Biochemical and Biologic Properties of rt-PA del(K296-G302), a Recombinant Human Tissue-Type Plasminogen Activator Deletion Mutant Resistant to Plasminogen Activator Inhibitor-1

By X.-K. Li, H.R. Lijnen, L. Nelles, B. Van Hoef, J.M. Stassen, and D. Collen

A mutant of recombinant tissue-type plasminogen activator (rt-PA), obtained by deletion of residues Lys296 to Gly302 (rt-PA del[K296-G302]), was previously shown to be resistant to inhibition by plasminogen activator inhibitor-1 (PAI-1) (Madison et al., Nature 339:721, 1989). This mutant was obtained by expression of its cDNA in Chinese hamster ovary cells and purification to homogeneity from conditioned cell culture medium. It was obtained as a single chain molecule with amidolytic activity, specific fibrinolytic activity, and binding to fibrin and lysine, which were comparable or somewhat lower than those of wild-type rt-PA obtained in the same expression system. The plasminogen-activating potential of rt-PA del[K296-G302] in the presence of CNBr-digested fibrinogen was about twofold lower than that of wild-type rt-PA. The inhibition rate of rt-PA del[K296-G302] by recombinant PAI-1 (rPAI-1) was more than 500-fold lower than that of wild-type rt-PA. In a human plasma milieu in vitro, rt-PA del[K296-G302] induced dose-dependent lysis of a labeled plasma clot; equi-effective concentrations (causing 50% clot lysis in 2 hours) were 0.28 µg/mL and 0.36 µg/mL for mutant and wild-type rt-PA, respectively. In this system, addition of rPAI-1 to the plasma resulted in a concentration-dependent reduction of the fibrinolytic potency of rt-PA del[K296-G302] and of rt-PA; a 50% reduction required 2.4 µg/mL rPAI-1, respectively. Continuous infusion of mutant or wild-type rt-PA over 60 minutes in hamsters with a 125I-labeled plasma clot in the pulmonary artery resulted in dose-dependent clot lysis, with a thrombolytic potency (percent clot lysis per milligram of compound administered per kilogram of body weight) and a specific thrombolytic activity (percent clot lysis per microgram per milliliter steady state rt-PA–related antigen level in plasma) that were not significantly different. Bolus injection in hamsters of 1 mg/kg rPAI-1 followed by bolus injection of 1 mg/kg rt-PA del[K296-G302] or wild-type rt-PA resulted in neutralization of the thrombolytic potency of wild-type rt-PA, while the mutant retained approximately half of its thrombolytic potency. These results indicate that rt-PA del[K296-G302], with a known resistance to inhibition by rPAI-1 in purified systems, maintains this property both in a plasma milieu in vitro and in an experimental animal model of thrombolyis in vivo. In the absence of added rPAI-1, the thrombolytic potency and specific thrombolytic activity of rt-PA del[K296-G302] is comparable with that of wild-type rt-PA. Thus, rt-PA del[K296-G302] may offer advantages as a thrombolytic agent in clinical conditions in which high PAI-1 levels may contribute to resistance to thrombolysis or predispose to reocclusion.

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Proteins and reagents. Recombinant single-chain human t-PA (Actilyse) was obtained from Boehringer Ingelheim (Ingelheim, Germany), or was homemade by expression of t-PA cDNA in Chinese hamster ovary (CHO) cells as described elsewhere.\(^5\) Two-chain rt-PA was prepared by overnight treatment of rt-PA (final concentration, 25 \(\mu\)mol/L) at 4°C in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl, 0.1 mol/L arginine, and 0.01% Tween 80, with plasmin-sepharose (5% molar ratio), which was removed by filtration. Plasmin-sepharose was prepared from plasminogen-sepharose as described elsewhere.\(^6\) Human plasminogen, fibrinogen, and CNNBr-digested fibrinogen were obtained and characterized as described elsewhere.\(^11\) Recombinant PAI-1 (rPAI-1) was obtained and characterized as described previously.\(^8\) It was reactivated by incubation with 4 \(\mu\)mol/L guanidinium chloride for 20 minutes at 37°C, followed by overnight dialysis at 4°C against phosphate-buffered saline (PBS). Its specific activity, as determined by titration against rt-PA (Actilyse), ranged between 100,000 and 220,000 U/mg for different preparations. rt-PA-related concentrations reported in this study represent active protein.

Human plasma was pooled fresh frozen plasma from at least five healthy blood donors. Aprotinin (Trasylo) was purchased from Bayer (Leverkusen, Germany), D-Val-Leu-Lys-p-nitroanilide (S-2251) and D-Ile-Pro-Arg-p-nitroanilide (S-2288) from KabiVitrum (Brussels, Belgium), and D-Phe-Pro-Arg-CH\(_2\)Cl (PPACK) from Calbiochem (La Jolla, CA).\(^9\) PPACK was dissolved in 1 mmol/L HCl and its concentration was determined by amino acid analysis.\(^9\) \(\beta\)-labeled fibrinogen was purchased from Amersham (Buckinghamshire, UK). Restriction and DNA modifying enzymes were obtained from Gibco/BRL (Ghent, Belgium), Boehringer Pharma (Brussels, Belgium), or New England Biolabs (Boston, MA).

Construction and expression of rt-PA del(K296-G302) cDNA. The isolation of t-PA cDNA and the construction of pT-PAsh, containing the complete coding sequence, 9 nucleotides of 5'-untranslated sequence, and 61 nucleotides of 3'-untranslated sequence, has been described elsewhere.\(^8\) This t-PA cDNA was subcloned in Mi13mp18 and used as template DNA for subsequent mutagenesis. The chemically synthesized deletion oligonucleotide, 5'-ACAGGAAACCGCTCGGCAAAGATGGC-3' (complementary to nucleotides 1063 to 1074 and 1096 to 1108 of the t-PA cDNA sequence) was phosphorylated using T4 polynucleotide kinase. Mutagenesis and selection for mutant phage by in situ hybridization were performed as described.\(^11\) Positive plaques were selected for restriction enzyme digestion and sequencing to verify the mutation and to rule out any undesired mutations.

The expression plasmid containing the cDNA coding for rt-PA del(K296-G302) was used for stable transfection of CHO cells as described.\(^11\) Transfected cells were selected with 400 \(\mu\)g/mL Geneticin (Gibco/BRL). Geneticin-resistant colonies were screened for rt-PA-related antigen secretion by enzyme-linked immunosorbent assay (ELISA) and a suitable cell line was chosen for large scale production, as described previously.\(^11\)

Purification of rt-PA del(K296-G302) from conditioned cell culture medium. rt-PA del(K296-G302) was purified by chromatography on zinc chelate-sepharose essentially as described,\(^11\) followed by immunoadsorption on an insolubilized murine monoclonal antibody (MoAb) against t-PA (MA-1C8).\(^11\) Elution was performed with 1.6 mol/L KSCN and the fractions containing t-PA-related antigen were pooled and dialyzed against 0.3 mol/L NaCl, 0.1 mol/L arginine, 0.02 mol/L Tris-HCl buffer, pH 7.4, containing 0.01% Tween 80 and 10 KIU/mL aprotinin. Before use, aprotinin was removed by extensive washing on a Centricon 30 microconcentrator (Amicon, Danvers, MA) with 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl, 0.1 mol/L arginine and 0.01% Tween 80.

