Inhibition of one-chain and two-chain forms of human tissue-type plasminogen activator by the fast-acting inhibitor of plasminogen activator in vitro and in vivo

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The inhibition of one-chain and two-chain molecular forms of human tissue-type plasminogen activator (t-PA) by the fast-acting inhibitor of plasminogen activator (PA-inhibitor) present in plasma was studied in vitro and in vivo in rabbits. In vitro, both one-chain and two-chain forms of t-PA were neutralized very rapidly in rabbit plasma with high levels of PA-inhibitor. The rate constant of the interaction between two-chain t-PA and PA-inhibitor was estimated to be 3.10^7 L/mol/sec. The presence of CNBr-digested fibrinogen, which mimics the effect of fibrin on the activation of plasminogen by t-PA, did not influence the rate constant. Moreover, PA-inhibitor–rich plasma inhibited in a very similar way in vitro thrombolysis by one-chain or two-chain t-PA incorporated into the clot. Injection of one-chain or two-chain t-PA into rabbits with increased levels of PA-inhibitor, induced by endotoxin, resulted in very rapid inhibition of t-PA activity. Within 30 seconds after injection, no residual free t-PA could be demonstrated. Gel filtration analysis showed that the disappearance of t-PA activity was associated with the generation of t-PA–PA-inhibitor complex with an apparent M, of 100,000. This enzyme-inhibitor complex, like free t-PA, was cleared from the circulation with a half-life of ~2 minutes, mainly via the liver. It is concluded that PA-inhibitor neutralizes one-chain and two-chain molecular forms of t-PA in plasma at very similar rates, both in vitro and in vivo. In this respect, the PA-inhibitor of plasma is different from that isolated from placenta tissue. (J LAB CLIN MED 1986; 108: 53-59)

Abbreviations: LPS = lipopolysaccharide; M_r = relative molecular mass; PA-inhibitor = fast-acting inhibitor of tissue-type plasminogen activator; t-PA = tissue-type plasminogen activator

Tissue-type plasminogen activator is a serine protease that converts plasminogen into plasmin, a relatively aspecific proteolytic enzyme that degrades fibrinogen, fibrin, and other plasma proteins.[1] Two molecular forms of t-PA have been isolated: a one-chain t-PA with M_r 70,000, which is the native form, and a two-chain proteolytic derivative, obtained by hydrolysis of the Arg 275–Ile 276 peptide bond.[2] In vitro and in vivo studies have shown that the two molecular forms of t-PA have very similar enzymatic,[3] turnover,[4] and thrombolytic properties.[5] Thus, unlike other serine proteases in which the single-chain form is the inactive precursor of the two-chain enzyme, one-chain t-PA has full enzymatic activity. It has, however, been reported that conversion of the one-chain form of t-PA into the two-chain form is associated with an increase of amidolytic activity.[6] Moreover, the rate of inhibition of two-

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chain t-PA by some synthetic inhibitors and by the plasma protease inhibitors α_2-antiplasmin and α_2-macroglobulin is faster than the rate of inhibition of one-chain t-PA.[4,6,7] Secretion of t-PA into the blood does not result in activation of the fibrinolytic system because, in the absence of fibrin, it has a very low affinity for plasminogen.[8] When an intravascular clot is formed, t-PA and plasminogen bind sequentially to fibrin resulting in very efficient generation of plasmin.[1,8]

PA-inhibitors have been identified in plasma,[9-13] in the culture medium of endothelial cells,[14-17] and in extracts of the placenta.[18-20] The plasma and endothelial cell PA-inhibitors have similar physicochemical properties[12] and are immunologically related,[21] but appear to be different from the placental PA-inhibitor.[12] All PA-inhibitors form relatively stable, inactive 1:1 molar complexes with t-PA.

Lecander et al.[22] showed that inhibition of one-chain t-PA by the placental PA-inhibitor occurs at a markedly slower rate than inhibition of the two-chain form, and suggested that this differential reactivity may play a role in the regulation of fibrinolysis.

