2, kindly supplied by Pharmacia Fine Chemicals, Sweden) were packed with Lysine Sepharose® 4B (bed volume 0.5 ml) or immobilized plasminogen (bed volume 2 ml).

Blood fractions: Human normal serum (HNS) and normal plasma kindly supplied from the Blood Bank, University Hospital, Uppsala, Sweden, pooled from 30 apparently healthy persons were used as references. Serum and plasma were obtained from the 20 patients on the day of operation and postoperatively. The blood fractions were stored at -70°C until used.

METHODS

Fibrinolysis inhibition activity (FIA): A clot lysis method adapted from Paraskevas *et.al.* (11) was employed. Serum or serum fractions were diluted with phosphate-buffered saline (PBS: 0.02 M phosphate, 0.15 M NaCl, pH 7.4). Dilutions: serum 1:2 and 1:4, plasminogen-depleted serum 1:10 and 1:20, fraction not bound to the plasminogen matrix 1:5, serum protein fraction from gel chromatography not diluted and 1:2, low molecular weight (LMW) fraction, LMW-fraction + serum protein fraction and LMW-fraction + HNS were not further diluted. To 0.5 ml of the sample were then added 0.2 ml of 0.5 % fibrinogen, 0.1 ml of urokinase (150 PU/ml) and 0.1 ml of thrombin (20 NIH/ml), in that order. The plasminogen was derived from the thrombin and fibrinogen. The time from the addition of urokinase to the complete absence of any visible fibrin fragments was recorded.

As standards, a series of AMCA solutions, ranging from 50 to 500 mg AMCA/l PBS and resulting in lysis time between approximately 15 and 200 min., were used.

The FIA of the sample was expressed as the concentration of the AMCA solution (mg/l) giving the same delay in clot lysis time, taking into account the dilution of the sample.

Fast-reacting antiplasmin (AP): The inhibition of plasmin by serum or serum fractions was measured by an enzymatic assay, using a chromogenic substrate (S-2251) for plasmin and is expressed in per cent of that in HNS. The end-point determination method described in the manual S-2251 (Kabi Diagnostica, Sweden) was used.

PFI, α_1-antitrypsin (α_1-AT), α_2-macroglobulin (α_2M) and plasminogen (Plg): These concentrations in serum or serum fractions were assayed by immunodiffusion as described by Mancini *et.al.* (9) and are expressed in per cent of HNS.

Plasminogen activators (PA): The fibrinolytic activity of the euglobulin fraction in plasma was determined before and after venous occlusion (release test) according to the method of Walker *et.al.* (14) and is expressed as lysis area (mm^2) on fibrin plates.

Significance analysis: All reported changes of mean values were derived by Student's t-test.

Correlation analysis: The traditional least squares method was used.

EXPERIMENTAL PROCEDURES

Sera (and plasma) from the 20 patients were sampled on day 0 (before surgery on the day of operation) and days 1,2,3,6 and 7 after surgery.

The two-step affinity chromatography procedure

I. Removal of plasminogen on lysine-Sepharose® 4B

Applied fraction	1 ml serum, incubation for 1 h at +4°C
Elution buffer	2 ml 0.05 M phosphate, pH 7.5
Non-bound fraction (EI)	3 ml plasminogen-depleted serum
Bound fraction	Plasminogen desorbed with 3 ml of 0.2 M EACA
Equilibration buffer	0.05 M phosphate, pH 7.5

II. Adsorption to immobilized plasminogen

Applied fraction	2 ml of plasminogen-depleted serum (EI), incubation for 30 min +4°C
Elution buffer	2 ml of PBS
Non-bound fraction (EII)	4 ml containing PFIβ
Bound fraction	PFIα desorbed with 3 ml of 0.02 M Tris, 1.0 M NaCl, pH 8.0
Equilibration buffer	PBS

FIA and PFI were determined in serum and serum fractions. PFIα was calculated as applied minus eluted amounts of antigen, as the analytical scale together with the desorbent procedure rendered direct quantitation difficult. The plasminogen matrix amounted to about twenty-fold excess as related to the inhibitor in HNS.