Assay techniques. DNA sequence analysis was performed according to Sanger et al.\(^11\) Amino acid analysis, after removal of arginine by extensive washing with 10% acetic acid on a Centricon 30 microconcentrator, was performed on a Beckman 119CL amino acid analyzer after 20 hours of hydrolysis in 6 mol/L HCl at 110°C in sealed ampoules. Specific fibrinolytic activities were determined on bovine fibrin plates\(^26\) by comparison with the 2nd International Reference Preparation for t-PA (code 86/670),\(^26\) obtained from the National Institute for Biological Standards and Control (London, UK). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% to 15% gradient gels without reduction or after reduction of the samples with dithioerythritol, using the Phast System (Pharmacia, Uppsala, Sweden). Total t-PA antigen was measured with a specific ELISA\(^26\) calibrated with rt-PA moieties of which the protein concentration was determined by amino acid analysis. Alternatively, t-PA protein concentration was determined with the Bradford assay.\(^25\) PAI-1 antigen, PAI-1 activity, and t-PA/PAI-1 complexes were determined with specific MoAb-based ELISAs.\(^27\) Fibrinogen levels in plasma samples were determined with a clotting rate assay\(^28\) and \(\alpha_2\)-antiplasmin levels with a chromogenic substrate method.\(^29\)

Activation of plasminogen. The initial rate of the activation of native plasminogen (final concentration, 5.0 \(\mu\)mol/L) by the rt-PA moieties (final concentration, 10 to 80 nmol/L) was measured by incubation at 37°C in 0.1 mol/L phosphate buffer, pH 7.3. The generated plasmin at different time intervals (0 to 5 minutes) was measured with S-2251 (final concentration, 0.3 \(\mu\)mol/L) after 25-fold dilution of the sample. Initial activation rates (\(v\), expressed as nanomoles per liter per second) were obtained from plots of the concentration of generated plasmin versus incubation time.

The catalytic efficiency for plasminogen activation by t-PA (\(k_v/K_m\)) was calculated from the formula

\[
v = \frac{k_v}{K_m + [S]} \cdot [S] \cdot [E]
\]

in which [S] and [E] are the plasminogen and t-PA concentrations, assuming that the [S] used (5.0 \(\mu\)mol/L plasminogen) can be neglected as compared to \(K_m\) (which is of the order of 100 \(\mu\)mol/L).\(^31\)

The effect of fibrin on the initial activation rate of plasminogen by the rt-PA moieties was evaluated by incubation of plasminogen (final concentration, 5.0 \(\mu\)mol/L) at 37°C in 0.1 mol/L phosphate buffer, pH 7.3, with CNNBr-digested fibrinogen (final concentration, 0.05 to 2.0 \(\mu\)mol/L) before the addition of the enzyme (final concentration, 20 nmol/L). Before use, arginine was removed from the rt-PA moieties by extensive dialysis. The plasmin generated (0 to 5 minutes) was measured as described above after 20-fold dilution of the sample, and initial activation rates were expressed as nanomoles per liter per second.

Alternatively, activation of plasminogen (final concentration, 1.0 \(\mu\)mol/L) by the rt-PA moieties (final concentration, 5 nmol/L) in the presence of CNNBr-digested fibrinogen (final concentration, 2.0 \(\mu\)mol/L) was monitored over a 30-minute period, as described above.
Kinetics of the inhibition of rt-PA del(K296-G302) by rPAI-1 or PPACK in purified systems. The inhibition rate of single-chain or two-chain forms of rt-PA del(K296-G302), wild-type rt-PA, or Actilyse by rPAI-1 was measured in the presence of S-2288 by continuous monitoring of the absorbance at 405 nm, as described by Chmielewska et al.27 All measurements were performed at 37°C in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl and 0.01% Tween 80 using S-2288 at a final concentration of 0.5 mmol/L. For the single-chain rt-PA moieties the concentrations of reagents used were 4 to 5 nmol/L enzyme with 5 to 6 mmol/L rPAI-1 for Actilyse and wild-type rt-PA and 120 nmol/L rPAI-1 for rt-PA del(K296-G302). For the two-chain rt-PA moieties, 7.0 nmol/L enzyme was used with 9 nmol/L rPAI-1 for Actilyse and wild-type rt-PA, and 2.6 nmol/L enzyme with 65 nmol/L rPAI-1 for rt-PA del(K296-G302). The second order association rate constant of the reactions (k2) was determined by using the classical equation for second order reactions (assuming a 1:1 stoichiometric interaction between rPAI-1 and enzyme) as described previously,27 or, for the experiments under pseudo-first order conditions, as the ratio of the pseudo-first order rate constant (determined from semilogarithmic plots of residual enzyme activity versus time) and the inhibitor concentrations used. Residual enzyme activity, determined at different time intervals, were corrected for the effect of S-2288 on the inhibition reaction,27 using the formula \[ [E] = [E]_i + b/[E]-b + [I]/K_{II}, \] with [E] = enzyme concentration at time t, [E]_i = initial enzyme concentration, b = slope of the absorbance versus time curve at time t, T_i = the initial slope; [S] = the substrate concentration; and K_{II} = the Michaelis constant of S-2288 for the different rt-PA moieties.

The inhibition rate of single-chain or two-chain forms of rt-PA del(K296-G302), wild-type rt-PA, or Actilyse by PPACK was measured under pseudo-first order conditions at 37°C in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl and 0.01% Tween 80. The enzymes were inactivated by the addition of D-Ile-Pro-Arg-CH,C1 (final concentration, 10 mmol/L) after 1 minute of incubation at 37°C, thrombin was inactivated by the addition of D-Ile-Pro-Arg-CH,C1 (final concentration, 10 mmol/L), and the samples were centrifuged for 1 minute at 10,000g. The concentration of rt-PA-related antigen in the supernatants was determined by ELISA.

Binding to lysine-Sepharose. The different rt-PA moieties (final concentration, 200 ng/mL), dialyzed extensively to remove arginine, were incubated on a tilting table for 2 hours at room temperature in PBS containing 1 mg/mL albumin and 0.01% Tween 80, with different amounts (0 to 333 μL) of a suspension of lysine-Sepharose (300 mg dry lysine-Sepharose/mL or 30%, wt/vol) in a total volume of 0.5 mL. After centrifugation, residual rt-PA antigen in the supernatants was measured by ELISA.