Injection of low doses of endotoxin (1 μg/kg body weight) in rabbits induces a dramatic (10- to 40-fold) increase of PA-inhibitor activity in blood without being associated with significant changes in platelet count, activated partial thromboplastin time, or plasma fibrinogen levels.[23] The endotoxin-induced PA-inhibiting activity in blood is a result of the presence of a fast-acting protease inhibitor, as indicated by the following observations[23]: (1) inhibition of t-PA is associated with the formation of an inactive t-PA–PA-inhibitor complex with an apparent molecular weight of 100,000; (2) adsorption of plasma with t-PA–Sepharose results in disappearance of PA-inhibitor activity as demonstrated by functional assay and by gel filtration analysis. Finally, active site–blocked t-PA no longer binds to endotoxin-induced PA-inhibitor. The PA-inhibitor present in the blood of endotoxin-treated animals is most probably similar to the PA-inhibitor produced by endothelial cells, because endotoxin also enhances the accumulation of PA-inhibitor in the culture medium of cultured human endothelial cells.[23]

We have used this endotoxin-induced PA-inhibitor response to investigate the rate of inhibition of the two molecular forms of t-PA by PA-inhibitor in rabbit plasma in vitro and in vivo.

METHODS

Materials. One-chain and two-chain molecular forms of human t-PA were purified as described elsewhere.[24] Depending on the conditions of purification (presence of aprotinin), either one-chain or two-chain t-PA is obtained. Recombinant human one-chain and two-chain t-PA, obtained by expression of the t-PA gene in a mammalian cell system, was a gift from Genentech Inc., South San Francisco, Calif. (courtesy of Dr. C. F. Hoyng). The t-PA preparations used in the present study had a specific activity of 500,000 IU/mg as calculated by comparison with the International Reference Preparation for t-PA (83/517).[25] This specific activity is five times higher than that reported in our previous studies, where the International Reference Preparation of urokinase (66/46) was used as standard.

Human fibrinogen was purified and digested with CNBr as previously described.[26,27] Fibrinogen was labeled with iodine 125 by the iodo-gen procedure.[28] *Escherichia coli* O111:B4 LPS W was purchased from Difco Laboratories Inc., Detroit. The synthetic substrate D-Val-Leu-Lys-*p*NA (S-2251) was purchased from KabiVitrum, Amsterdam.

Irreversible blocking of the active-site histidine of t-PA was achieved by incubation (30 minutes at room temperature) with 2.10^{-4} mol/L (final concentration) D-Ile-Pro-Arg-CH_2Cl, a synthetic inhibitor of t-PA.[29]

In vitro experiments. Aliquots (0.5 ml) of citrated rabbit plasma were placed in a water bath at 37° C. t-PA (50 IU/ml final concentration) was added to each sample, followed, at fixed time intervals, by the addition of D-Ile-Pro-Arg-CH_2 Cl (2.10^{-4} mol/L final concentration) to stop the reaction between t-PA and PA-inhibitor. The presence of free t-PA and t-PA–PA-inhibitor complex was then monitored by gel filtration on Ultrogel AcA 44 (LKB, Bromma, Sweden). In some experiments, 1.5 μmol/L (final concentration) of human fibrinogen digested with CNBr was added to plasma 5 minutes before addition of t-PA.

PA-inhibitor–rich plasma was obtained from endotoxin-treated rabbits 3 hours after injection of 1 μg/kg body weight of *E. coli* LPS. A pool of plasma derived from six donor rabbits was prepared and stored at −20° C in 10 ml aliquots. PA-inhibitor levels, assayed as described below, were 15 U/ml in control and 170 U/ml in PA-inhibitor–rich plasma. No differences in plasminogen, fibrinogen, and α_2-antiplasmin were observed between control plasma and plasma obtained from endotoxin-treated animals.