Quenching of serum and plasminogen-depleted serum with antibodies against PFI

100 µl of the sample and 20 µl of anti-PFI (IgG) or PBS (reference) were incubated for 30 min at +37°C and diluted 1:10 and 1:20 (serum) or 1:25 and 1:50 (plasminogen-depleted serum) with PBS. The FIA of the sample was determined as previously described, with the exception of the concentration of urokinase

(50 PU/ml PBS) and is expressed in clot lysis time.

Fractioning of serum to serum protein fraction and low molecular fraction on Sephadex® G-25

One ml of serum was passed through the PD-10 column, followed by 8 ml of PBS in portions of 1.5, 2.5 and 4.0 ml. Three fractions were obtained: 1) PBS (2.5 ml), 2) serum protein (2.5 ml) and 3) LMW (4.0 ml). FIA was determined as previously described. The compositions of the mixtures of LMW-fraction were as follows: 0.3 ml of LMW-fraction + 0.2 ml of serum protein fraction, resulting in almost the same relationship between the fractions as in the source serum (retitration); 0.3 ml of LMW-fraction + 0.2 ml of HNS were mixed in order to study the additive effect of LMW-fraction at a constant level of other inhibitors in a serum milieu.

RESULTS

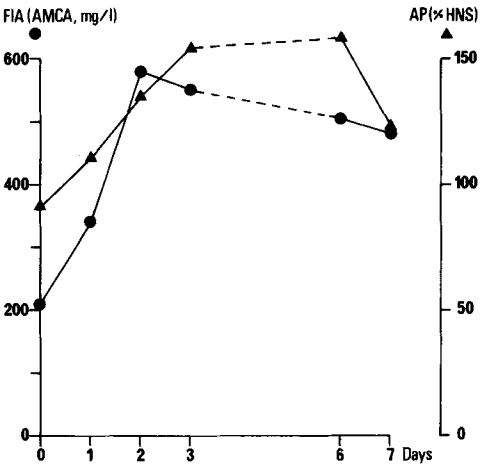

Fig.1 FIA (mean of 19 patients, S.E.M. about 15%) and AP (10 patients, S.E.M. about 10%) after the surgical trauma. FIA is expressed as the concentration of AMCA giving the corresponding clot lysis time. AP is expressed as the inhibition of plasmin activity by human normal serum (HNS).

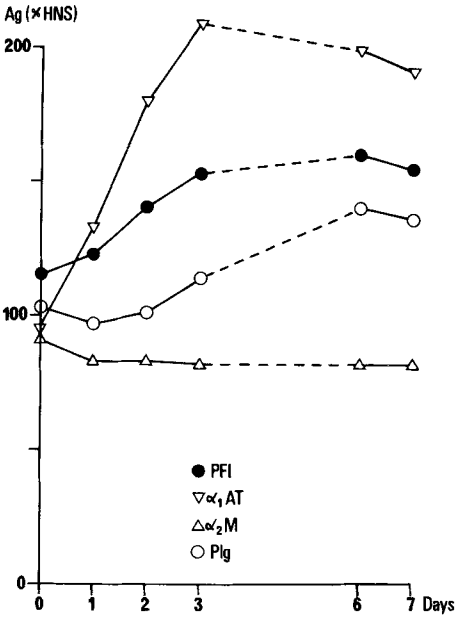

Fig.2 Posttraumatic response of fibrinolysis inhibitors and plasminogen. Mean of antigen concentration (Ag) in percentage of human normal serum (HNS).

Fibrinolytic parameters in serum: It was found that both FIA and AP (10 patients) were increased after the surgical trauma (Fig.1).

α_1AT was strongly and PFI moderately increased posttraumatically, while α_2M decreased slightly to a constant level (Fig.2).

Plg was increased after day 3.

Quenching of serum with antibodies against PFI abolished the FIA (Fig.3) to a lysis time below that in the samples containing buffer alone, due to the endogenous plasminogen. It was specially noted that the FIA of the quenched serum was independent of the level of FIA in the untreated serum.