Fibrinolytic and fibrinogenolytic properties in a human plasma milieu in vitro. The relative fibrinolytic potency and fibrin specificity of the single-chain rt-PA moieties (final concentration, 0.025 to 6.4 μg/mL) was measured in a system composed of a 60 μL 125I-fibrin-labeled human plasma clot, suspended in 0.5 mL citrated human plasma, essentially as described elsewhere.28 The concentration of PA required to obtain 50% clot lysis in 2 hours (C_{50}) was determined from plots of percent lysis versus the concentration of PA. Residual fibrinogen levels at C_{50} were determined from plots of residual fibrinogen at 2 hours versus the concentration of PA. Fibrin clot lysis induced by an equipotent concentration of rt-PA moiety was also evaluated in human plasma supplemented with reactivated rPAI-1 (final concentration, 0.032 to 10 μg/mL).

In addition, systemic activation of the fibrinolytic system in normal human plasma by the single-chain rt-PA moieties (final concentration, 0.8 to 100 μg/mL) in the absence of fibrin was monitored by measuring residual fibrinogen levels. The concentration of PA required to obtain 50% fibrinogen degradation within 2 hours was determined graphically from dose-response curves.

Thrombolytic properties in hamsters with pulmonary embolism. The thrombolytic potency of single-chain rt-PA del(K296-G302) was compared with that of single-chain wild-type rt-PA and Actilyse in hamsters with experimental pulmonary embolism.29 In brief, a 25 μL 125I-fibrin-labeled human plasma clot was injected via the left jugular vein and clot lysis was performed by intravenous infusion over 60 minutes of PA at different doses, using a constant rate infusion pump. Thirty minutes were then allowed before the end of the infusion, the extent of clot lysis was determined as the difference between the radioactivity initially incorporated in the clot and that recovered ex vivo in the heart and lungs. An isotope recovery balance was made by adding the radioisotope content recovered in the heart and lungs and that in the blood (multiplied with a factor 3 to correct for extravasular distribution) at the end of the experiment. Blood samples of 0.2 mL were drawn into trisodium citrate (0.011 mol/L final concentration) for measurement of fibrinogen (at 0 and 90 minutes), α1-antiplasmin (at 0 and 90 minutes), and rt-PA-related antigen (at 60 minutes).

The thrombolytic potency (clot lysis versus dose) and the specific thrombolytic activity (clot lysis versus plasma antigen level) of the agents were determined as follows. The individual values of the percent lysis (parameter x) versus dose in milligrams of compound per kilogram of body weight (parameter x), or of the percent lysis (parameter y) versus steady state plasma level in micrograms per milliliter (parameter x) were fitted with an exponentially transformed sigmoidal function.

\[
y = \frac{100c}{1 + e^{-ax-b^2}}
\]
using the statistical program GraFit (Erithacus Ltd, Middlesex, UK). In this equation, the parameter a represents the reciprocal of the dose administered (for the thrombolytic potency) or of the plasma antigen level (for the specific thrombolytic activity). This procedure allows adequate fitting of exponential dose-response curves and furthermore allows to determine the mean plasma antigen level in micrograms per milliliter at which the rate of clot lysis is maximal and 

\[ z = \frac{ac}{4} \cdot c^b \]

(maximal rate of clot lysis, expressed as percent lysis per milligram of compound administered per kilogram of body weight or plasma antigen level in micrograms per milliliter at which the rate of clot lysis is maximal) and 

by external gamma counting, using a sodium iodide/thallium crystal. Blood samples for determination of t-PA-related antigen, rPAI-related antigen, or t-PA/PAI-I complex were collected on trisodium citrate (0.01 mol/L final concentration) containing PPACK (final concentration, 5 x 10^-5 mol/L) after 0.5, 1, 5, 10, 30, and 60 minutes. Blood samples without addition of PPACK were collected at time 0 and 90 minutes for determination of fibrinogen and α-antiplasmin levels.

**Pharmacokinetic properties in hamsters.** The pharmacokinetics of single-chain rt-PA del(K296-G302) were compared with those of wild-type rt-PA and Actilyse after bolus injection of 0.1 mg/kg in groups of three hamsters. t-PA-related antigen concentrations in plasma were measured at different time intervals and the results were plotted on semilogarithmic paper and fitted with a sum of two exponential terms, \( C(t) = Ae^{-k_1t} + Be^{-k_2t} \) by graphical curve peeling. Therefore, the linear terminal portion of the curves representing the logarithm of the antigen concentration versus time was extrapolated to yield the ordinate intercept B. This line had a slope \(-a\). The extrapolated values were subtracted from the initial values and these corrected data were fitted with a line that had a slope \(-a\) and an ordinate intercept A.

The following drug disposition parameters were calculated from the coefficients (A and B) and exponents (α and β) describing the disposition of t-PA-related antigen from plasma, using standard formulas derived by Gibaldi and Perrier: volume of the central compartment (in milliliters) \( V_c = (A + B) \); extrapolated area

| Table 1. Comparative Enzymatic Properties of Single-Chain and Two-Chain Forms of rt-PA del(K296-G302), Wild-Type rt-PA, and Actilyse |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Agent                      | Specific activity* (IU/mg)  | \( k_2 \) (s^-1)            | \( k_{2s} \) (mmol/L)       | \( k_2/K_{2s} \) (L·mmol^-1·s^-1) | \( v \) (nmol/L·s^-1) | Initial Rate of Plasminogen Activation† |
|                            |                             |                             |                             |                             |                             | No Fibrin | Fibrin | Stimulation Factor |
| Single-chain rt-PA del(K296-G302) | 120,000 ± 18,000           | 9                           | 1.0                         | 10                          | 0.017 ± 0.001               | 1.7         | 100    |
| Two-chain rt-PA del(K296-G302)   | 110,000 ± 24,000           | 21                          | 0.64                        | 33                          | 0.025 ± 0.001               | 1.8         | 73     |
| Single-chain rt-PA            | 210,000 ± 38,000           | 4                           | 0.81                        | 5                           | 0.23 ± 0.009                | 4.1         | 18     |
| Two-chain rt-PA               | 130,000 ± 10,000           | 7                           | 0.67                        | 11                          | 0.13 ± 0.003                | 1.0         | 8      |
| Single-chain Actilyse        | 460,000 ± 29,000           | 12                          | 0.42                        | 12                          | 0.54 ± 0.026                | 11          | 21     |
| Two-chain Actilyse            | 470,000 ± 11,000           | 21                          | 0.42                        | 50                          | 0.59 ± 0.048                | 8.6         | 15     |

*Specific activity determined on fibrin plates. Results are mean ± SEM of six determinations with two different preparations.
†Amidolytic activity against S-2298; the data are obtained by linear regression analysis (r > .99) using five or six substrate concentrations.
‡The initial rate (mean ± SEM of three determinations) of plasminogen activation (5.0 μmol/L) is expressed per 20 nmol/L of enzyme. The data at infinite fibrin concentration (= fibrin) are obtained by linear regression analysis (r > .96) using six or seven concentrations of CNBr-digested fibrinogen.