The inhibition of the thrombolytic activity of fibrin-associated t-PA by PA-inhibitor was determined in an in vitro system consisting of a test tube containing a radiolabeled blood clot immersed in citrated rabbit plasma (2 ml). The labeled blood clot was produced as follows. Aliquots of 2 ml whole rabbit blood containing ^{125}I-fibrinogen (~200,000 cpm) was mixed with t-PA (40 IU/ml final concentration) and with 4 IU thrombin (Topostasin; Roche, Brussels). The mixture was quickly aspirated in silicon tubing (internal diameter 4 mm), and allowed to clot for 30 minutes at 37° C. Then pieces of 1.5 cm length were cut off, yielding labeled blood clots of ~0.2 ml. The clots were poured into Petri dishes, washed at room temperature for 15 minutes in 0.15 mol/L NaCl with several changes, and then immersed in control or PA-inhibitor–rich plasma. Alternatively, t-PA–free blood clots were prepared as outlined above but omitting t-PA. In these experiments, t-PA was added to the plasma surrounding the clot to a final concentration of 100 IU/ml. After isotope counting, the test tubes were placed in a water bath at 37° C. At intervals, 0.2 ml plasma samples were removed for radioisotope counting. The extent of thrombolysis was calculated from the amount of radioactivity released from the clot with correction

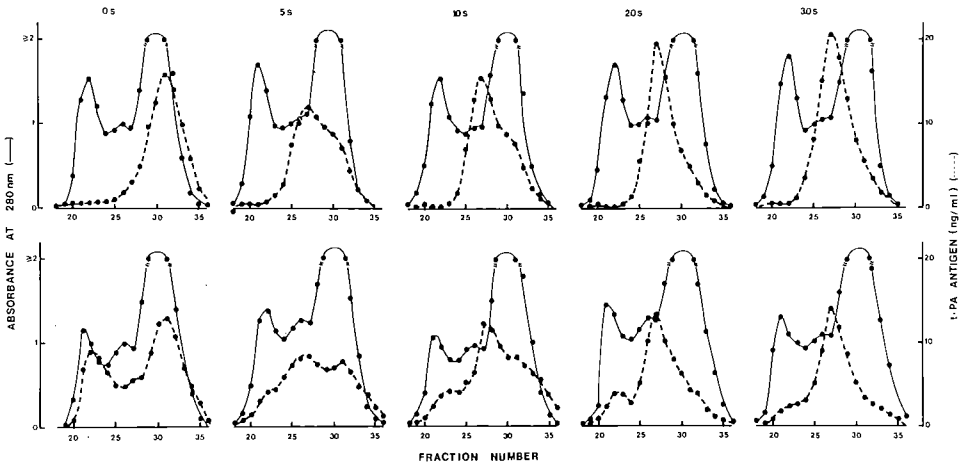

Fig. 1. Elution pattern on Ultrogel AcA 44 of PA-inhibitor–rich (170 U/ml) plasma incubated for different time intervals with two-chain *(upper panel)* or one-chain *(lower panel)* t-PA (50 IU/ml final concentration). After indicated incubation time (0 to 30 seconds), reaction was arrested by addition of active site inhibitor D-Ile-Pro-Arg-CH_2Cl to a final concentration of 2.10^{-4} mol/L. Similar elution patterns were obtained in each of three experiments. First peak at $A_{280\ nm}$ corresponds to void volume (macroglobulins), and main peak around fraction 30 to albumin.

for the reduction in volume and loss of radioactivity after removal of each sample.