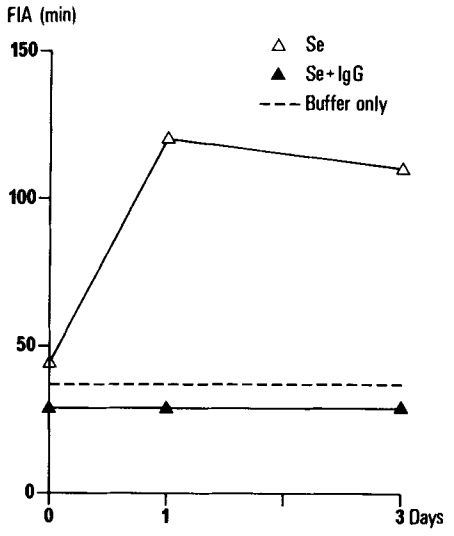

Fig.3 FIA in serum from one patient after quenching of the serum with antibodies (IgG) against PFI. FIA is expressed as lysis time, as the quenched sera lysed faster than the samples containing buffer alone.

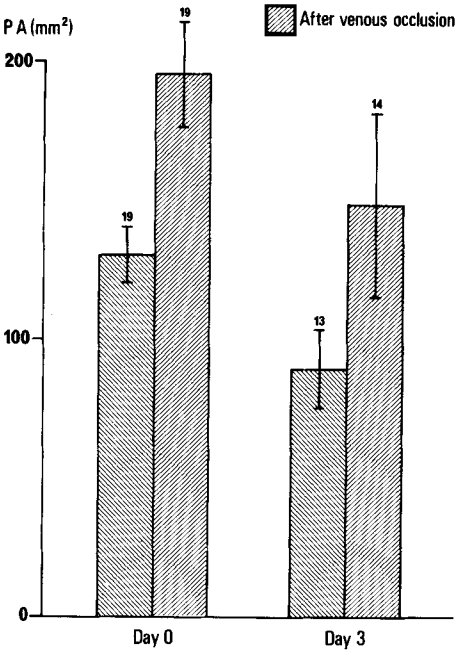

Fig. 4 Plasminogen activators (PA) before and three days after surgery. Mean ± S.E.M. of lysis area. The release is presented as the increase after venous occlusion.

PA in plasma was determined on day 0 (before surgery) and on the third day postoperatively. The level on day 3 was depressed, while the release due to venous occlusion was unaltered (Fig.4).

FIA of the Low Molecular Weight Fraction in Serum

Sera from nine patients were separated on Sephadex® G-25 Medium to a serum protein fraction and a low molecular weight fraction.

Whereas the protein fraction showed consistently lower FIA than the corresponding untreated serum (Fig.5) the posttraumatic increase was unaltered. Further, correlation analysis of FIA in serum versus FIA in serum protein fraction revealed that the loss of FIA accounted to about 40% (Fig.6).

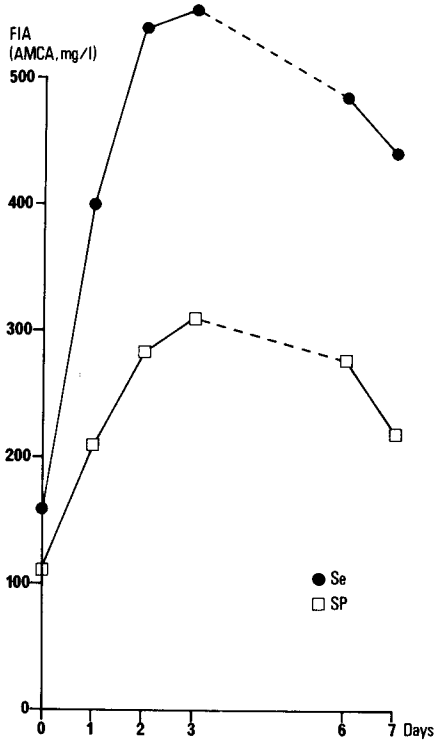

Fig.5 Mean of FIA in serum (Se) and serum protein fractions (SP) obtained after gel filtration on Sephadex® G-25 Medium.