Fig 1. SDS-PAGE on 10% to 15% gradient gels without reduction (A) or after reduction with dithioerythitol (B). Lane 2, single-chain rt-PA del(K296-G302); lane 3, two-chain rt-PA del(K296-G302); lane 4, single-chain rt-PA; lane 5, two-chain rt-PA; lane 6, single-chain Actilyse; lane 7, two-chain Actilyse; lanes 1 and 8, protein calibration mixture consisting of phosphorylase b (M, 97,000), albumin (M, 67,000), ovalbumin (M, 45,000), carbonic anhydrase (M, 30,000), trypsin inhibitor (M, 20,100), and α-lactalbumin (M, 14,400).
PAI-1 RESISTANT t-PA

Fig 2. Activation of plasminogen (final concentration, 1.0 μmol/L) by t-PA moieties (final concentration, 5 nmol/L) in the presence of CNBr-digested fibrinogen (final concentration, 2.0 μmol/L). The concentration of generated plasmin is plotted versus time. (●) Single-chain rt-PA del(K296-G302); (△) two-chain rt-PA del(K296-G302); (■) single-chain wild-type rt-PA; (□) two-chain wild-type rt-PA; (◇) single-chain Actilyse; (∆) two-chain Actilyse.

under the curve (in μg · min · mL⁻¹) AUC = A/α + B/β; and plasma clearance (in mL · min⁻¹) Clₚ = dose/AUC.

The clearance (in mL · min⁻¹) during the steady state phase accompanying continuous intravenous infusion of the rt-PA moieties was also calculated from the ratio between the infusion rate (in nanograms per minute) and the steady state plasma concentration (in nanograms per milliliter), assuming a body weight of 100 g.

RESULTS

Construction and expression of rt-PA del(K296-G302) cDNA. The rt-PA del(K296-G302) cDNA was produced by oligonucleotide directed deletion mutagenesis as described under Materials and Methods. The mutation was confirmed by DNA sequence analysis. Then the cDNA for rt-PA del(K296-G302) was inserted into the eukaryotic expression vector pCMpneo, as described.* The sequence extending at least 80 bp on each side of the mutation was extended at least 80 bp on each side of the mutation was isolated and screened for t-PA-related antigen production by ELISA. The cell line secreting the highest amount of t-PA-related antigen was selected for large-scale production.

Purification and physicochemical characterization of rt-PA del(K296-G302). Chromatography of 10 or 34 L of conditioned medium (start concentration, 0.25 mg/L) on zinc chelate-sepharose yielded a 100- to 200-fold volume reduction. After immunoadsorption on MA-IC8-sepharose, the yield (mean of two preparations) was 0.12 mg/L, corresponding to an average recovery of 48%. Nearly homogeneous preparations with Mᵋ of about 70,000 were obtained, as shown by SDS-PAGE without reduction (Fig 1A, lane 2). The purified protein was obtained primarily in its single-chain form, as evidenced by SDS-PAGE after reduction with dithioerythritol (Fig 1B, lane 2). The amino acid composition of rt-PA del(K296-G302) was compatible with the published amino acid sequence (not shown). The specific fibrinolytic activities measured on fibrin plates were (mean ± SEM of six determinations with two preparations) 120,000 ± 18,000 IU/mg or 110,000 ± 24,000 IU/mg for single-chain or two-chain forms of rt-PA del(K296-G302), as compared with 210,000 ± 38,000 IU/mg or 130,000 ± 10,000 IU/mg for single-chain or two-chain forms of wild-type rt-PA expressed in the same system and to 460,000 ±

<table>
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<th>Agent</th>
<th>Time (min)</th>
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<td>26</td>
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<tr>
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<td>240</td>
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<tr>
<td>Two-chain Actilyse</td>
<td></td>
<td>140</td>
<td>190</td>
<td>185</td>
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<td>185</td>
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</table>

Table 3. Generation of t-PA/PAI-1 Complex (in Picomoles per Liter) After Addition of Single-Chain or Two-Chain rt-PA Moieties (Final Concentration, 1 nmol/L) to Normal Human Plasma Supplemented With rPAI-1 (Final Concentration, 0.7 nmol/L)

Table 2. Second-Order Association Rate Constant (kᵣ) for the Inhibition of Single-Chain or Two-Chain Forms of rt-PA del(K296-G302), Wild-Type rt-PA, and Actilyse by rPAI-1 or by PPACK

<table>
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<th>Agent</th>
<th>rPAI-1</th>
<th>PPACK</th>
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<tbody>
<tr>
<td>Single-chain rt-PA</td>
<td>del(K296-G302)</td>
<td>4.0 ± 0.58 x 10⁻⁵</td>
</tr>
<tr>
<td>Two-chain rt-PA</td>
<td>del(K296-G302)</td>
<td>14 ± 1.0 x 10⁻⁴</td>
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<tr>
<td>Single-chain rt-PA</td>
<td>del(K296-G302)</td>
<td>2.2 ± 0.64 x 10⁻⁴</td>
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<tr>
<td>Two-chain rt-PA</td>
<td>del(K296-G302)</td>
<td>1.6 ± 0.08 x 10⁻³</td>
</tr>
<tr>
<td>Single-chain Actilyse</td>
<td>del(K296-G302)</td>
<td>3.4 ± 0.25 x 10⁻⁴</td>
</tr>
<tr>
<td>Two-chain Actilyse</td>
<td>del(K296-G302)</td>
<td>4.1 ± 0.20 x 10⁻³</td>
</tr>
</tbody>
</table>

kᵣ is determined at 37°C in 0.05 mol/L Tris-HCl buffer pH 7.4 containing 0.038 mol/L NaCl and 0.01% Tween 80. The data are mean ± SD of the number of determinations indicated in parentheses.

Fig 3. Binding of single-chain rt-PA moieties to fibrin clots. (●) rt-PA del(K296-G302); (■) wild-type rt-PA; (◇) Actilyse. The results, expressed as percent t-PA-related antigen bound to the fibrin clot, represent mean values ± SD of three to five separate experiments.
29,000 IU/mg or 470,000 ± 11,000 IU/mg for single-chain or two-chain forms of Actilyse (Table 1). Our two-chain wild-type rt-PA thus has a lower specific activity than the single-chain wild-type rt-PA (P = .02), whereas this difference is not significant between single-chain and two-chain forms of rt-PA del(K296-G302) or Actilyse. Possibly, some rt-PA activity has been lost during plasmin treatment.