In vivo experiments. New Zealand rabbits, weighing 1.9 to 2.5 kg, were anesthetized by intramuscular injection of 0.4 ml/kg body weight of Hypnorm (Duphar, Amsterdam) containing 10 mg/ml fluanisone and 0.2 mg/ml fentanyl. A catheter was introduced in a femoral vein for blood sampling. t-PA (7,500 IU/kg body weight) was injected via a marginal ear vein. At fixed time intervals, 2 ml blood samples were collected on trisodium citrate (final concentration 0.4%), and on citrate plus D-Ile-Pro-Arg-CH_2Cl (2.10^{-4} mol/L final concentration). Plasma was immediately prepared by short centrifugation (2 minutes at 12,000 × g) and kept on ice until tested. Functional hepatectomy was achieved by ligation of the hepatic artery and the portal vein. *E. coli* LPS (1 μg/kg body weight) was injected intravenously into conscious rabbits 3 hours before injection of t-PA.

Other assays and procedures. t-PA activity in plasma was measured by the fibrin plate method[30] as reported,[31] and t-PA–related antigen by using a two-site immunoradiometric assay.[32] PA-inhibitor activity was assayed by an amidolytic method as described elsewhere[23] and expressed in units of t-PA inhibited per milliliter. Gel filtration of plasma samples (0.5 ml) was performed on an Ultrogel AcA 44 column (30 × 1.6 cm) equilibrated with 0.02 mol/L Tris HCl, 0.3 mol/L NaCl buffer, pH 7.4, containing 0.01 mol/L trisodium citrate, and 0.02% Tween 80. The flow rate was 10 ml/hour, and fractions of 1 ml were collected. Adsorption of PA-inhibitor–rich plasma with t-PA–Sepharose was carried out as described elsewhere.[23]

RESULTS

All results reported below were obtained with recombinant t-PA, but very similar or identical results were obtained with natural t-PA isolated from melanoma cell culture medium.

In vitro studies. The inhibition of one-chain and two-chain t-PA, by PA-inhibitor–rich plasma in vitro, is illustrated in Fig. 1. When two-chain t-PA (50 IU/ml final concentration) was added to PA-inhibitor–rich plasma (170 U/ml) a very fast disappearance of free t-PA, eluting with an apparent M_r of 70,000, was observed on gel filtration. This was accompanied by the formation of t-PA–PA-inhibitor complex eluting with an apparent M_r of 100,000, which was identified by t-PA antigen assay. After 5 seconds, ~60% of t-PA–related antigen eluted as complex. Within 20 to 30 seconds virtually all t-PA was bound to the inhibitor. The disappearance rate of free t-PA was measured by planimetry of the surface under the curve representing t-PA antigen eluting in the position of free t-PA. Fig. 2 represents a plot of the residual free t-PA concentration vs. time. A semilogarithmic disappearance rate of free t-PA is observed as expected from the initial pseudo-first-order conditions. The half-life of t-PA was estimated to be ~5 seconds. No significant change in the elution pattern of two-chain t-PA was observed when the activator was added to normal plasma or to PA-inhibitor–rich plasma previously adsorbed with t-PA–Sepharose, or when active site–blocked t-PA was added to PA-inhibitor–rich plasma (not shown), establishing the validity of the applied method. These findings also demonstrate that, under our experimental conditions, no complex is formed between t-PA and other plasma protease inhibitors (e.g. α_2-antiplasmin, α_2-macroglobulin) or other plasma components.

The elution pattern of t-PA antigen after addition of one-chain t-PA to PA-inhibitor–rich plasma simultaneously with D-Ile-Pro-Arg-CH_2Cl (time 0) consisted