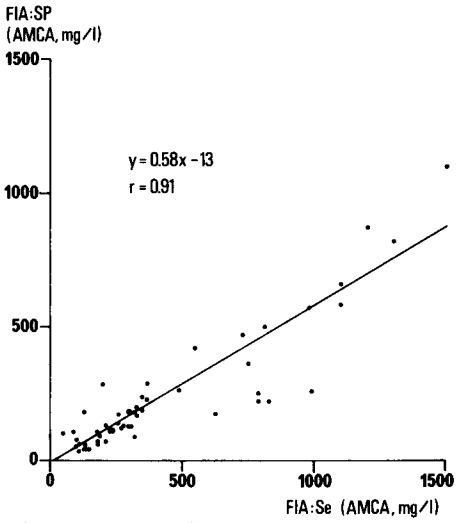

Fig.6 Correlation between FIA in serum (Se) and in serum protein fractions (SP).

Determinations of FIA were made in 1) LMW-fraction (alone), 2) LMW-fraction + serum protein fraction (retitration) and 3) LMW-fraction + HNS. No significant FIA was detectable in the LMW-fraction alone. The retitration with serum protein fraction resulted in restoration of FIA to almost the same level as that in the source serum. Addition of the LMW-fraction to HNS resulted in a constantly higher FIA (about 20%), as compared with HNS alone, but no post-

traumatic increase was noted.

Sera from seven patients were used.

Distributions and posttraumatic changes of FIA and PFI

At the first step the endogenous plasminogen was removed, resulting in a large increase in FIA in the eluted fraction (Fig.7). The posttraumatic rise occurred on the third day and was significant as compared to that in native serum ($p < 0.0005$). After adsorption to PFIα of the immobilized plasminogen, the FIA was reduced in the unbound fraction ($p < 0.005$) and was not significantly increased after trauma.

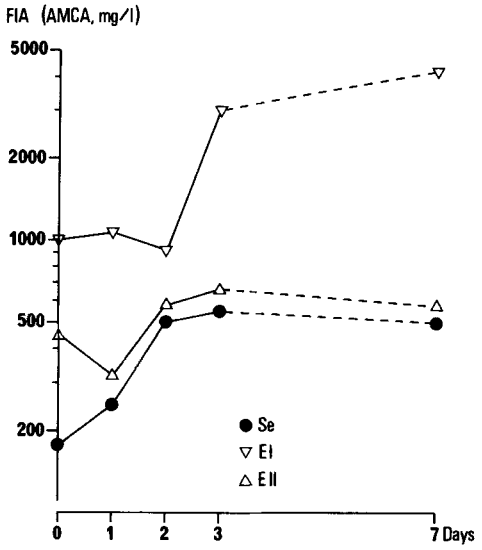

Fig.7 Posttraumatic patterns of FIA (Mean of seven patients) in serum (Se), plasminogen-depleted serum (EI) and the fraction not bound to the plasminogen (EII). Note the logarithmic scale of FIA.

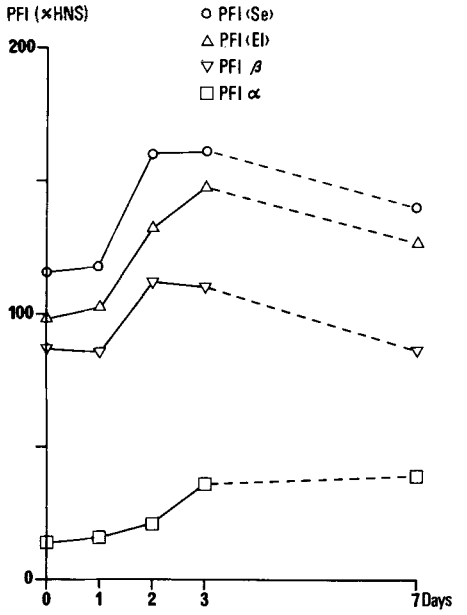

Fig.8 PFI in serum (Se) and plasminogen-depleted serum (EI), PFIβ not bound and PFIα bound to the immobilized plasminogen. In all fractions PFI is related to HNS. Mean of seven patients during the seven-day period after surgery.