Amidolytic activity. The Kₐ and kₐ values for hydrolysis of the chromogenic substrate S-2288 by single-chain or two-chain rt-PA moieties, as determined by linear regression analysis of the data after Lineweaver-Burk transformation (r > .99), are summarized in Table 1. The affinity of all rt-PA moieties for S-2288 was comparable, as shown by Kₐ values ranging between 0.81 and 1.0 mmol/L for the single-chain molecules and between 0.42 and 0.67 mmol/L for the two-chain molecules. The catalytic efficiency (kₐ/Kₐ) of all two-chain forms was twofold to fourfold higher than that of the single-chain counterparts.

Activation of plasminogen. The catalytic efficiencies (kₐ/Kₐ) for plasminogen activation, calculated from the initial activation rates and the concentrations of enzyme and substrate used (Table 1), were (mean ± SEM; n = 3) 2.3 ± 0.09 × 10⁻³ or 5.4 ± 0.3 × 10⁻³ L · μmol⁻¹ · s⁻¹ for single-chain forms of wild-type rt-PA or Actilyse, respectively, as compared with 1.3 ± 0.03 × 10⁻³ or 5.9 ± 0.5 × 10⁻³ L · μmol⁻¹ · s⁻¹ for the corresponding two-chain forms. The catalytic efficiency for plasminogen activation of both single-chain rt-PA del(K296-G302) (0.17 ± 0.01 × 10⁻³ L · μmol⁻¹ · s⁻¹) and of its two-chain derivative (0.25 ± 0.01 L · μmol⁻¹ · s⁻¹) was fivefold to 10-fold lower than that of wild-type rt-PA. Addition of CNBr-digested fibrinogen resulted in a concentration-dependent stimulation of the initial activation rate of plasminogen by all rt-PA variants (not shown). The activation rate at infinite concentration of fibrin is determined from the inverse of the ordinate intercept in a double reciprocal plot of the activation rate versus the concentration of CNBr-digested fibrinogen (not shown). Table 1 summarizes these data and also shows the stimulation factor of CNBr-digested fibrinogen on plasminogen activation, obtained as the ratio of the initial activation rate in the presence of infinite fibrin concentration and the initial activation rate in the absence of fibrin. The initial rate of plasminogen activation in the presence of CNBr-digested fibrinogen was somewhat lower for rt-PA del(K296-G302) than for wild-type rt-PA, and was about fivefold lower than that of Actilyse.

When plasminogen activation in the presence of CNBr-digested fibrinogen was monitored over a longer time period, it appeared that both single-chain and two-chain rt-PA del(K296-G302) showed a clear lag phase (Fig 2). Maximal activation was obtained at later time points and was twofold to threefold lower than that of wild-type rt-PA or Actilyse, respectively.

Kinetics of the inhibition of rt-PA del(K296-G302) by rPAI-1 or PPACK in purified systems. The second-order rate constant for the inhibition of single-chain rt-PA del(K296-G302) by rPAI-1 was 560- or 850-fold lower than that for single-chain wild-type rt-PA or Actilyse, respectively; for the two-chain moiety these values were, respectively, 120- or 300-fold lower (Table 2). Inhibition of
were 120 minutes, 120 minutes, or 60 minutes for single-chain forms.

The inhibition rates were comparable with those of rt-PA and Actilyse.

The data represent mean ± SD of three separate experiments.

Inhibition of rt-PA del(K296-G302) in human plasma in vitro. After the addition to normal human plasma of single-chain or two-chain rt-PA moieties to a final concentration of 1 μg/mL, the plasma fibrinolytic activity, as measured in cuglobulin fractions on fibrin plates, decreased slowly as a function of time. Semilogarithmic plots of residual rt-PA activity (mean of three independent determinations) versus time were linear (not shown): half-life times for single-chain and two-chain rt-PA del(K296-G302), wild-type rt-PA, or Actilyse, respectively, as compared with 90 minutes, 90 minutes, or 50 minutes for the corresponding two-chain forms.

Addition of 1 nmol/L of the single-chain or two-chain rt-PA moieties to human plasma supplemented with 0.7 nmol/L rPAI-1 resulted in rapid generation of t-PA/PAI-1 complexes for single-chain and two-chain rt-PA or Actilyse, whereas for single-chain and two-chain rt-PA del(K296-G302), virtually no complex was detected after 15 minutes [10 pmol/L or 26 pmol/L for single-chain or two-chain rt-PA del(K296-G302) as compared with 8 pmol/L in the plasma before addition of PA] (Table 3).

Binding to fibrin. All single-chain rt-PA moieties showed a concentration-dependent binding to fibrin (Fig 3). Specificity of the binding is evidenced by the absence of binding of single-chain urokinase-type PA (not shown). At a fibrin concentration of 3.4 mg/mL, binding was (mean ± SD; n = 3) 97% ± 2% for rt-PA del(K296-G302) as compared with 91% ± 1% for rt-PA and 99% ± 2% for Actilyse.

Binding to lysine-sepharose. Figure 4 shows that all rt-PA moieties bind in a concentration-dependent way to lysine-sepharose with a maximal binding of about 60% at a comparable concentration of lysine-sepharose. Specificity of the binding is evidenced by the absence of binding of α2-antiplasmin (not shown).

Fibrinolytic and fibrinogenolytic properties in a human plasma milieu in vitro. All single-chain rt-PA moieties tested induced a time- and concentration-dependent lysis of a 125I-fibrin-labeled human plasma clot immersed in human plasma. Equi-effective concentrations (causing 50% clot lysis in 2 hours) (Fig 5A) were (mean ± SEM) 0.04 ± 0.004 μg/mL (n = 5) for rt-PA del(K296-G302), as compared with 0.36 ± 0.01 μg/mL (n = 3) for rt-PA and with 0.17 ± 0.006 μg/mL (n = 4) for Actilyse. At these concentrations of PA, residual fibrinogen levels after 2 hours were 93% ± 2%, 98% ± 1% or 90% ± 2% for rt-PA del(K296-G302), wild-type rt-PA, or Actilyse (Fig 5B).

Upon addition of reactivated rPAI-1 to human plasma, the fibrinolytic potency of an equi-effective dose of PA (yielding 75% clot lysis in 2 hours) was reduced to 50% with (mean of three determinations) 0.15 μg/mL rPAI-1 for wild-type rt-PA and with 0.16 μg/mL rPAI-1 for Actilyse, but this required 2.4 μg/mL rPAI-1 for rt-PA del(K296-G302) (Fig 6).