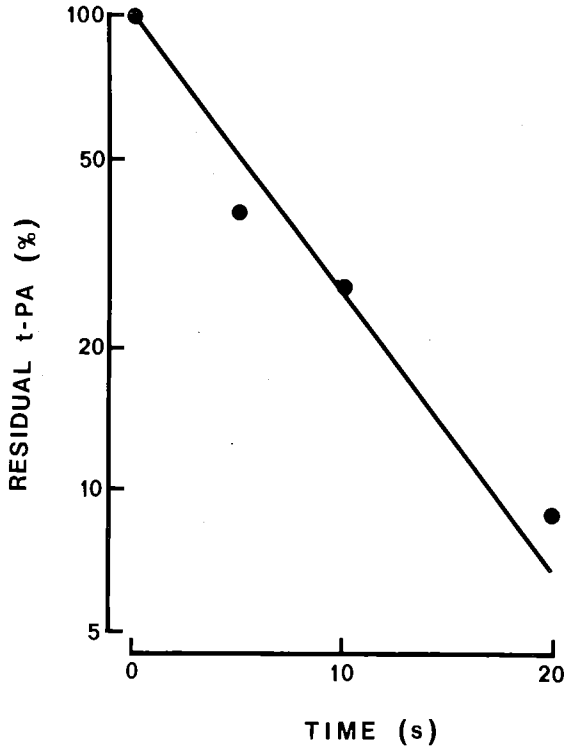

Fig. 2. In vitro disappearance rate of two-chain t-PA in PA-inhibitor–rich (5 nmol/L) rabbit plasma measured by gel filtration. Residual free t-PA at each time interval was calculated from area under curve (compare Fig. 1) and plotted against time. A half-life of 5 seconds corresponding to a second-order rate constant of 3.10^7 L/mol/sec is thus obtained.

of two main peaks, one eluting in the void volume of the column and one eluting in the albumin region. A similar profile was observed when one-chain t-PA was added to normal plasma, or when active site–blocked t-PA was added to PA-inhibitor–rich plasma (not shown). Incubation of active t-PA with PA-inhibitor–rich plasma, before the addition of the active site titrant, resulted in progressive disappearance of both peaks and formation of a new component eluting with an apparent M_r of 100,000. After 30 seconds, virtually all t-PA antigen eluted in this region. The rate of interaction between either molecular form of t-PA and PA-inhibitor in plasma was unchanged in the presence of 1.5 μmol/L CNBr-digested fibrinogen (not shown).

The inhibition, by PA-inhibitor, of in vitro thrombolysis induced by one-chain or two-chain t-PA, incorporated into blood clots, is illustrated in Fig. 3. When t-PA–containing clots were immersed in PA-inhibitor–rich plasma, significant thrombolysis was observed. The extent of clot lysis was, however, markedly reduced as compared with that of blood clots immersed in normal plasma. The delay of clot lysis induced by PA-inhibitor–rich plasma was similar for either molecular form of t-PA. When t-PA–free blood clots were immersed in PA-inhibitor–rich plasma and t-PA (one chain or two chain, 100 IU/ml) was subsequently added to plasma, no lysis was observed (<10% after 20 hours). In control plasma the same concentration of t-PA yielded 55.7% ± 5.3% lysis after 4 hours.

In vivo studies. Fig. 4 illustrates the disappearance rate of t-PA (antigen and activity) in rabbit plasma after injection of 7,500 IU of t-PA per kilogram of body weight (one-chain and two-chain forms). In control animals, fibrinolytic activity and t-PA antigen of both molecular forms of t-PA declined, with a half-life of about ~2 minutes. The results obtained with one-chain t-PA are represented in Fig. 4,*A*. In endotoxin-treated rabbits, t-PA antigen disappeared from plasma at a similar rate to that in control rabbits, but no residual activity (<1 IU/ml) could be measured 30 seconds after injection of either one-chain (Fig. 4,*B*) or two-chain t-PA. Injection of t-PA in endotoxin-treated rabbits with functional hepatectomy resulted in a very rapid disappearance rate of plasma fibrinolytic activity but not of t-PA antigen (Fig. 4,*C*).