The major part of PFI was not bound to the lysine matrix and the amount of trapped antigen was almost constant. Most of the PFI was not bound to the plasminogen matrix either. This form (PFIβ was moderately increased posttraumatically (Fig.8) but was not correlated to the FIA in plasminogen-depleted serum ($r = 0.1$).

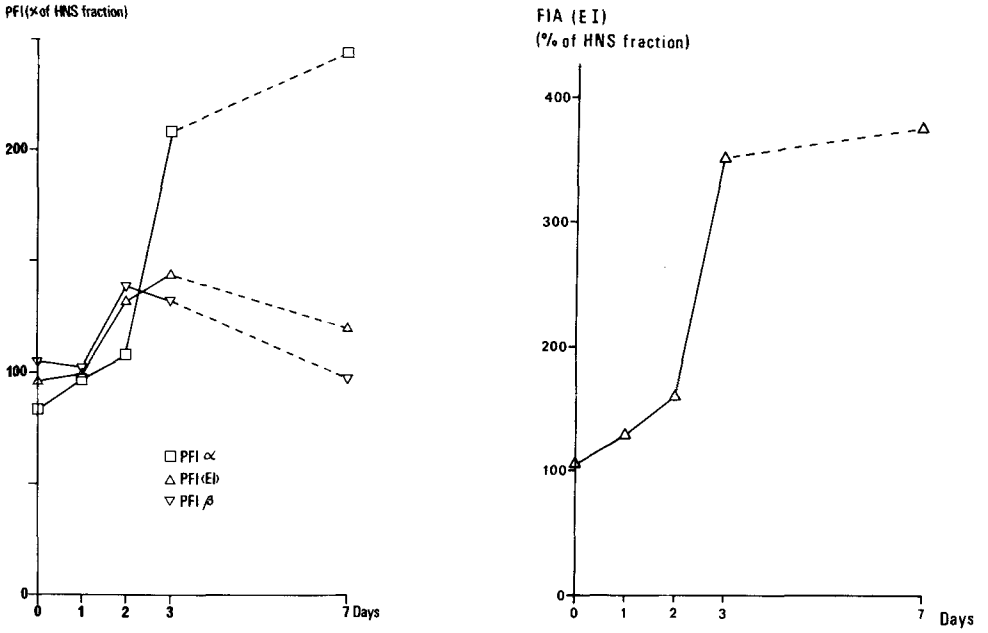

Fig.9 PFI and FIA in serum fractions after the surgical trauma as related to those in the corresponding fractions of HNS. Mean of seven patients.

A. PFI in plasminogen-depleted serum (EI), PFIβ and PFIα

B. FIA in plasminogen-depleted serum (EI)

Note the similar patterns of PFIα and FIA in the plasminogen-depleted serum (EI).

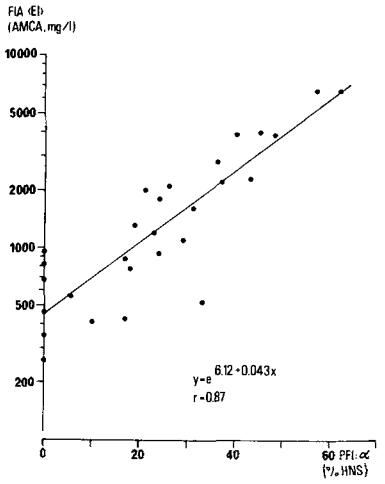

Fig.10 Correlation between log FIA in plasminogen-depleted serum (EI) and PFIα. Note the logarithmic scale of FIA and that PFIα is expressed in per cent of total PFI in HNS.