In the absence of fibrin, the addition of all rt-PA moieties

### Table 4. Clot Lysis and Hemostasis Parameters After Continuous Intravenous Infusion Over 60 Minutes of Single-Chain Forms of rt-PA Del(K296-G302), Wild-Type rt-PA, or Actilyse in Hamsters With Pulmonary Embolism

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (mg/kg)</th>
<th>Clot Lysis at 90 min (%)</th>
<th>Residual Fibrinogen (%)</th>
<th>Residual α2-Antiplasmin (%)</th>
<th>t-PA-Related Antigen (mg/mL)</th>
<th>CTP (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>—</td>
<td>20 ± 2 (16)</td>
<td>150 ± 10 (13)</td>
<td>110 ± 5 (10)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>rt-PA del(K296-G302)</td>
<td>0.25</td>
<td>37 ± 10 (3)</td>
<td>130 (1)</td>
<td>120 (1)</td>
<td>190 ± 18 (3)</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>rt-PA</td>
<td>0.50</td>
<td>52 ± 4 (4)</td>
<td>140 ± 12 (5)</td>
<td>130 ± 21 (4)</td>
<td>380 ± 36 (5)</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>59 ± 11 (5)</td>
<td>130 ± 10 (4)</td>
<td>100 ± 12 (4)</td>
<td>800 ± 130 (5)</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>0.003</td>
<td>33 ± 5 (3)</td>
<td>140 ± 5 (3)</td>
<td>80 ± 13 (3)</td>
<td>50 ± 15 (3)</td>
<td>2.8 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>42 ± 5 (4)</td>
<td>130 ± 15 (2)</td>
<td>120 ± 15 (4)</td>
<td>160 ± 46 (4)</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>0.250</td>
<td>50 ± 8 (4)</td>
<td>140 ± 28 (3)</td>
<td>100 ± 6 (2)</td>
<td>340 ± 96 (3)</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Actilyse</td>
<td>0.500</td>
<td>66 ± 5 (4)</td>
<td>120 ± 3 (4)</td>
<td>120 ± 25 (4)</td>
<td>470 ± 2 (4)</td>
<td>1.8 ± 0.1</td>
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<tr>
<td></td>
<td>0.032</td>
<td>34 ± 4 (9)</td>
<td>140 ± 9 (8)</td>
<td>110 ± 10 (7)</td>
<td>25 ± 3 (8)</td>
<td>2.3 ± 0.3</td>
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<tr>
<td></td>
<td>0.063</td>
<td>43 ± 3 (13)</td>
<td>120 ± 8 (10)</td>
<td>87 ± 7 (7)</td>
<td>37 ± 3 (11)</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>57 ± 4 (9)</td>
<td>150 ± 25 (7)</td>
<td>120 ± 9 (8)</td>
<td>90 ± 8 (9)</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0.250</td>
<td>76 ± 6 (8)</td>
<td>140 ± 6 (8)</td>
<td>120 ± 22 (6)</td>
<td>180 ± 26 (8)</td>
<td>2.9 ± 0.6</td>
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<tr>
<td></td>
<td>0.500</td>
<td>84 ± 6 (4)</td>
<td>120 ± 19 (4)</td>
<td>100 ± 8 (3)</td>
<td>320 ± 61 (4)</td>
<td>2.8 ± 0.4</td>
</tr>
</tbody>
</table>

The data represent mean ± SEM of the number of experiments indicated in parentheses.
induced a time- and concentration-dependent fibrinogen breakdown (not shown), with 50% fibrinogen degradation in 2 hours with (mean of three determinations) 28 µg/mL for rt-PA del(K296-G302), as compared with 5.6 µg/mL for rt-PA and with 5.2 µg/mL for Actilyse.

**Thrombolytic properties in hamsters with pulmonary embolism.** Dose-response curves of clot lysis after intravenous infusion over 60 minutes of single-chain forms of rt-PA del(K296-G302), wild-type rt-PA, or Actilyse in hamsters with pulmonary embolism induced with a human plasma clot are summarized in Table 4. All three PAs caused a progressive dose-dependent degree of clot lysis. Steady state t-PA-related antigen concentrations in plasma ranged between 0.19 and 0.80 µg/mL for rt-PA del(K296-G302) at doses between 0.25 and 1.0 mg/kg, as compared with 0.05 to 0.47 µg/mL with 0.063 to 0.50 mg/kg wild-type rt-PA and with 0.025 to 0.32 µg/mL with 0.032 to 0.50 mg/kg Actilyse. This resulted in 37% ± 10% to 59% ± 11% clot lysis with rt-PA del(K296-G302), 33% ± 5% to 66% ± 5% with wild-type rt-PA, and 34% ± 4% to 84% ± 6% with Actilyse. The isotope recovery balance ranged between 93% ± 6% and 120% ± 19% in all experimental groups, confirming that no significant parts of the thrombus were lost by embolization.

Fitting of the dose-response data with the individual values (clot lysis in percent versus dose in milligrams of compound administered per kilogram of body weight, or clot lysis in percent versus steady state plasma level of t-PA in micrograms per milliliter) with the exponentially transformed sigmoidal function (Fig 7) yielded values for c (maximal lysis achieved), b (dose or plasma antigen level at which maximal lysis occurs), and z (maximal rate of clot lysis), as summarized in Table 5. The thrombolytic potency of single-chain rt-PA del(K296-G302) was somewhat but not significantly lower than that of wild-type rt-PA, as shown by both a lower z-value (69% ± 20% v 150% ± 36% lysis per milligram of compound administered per kilogram of body weight; P = .045) and a higher b-value (0.151 ± 0.077 v 0.086 ± 0.028 mg/kg; P = .466). The specific thrombolytic activity of single-chain rt-PA del(K296-G302) was also somewhat lower than that of wild-type rt-PA, as shown by both a lower z-value (88% ± 23% v 160% ± 49% lysis per microgram per milliliter plasma level; P = .161) and a higher b-value (0.129 ± 0.065 v 0.065 ± 0.029 µg/mL; P = .413).

Actilyse had an approximately twofold higher thrombolytic potency than our homemade wild-type rt-PA (z = 310% ± 42% lysis per milligram of compound administered per kilogram of body weight [P = .025]; b = 0.061 ± 0.011 mg/kg [P = .316]) and an approximately threefold higher specific thrombolytic activity (z = 500% ± 79% lysis per microgram per milliliter plasma level [P = .100]; b = 0.032 ± 0.007 µg/mL [P = .126]). This difference, which is statistically not very significant, may be due to the different production and/or purification procedures.