Fig. 5 shows the elution patterns on Ultrogel AcA 44 of plasma samples obtained after injection of t-PA in control and endotoxin-treated animals. Plasma was obtained from blood samples collected on D-Ile-Pro-Arg-CH_2Cl to prevent in vitro interaction between t-PA and PA-inhibitor. Thirty seconds after injection of two-chain t-PA in control rabbits, t-PA antigen eluted as a single main peak in the albumin region, corresponding to unchanged activator with an M_r of ~70,000 (Fig. 5,*A*, upper panel). A similar elution pattern was obtained after injection of active site–blocked t-PA (not shown). In animals with high blood levels of PA-inhibitor, the two-chain t-PA eluted almost exclusively with an apparent M_r of 100,000 (Fig. 5,*B*, upper panel). However, when blocked activator was injected in endotoxin-treated animals, t-PA antigen eluted as a single peak in the albumin region (not shown), indicating that the active site is required for interaction with PA-inhibitor. Thirty seconds after injection of one-chain t-PA, the antigen eluted as two main peaks, one in the void volume of the column and one in the albumin region in control rabbits, but essentially as a single peak in the 100,000 M_r region in endotoxin-treated animals (Fig. 5, lower panels). Active site–blocked one-chain t-PA gave similar elution profiles (consisting of two peaks) in control and endotoxin-treated rabbits.

Similar elution profiles were obtained with serial plasma samples derived from hepatectomized rabbits (control and endotoxin treated) injected with either form of t-PA (not shown).

DISCUSSION

The data presented here indicate that the fast-acting PA-inhibitor present in plasma inhibits both one-chain

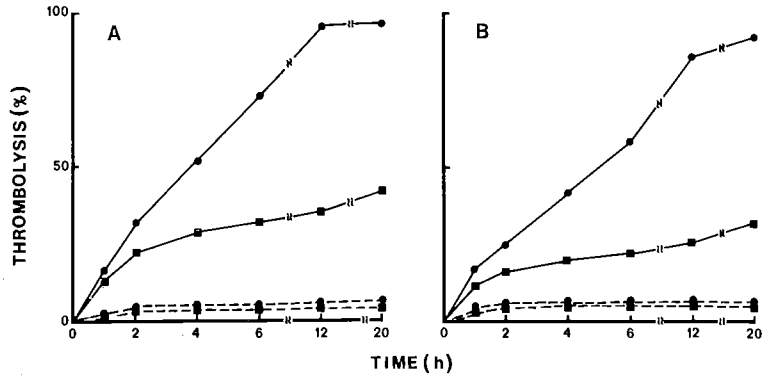

Fig. 3. In vitro thrombolysis induced by two-chain (**A**) or one-chain (**B**) t-PA incorporated into blood clots immersed in control (●) or PA-inhibitor–rich plasma (■) (see Methods for details). - - - -, Control experiments without added t-PA. Data represent mean values of two experiments.

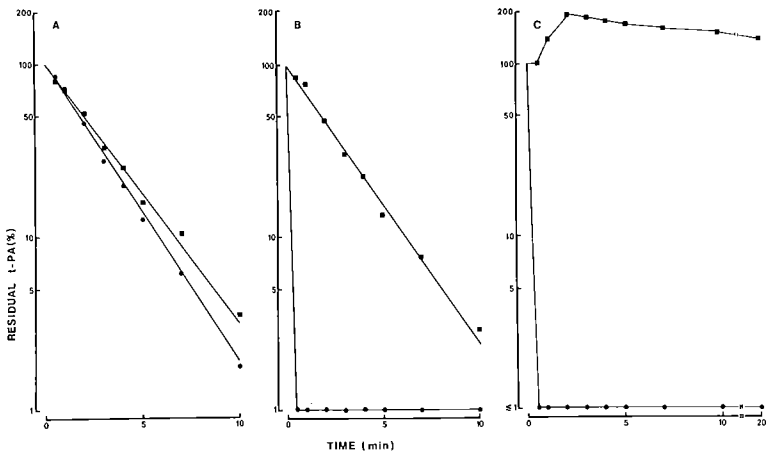

Fig. 4. Plasma disappearance curves of t-PA–related antigen (■) and activity (●) in rabbits injected with one-chain t-PA (7500 IU/kg body weight). **A,** Control rabbits. **B,** Endotoxin-treated rabbits. **C,** Endotoxin-treated rabbits with functional hepatectomy. Data are mean of two experiments. Similar results were obtained with two-chain t-PA.