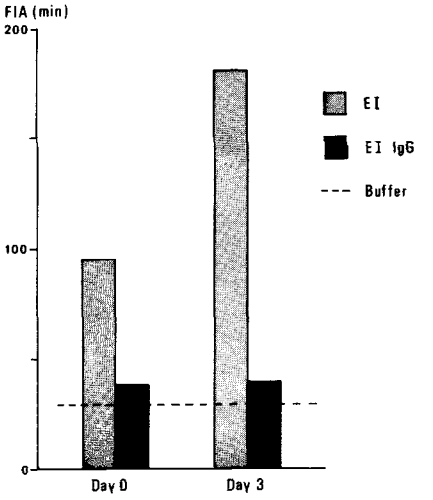

Fig.11 FIA in plasminogen-depleted serum (EI) from one patient before and after quenching with antibodies against PFI (IgG).

The minor, adsorbed part of the antigen (PFIα) showed the largest increment after trauma, which is particulary evident from Fig.9A ($p = 0.005$) and the pattern of the curve corresponded with that of the FIA (Fig.9B). PFIα was correlated to the logarithm of FIA (Fig.10).

Quenching of plasminogen-depleted serum with antibodies against the PFI almost abolished the FIA, even this was increased posttraumatically (Fig.11).

DISCUSSION

The findings reported above showed that the surgical trauma caused a strong inhibition of the fibrinolytic system, which was detectable both by determination of FIA and by measurement of AP.

On determination of the concentrations of the three probably most important fibrinolysis inhibitors (6), it was established that α_1AT was strongly and PFI moderately increased after trauma, while α_2M was slightly depressed.

It was found, however that the posttraumatic increase of FIA in serum and Plg-depleted serum was attributable to the PFI, as shown by the quenching with antibodies against this inhibitor.

This investigation has confirmed the existence of (at least) two forms of the PFI-related antigen, differing in affinity to immobilized plasminogen. The unbound PFIβ constituted the major part, showed weak FIA and small posttraumatic increase. The bound part, PFIα, showed the greatest increase after the surgical trauma and was obviously the main cause of the variations of FIA in plasminogen-depleted serum.

Comparison of the FIA in serum and plasminogen-depleted serum showed that the concentration of Plg strongly influenced the FIA assay, as was also indicated by the good correlation between PFIα and the logarithm of FIA in plasminogen-depleted serum.

Previously, Hedner and Nilsson (8) reported the probable existence of a low molecular weight component in serum acting as a fibrinolysis inhibitor. We found that serum depletion of the low molecular weight fraction resulted in a loss of FIA, but the posttraumatic increase in FIA was unaltered and mainly due to the increase in PFI. The approximately constant proportion of FIA lost after removal of the LMW-fraction indicated a potentiation of the increase in FIA derived from the PFI, due to the LMW-fraction.

Clemmensen has reported the finding of two different forms of α_2-antiplasmin (5) which is immunologically identical with the PFI. Precipitation of plasma with ammonium sulphate followed by ion exchange chromatography resulted in two

peaks of the inhibitor antigen, which differed in their affinity to plasminogen linked Sepharose and both of which had the ability to inactivate plasmin. About 40% of the total antigen was not bound to the matrix, which was less than our figure. This discrepancy can probably be explained by the different experimental conditions and not by insufficient binding of PFIα in this study, as we exploit less than 10 per cent of the capacity of the plasminogen matrix. The analytical scale of the chromatography procedures rendered direct determinations of the bound fractions difficult. However, the distributions of PFI and FIA were consistent with that of a preparative-scale procedure under equal conditions (2) where these parameters were quantified in all fractions.

Further studies on the two forms of PFI in patients at risk of developing the delayed microembolism syndrome following trauma or sepsis (3) have revealed that PFIα is mainly responsible for the total increase of the PFI-related antigen. It was also found that PFIα increased most strongly in the patients developing this syndrome and in these patients the serum level of PFIα could even exceed the level of PFIβ. The latter form, with low FIA, showed irregular and relatively small variations posttraumatically.

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