No significant systemic activation of the fibrinolytic system was induced by either agent. Fibrinogen levels at the end of the experiments were even somewhat increased both in the control groups and in the experimental groups (Table 4). This finding may be the result of some hemodilution due to chasing of the plasma clot in the jugular vein with 1 mL saline. The sample used to determine the baseline fibrin-
Thrombolytic properties in hamsters supplemented with rPAI-1. Table 6 summarizes the results of clot lysis and hemostasis analysis obtained with rt-PA del(K296-G302) and Actilyse in hamsters with pulmonary embolism supplemented with rPAI-1. Bolus injection of reactivated rPAI-1 at a dose of 1 mg/kg (total protein concentration about 3 mg/kg) decreased clot lysis induced by subsequent bolus injection of 1 mg/kg rt-PA del(K296-G302) from 44% ± 6% (in the absence of rPAI-1) to 21% ± 1% (P = .009). Under the same experimental conditions, clot lysis induced by bolus injection of 1 mg/kg Actilyse was decreased from 69% ± 10% to 6% ± 1% (n = 4) after prior bolus injection of rPAI-1 (P < .001).

Spontaneous clot lysis with injection of saline was decreased from 19% ± 2% to 3% ± 1% (P < .001) upon bolus injection of rPAI-1. Whereas in the absence of rPAI-1 clot lysis obtained with both rt-PA del(K296-G302) and Actilyse was significantly above the background of spontaneous clot lysis (P < .001 both), clot lysis after prior bolus injection of rPAI-1 was only significantly above background for rt-PA del(K296-G302) (P < .001) but not for Actilyse (P = .078). Thus, in the presence of rPAI-1, bolus injection of rt-PA del(K296-G302) at a dose of 1 mg/kg induced significantly more clot lysis than Actilyse at the same dose (P < .001), whereas in the absence of rPAI-1, bolus injection of 1 mg/kg rt-PA del(K296-G302) induced somewhat less clot lysis than Actilyse (P = .072).

The isotope recovery balance in all experimental groups ranged between 98% ± 12% and 112% ± 6%. Fibrinogen and α2-antiplasmin levels, determined 90 minutes after the bolus injections, were not significantly different from the baseline values in the different groups (Table 6).

Figure 8 shows the time course of clot lysis (decrease of radioactivity in the clots) for the experiments without (Fig 8A) or with (Fig 8B) bolus injection of rPAI-1, as monitored continuously by external gamma counting. The extent of clot lysis at 90 minutes in the absence of rPAI-1 was 16% ± 2% for the control experiments, as compared with 46% ± 12% or 25% ± 5% for bolus injection of 1 mg/kg Actilyse or rt-PA del(K296-G302), respectively. After prior bolus injection of 1 mg/kg reactivated rPAI-1, no lysis was detected in the experiments with saline or Actilyse. At 90 minutes, the measured radioactivity was actually somewhat higher than at the beginning of the experiments. Bolus injection of 1 mg/kg rt-PA del(K296-G302) after prior bolus injection of rPAI-1 still resulted in significant clot lysis as monitored by external gamma counting (15% ± 1% at 90 minutes).

Table 6 also summarizes the levels of t-PA–related antigen (in micrograms per milliliter), PAI-1–related antigen (in micrograms per milliliter), and t-PA/PAI-1 complex (in micrograms of PAI-1 per milliliter) measured 1 minute and 30 minutes after the bolus injections. Immediately after bolus injection of 1 mg/kg rt-PA del(K296-G302) PAI-1–related antigen increased to approximately 30 μg/mL; after subsequent bolus injection of 1 mg/kg rt-PA del(K296-G302) t-PA/PAI-1 complex increased to 69 ± 10 μg/mL.

The data represent mean ± SEM, derived from the data in Table 4. The individual data were used for curve fitting.

Table 6. Clot Lysis and Hemostasis Parameters After Bolus Injection of 1 mg/kg rt-PA del(K296-G302) or Actilyse in Hamsters With Pulmonary Embolism, With or Without Prior Bolus Injection of 1 mg/kg rPAI-1

<table>
<thead>
<tr>
<th>Agent</th>
<th>Clot lysis at 90 min (%)</th>
<th>Residual Fibrinogen (%)</th>
<th>Residual α2-Antiplasmin (%)</th>
<th>t-PA–Related Antigen (μg/mL)</th>
<th>PAI-1–Related Antigen (μg/mL)</th>
<th>t-PA/PAI-1 Antigen (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min</td>
<td>30 min</td>
<td>1 min</td>
<td>30 min</td>
<td>1 min</td>
<td>30 min</td>
</tr>
<tr>
<td>With rPAI-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>3 ± 1 (4)</td>
<td>110 ± 5 (4)</td>
<td>88 ± 21 (3)</td>
<td>—</td>
<td>34 ± 7.4 (4)</td>
<td>6.1 ± 1.1 (4)</td>
</tr>
<tr>
<td>rt-PA del(K296-G302)</td>
<td>21 ± 1 (4)</td>
<td>110 ± 6 (4)</td>
<td>110 ± 16 (4)</td>
<td>8.8 ± 0.43 (4)</td>
<td>0.16 ± 0.01 (4)</td>
<td>32 ± 3.5 (4)</td>
</tr>
<tr>
<td>Actilyse</td>
<td>6 ± 1 (4)</td>
<td>110 ± 9 (4)</td>
<td>76 ± 5 (4)</td>
<td>5.3 ± 0.76 (4)</td>
<td>0.10 ± 0.01 (4)</td>
<td>27 ± 3.3 (4)</td>
</tr>
<tr>
<td>Without rPAI-1</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>19 ± 2 (12)</td>
<td>140 ± 13 (6)</td>
<td>120 ± 8 (5)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>rt-PA del(K296-G302)</td>
<td>44 ± 6 (4)</td>
<td>100 ± 7 (3)</td>
<td>91 ± 7 (3)</td>
<td>11 ± 0.96 (4)</td>
<td>0.26 ± 0.08 (4)</td>
<td>—</td>
</tr>
<tr>
<td>Actilyse</td>
<td>69 ± 10 (3)</td>
<td>110 ± 7 (3)</td>
<td>94 ± 5 (3)</td>
<td>9.8 ± 2.4 (3)</td>
<td>0.27 (1)</td>
<td>—</td>
</tr>
</tbody>
</table>

The data represent mean ± SEM of the number of experiments indicated in parentheses.
* Determined by the ex vivo isotope recovery method.
† Expressed as micrograms per milliliter rPAI-1 in the complex.
Thus, on a molar basis, 1 minute after bolus injection all the t-PA-related antigen in plasma was complexed with rPAI-1 for Actilyse, but only 26% was complexed for rt-PA del(K296-G302). Figure 9 shows the t-PA-related and the t-PA/rPAI-1 antigen levels as a function of time (0 to 10 minutes) for bolus injections of rt-PA del(K296-G302) or Actilyse in hamsters supplemented with rPAI-1.

Pharmacokinetic properties in hamsters. Table 7 summarizes relevant pharmacokinetic parameters describing the disposition of single-chain rt-PA del(K296-G302), wild-type rt-PA, or Actilyse from blood after bolus injection at a dose of 0.10 mg/kg in groups of three hamsters.