and two-chain forms of t-PA at a similar rate. In vitro, the rate of inhibition of either molecular form of t-PA by PA-inhibitor–rich plasma is very rapid. In the present study, inhibition of t-PA was monitored by gel filtration analysis. This method, although not very accurate for kinetic analysis of the reaction rate, was selected because no simple methods are available for the very rapid quantitation of residual t-PA in plasma, and because the reaction between t-PA and PA-inhibitor can be stopped only by addition of a large excess of the active-site titrant D-Ile-Pro-Arg-CH_2Cl to plasma. Indeed, neither acidification nor dilution of plasma arrested the reaction efficiently.

When 50 IU of two-chain t-PA per milliliter was added to plasma containing 170 U/ml of PA-inhibitor, a half-life of t-PA activity of ~5 seconds was estimated. Thus the second-order rate constant of t-PA inhibition by the plasma PA-inhibitor is $\sim 3.10^7$ L/mol/sec, which is in good agreement with values reported earlier.[9,10] An estimate of the rate constant of the interaction between one-chain t-PA and PA-inhibitor is complicated by the heterogeneous elution profile of one-chain t-PA in rabbit plasma.[4] It is, however, very similar to that of two-chain t-PA, because complete neutralization of one-chain t-PA also occurs within 30 seconds. Under these experimental conditions there is no conversion of one-chain t-PA to the two-chain form. Previous studies have indeed shown that in plasma, at a t-PA concentration of 150 ng/ml, conversion of single-chain to two-chain t-PA does not occur within 1 hour.[3]

Estimation of the rate constant is only relevant if a single inhibitor is responsible for the neutralization of t-PA. Our data indicate that the protease inhibitors present in normal (PA-inhibitor–poor) plasma do not contribute to inhibition of t-PA. Indeed, neither inhibition of t-PA activity nor changes in its elution profile are

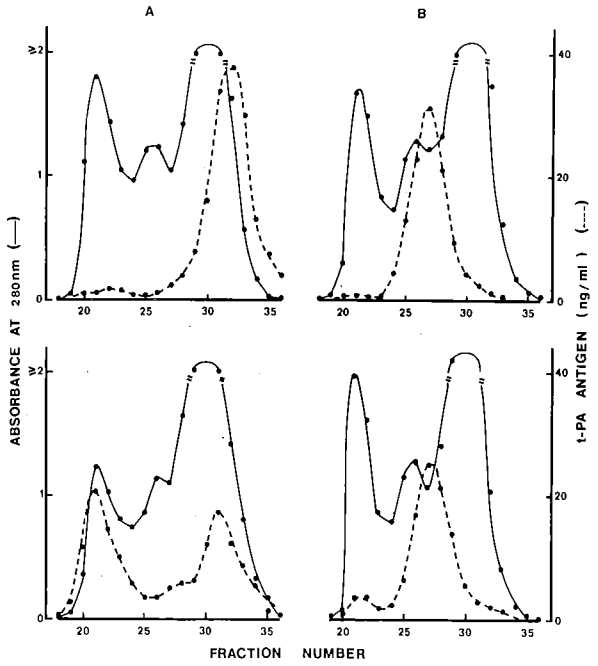

Fig. 5. Elution pattern on Ultrogel AcA 44 of plasma samples from control (**A**) and endotoxin-treated (**B**) rabbits 30 seconds after injection of two-chain *(upper panel)* or one-chain *(lower panel)* t-PA (7500 IU/kg body weight). Plasma was obtained from blood samples collected on D-Ile-Pro-Arg-CH_2Cl (2.10^{-4} mol/L final concentration). In three independent experiments, performed in each group, very similar patterns were obtained.

observed after addition of the activator to normal plasma or to PA-inhibitor–rich plasma adsorbed with t-PA–Sepharose,[23] a procedure that completely removes the PA-inhibitor activity but barely affects the antiplasmin activity. We observed that the endotoxin-induced PA-inhibitor activity is stable to acid pH and to sodium dodecyl sulfate treatment (data not shown), which is not characteristic of protease nexin.[33] Moreover, the observation that the half-lives of the two molecular forms of t-PA, in PA-inhibitor–rich plasma, are comparable makes it unlikely that a placental-like PA-inhibitor is induced by endotoxin. We therefore conclude that the endothelial cell type PA-inhibitor is responsible for the phenomena observed in the plasma of endotoxin-treated animals.

Fibrin has been shown to delay the inhibition of t-PA by PA-inhibitor.[9] The rate of inhibition of either molecular form of t-PA by PA-inhibitor–rich plasma in vitro, however, was not influenced by CNBr-digested fibrinogen, which contains fragments that mimic the effect of fibrin on the activation of plasminogen by t-PA.[27] This suggest that the interference with the inhibition of t-PA by PA-inhibitor requires a complex structure, present in the fibrin clot but not in the fibrinogen digest. Inhibition of t-PA incorporated into a blood clot was then studied in vitro by measuring the rate of lysis of labeled blood clots immersed in PA-inhibitor–rich plasma. Both one-chain and two-chain t-PA caused thrombolysis, although to a lesser extent than that observed in control plasma, suggesting that fibrin-bound t-PA is partially protected from inhibition by PA-inhibitor. The delay of thrombolysis induced by PA-inhibitor was similar for either molecular form of t-PA, indicating that the inhibition of the fibrin-associated activators occurs at a comparable rate. On the contrary, when t-PA was added to the plasma surrounding a blood clot (free of t-PA), no lysis was observed in the presence of PA-inhibitor, indicating that the fast inhibition of t-PA measured by gel filtration and by functional (amidolytic) assay is also associated with a loss of thrombolytic activity.

Intravenous injection of either molecular form of t-PA in rabbits with high blood levels of PA-inhibitor also revealed a very fast inhibition of t-PA, resulting in $<1\%$ residual free t-PA within 30 seconds. Indeed, gel filtration analysis of plasma obtained from blood samples collected on D-Ile-Pro-Arg-CH_2Cl, which efficiently arrests the t-PA–PA-inhibitor reaction, revealed that within 30 seconds after injection of t-PA (one-chain or two-chain), all t-PA–related antigen eluted with an apparent M_r of 100,000, corresponding to t-PA–PA-inhibitor complex.

The plasma disappearance rates of t-PA antigen in control and endotoxin-treated rabbits were very similar, indicating that the turnover rate of t-PA–PA-inhibitor complex is indistinguishable from that of the free enzyme. Moreover, clearance by the liver seems to be the main pathway of elimination of the complex, because functional hepatectomy markedly prolonged the half-life of t-PA antigen in endotoxin-treated rabbits. Under these conditions, t-PA antigen continued to circulate as a component with an apparent M_r of 100,000, indicating that no degradation or dissociation of t-PA–PA-inhibitor complex occurs in the circulation. Previous studies showed that active site–blocked t-PA is cleared at a similar rate to native t-PA.[3,34] These data, together with our present results, suggest that t-PA and t-PA–PA-inhibitor complex may clear via the same mechanism, involving determinants on the t-PA moiety independent of the active site of the enzyme.

In conclusion, our data indicate that both one-chain and two-chain molecular forms of either natural or recombinant human t-PA are inhibited very rapidly by the circulating PA-inhibitor. Rapid neutralization of t-PA in PA-inhibitor–rich plasma results in significant inhibition of thrombolysis by fibrin-bound t-PA in in vitro systems. Whether the presence of high levels of PA-inhibitor in plasma may interfere with physiologic or therapeutic thrombolysis in humans remains to be

further investigated. On the basis of our present findings we can, however, anticipate that the effect of PA-inhibitor on thrombolysis by t-PA will be similar for the one-chain and the two-chain forms of either natural or recombinant t-PA.

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