The plasma concentration of t-PA-related antigen after the bolus injection (C₀) increased to 1.3 ± 0.07 μg/mL for rt-PA del(K296-G302), as compared with 0.96 ± 0.12 or 1.2 ± 0.06 μg/mL for wild-type rt-PA or Actilyse, respectively. The disappearance rates of t-PA-related antigen (Fig 10) could be described by a sum of two exponential terms by graphical curve peeling. This yielded the coefficients and exponents reported in Table 7 together with the derived pharmacokinetic parameters. The results indicate that all three rt-PA moieties have a rapid clearance of about 2 mL/min as a result of a short initial t½ of 1.0 to 1.4 minutes.

Plasma clearances derived from the steady state t-PA-related antigen levels during the 60-minute infusion in the thrombolysis experiments ranged between 2.2 and 2.3 mL/min for different doses of single-chain rt-PA del(K296-G302), between 1.3 and 2.8 mL/min for wild-type rt-PA, and between 2.3 and 3.2 mL/min for Actilyse.

DISCUSSION

t-PA is rapidly inhibited in human plasma by a specific rapid reacting inhibitor, PAI-1, which is present in normal plasma at a very low concentration. In several pathologic conditions, including patients with coronary artery disease and acute myocardial infarction, significantly enhanced levels of PAI-1 have been reported. Plasma concentrations of t-PA achieved during thrombolytic ther-
apy in humans are several hundred fold higher than the plasma concentrations of PAI-1 activity. In addition, t-PA has a short half-life in humans, of the order of 5 minutes,\textsuperscript{15} mainly as a result of rapid hepatic clearance. These findings suggest that inhibition of t-PA by PAI-1 in circulating blood would not significantly affect the outcome of thrombolytic therapy. However, in as far as high PAI-1 levels may contribute to the occurrence of reocclusion,\textsuperscript{36} PAI-1-resistant mutants of t-PA may be useful for maintenance therapy. However, in as far as high PAI-1 levels may contribute to the occurrence of reocclusion,\textsuperscript{36} PAI-1-resistant mutants of t-PA may be useful for maintenance therapy.

Madison et al.\textsuperscript{7} have reported that substitutions or deletions in the region comprising residues 296 to 304 of t-PA confer resistance to PAI-1 as well as to the complex mixture of serpins in human plasma in vitro, without affecting the activity towards plasminogen. A mutant in which the three positively charged amino acids in this region are replaced by glutamic acid residues (t-PA K296E R298E R299E) was shown to be extremely resistant to inhibition by PAI-1.\textsuperscript{37} The region of PAI-1 that is proposed to interact with the contact between t-PA and PAI-1 is mediated by electrostatic interactions between loops of amino acids located on the surface of both proteins.\textsuperscript{38}

In the present study, we have constructed an rt-PA mutant, obtained by deletion of residues Lys\textsuperscript{395} to Gly\textsuperscript{392} [rt-PA del(K296-G302)], confirmed its biochemical properties, and evaluated its biologic properties in a plasma milieu in vitro and its thrombolytic and pharmacokinetic properties in vivo in a hamster pulmonary embolism model.

Purified rt-PA del(K296-G302) was obtained as a single-chain molecule with amidolytic activity against a chromogenic substrate for t-PA that is comparable with that of wild-type rt-PA. Its catalytic efficiency for plasminogen activation in the absence of fibrin and stimulation of the initial activation rate by fibrin were, however, significantly lower than those of wild-type rt-PA (Table 1). Prolonged activation of plasminogen (1 \textmu mol/L) in the presence of CNBr-digested fibrinogen (2 \textmu mol/L) showed a lag phase for both single-chain and two-chain rt-PA del(K296-G302) and a plasminogen activating potential that was twofold to threefold lower than that of wild-type rt-PA or Actilyse, respectively (Fig 2). This finding is in contrast with the report of Madison et al.\textsuperscript{7} that a similar mutant has unaltered affinity for plasminogen. These studies were, however, performed using Lys-plasminogen instead of native Glu-plasminogen, and with only one (saturating) concentration of desAAfibrin as stimulator. In agreement with the findings of Madison et al., the inhibition rate of rt-PA del(K296-G302), both in its single-chain and two-chain form, by PAI-1 in purified systems was several hundred fold lower than that of wild-type rt-PA. If added to plasma at low concentrations, complex formation of the mutant with PAI-1 was indeed found to be impaired as compared with wild-type rt-PA. However, when added to plasma at concentrations such as those achieved during thrombolytic therapy, the half-life of t-PA activity was not significantly different from that of wild-type rt-PA, indicating that under these conditions the mutant rt-PA is inhibited by the plasma protease inhibitors. These findings also suggest that rt-PA del(K296-G302) is a specific PAI-1-resistant variant that reacts normally with other serpins.

In a human plasma milieu in vitro, equi-effective concentrations (causing 50% clot lysis in 2 hours) were comparable for mutant and wild-type rt-PA (0.28 and 0.36 \textmu g/mL, respectively). Reduction of the fibrinolytic activity to 50% was achieved by the addition of 15-fold lower concentration of rPAI-1 for wild-type rt-PA as compared with rt-PA.

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**Table 7. Pharmacokinetic Parameters of the Disposition of Single-Chain Forms of rt-PA del(K296-G302), Wild-Type rt-PA, or Actilyse From Blood After Bolus Injections (100 \textmu g/kg) in Hamsters**

<table>
<thead>
<tr>
<th>Agent</th>
<th>(C_{p0}^\ast) (\textmu g/mL)</th>
<th>A (\textmu g/mL)</th>
<th>B (\textmu g/mL)</th>
<th>(\alpha) (min\textsuperscript{-1})</th>
<th>(\beta) (min\textsuperscript{-1})</th>
<th>(V_1) (mL)</th>
<th>(AUC) (\textmu g \cdot min \cdot mL\textsuperscript{-1})</th>
<th>(Cl_p) (mL \cdot min\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-PA del(K296-G302)</td>
<td>1.3 ± 0.07</td>
<td>1.10 ± 0.06</td>
<td>0.19 ± 0.01</td>
<td>1.4</td>
<td>10</td>
<td>0.50</td>
<td>0.069</td>
<td>7.8 ± 0.44</td>
</tr>
<tr>
<td>rt-PA</td>
<td>0.96 ± 0.12</td>
<td>0.77 ± 0.10</td>
<td>0.19 ± 0.02</td>
<td>1.0</td>
<td>15</td>
<td>0.69</td>
<td>0.046</td>
<td>11 ± 1.2</td>
</tr>
<tr>
<td>Actilyse</td>
<td>1.2 ± 0.06</td>
<td>0.82 ± 0.04</td>
<td>0.35 ± 0.02</td>
<td>1.4</td>
<td>6.0</td>
<td>0.50</td>
<td>0.12</td>
<td>8.7 ± 0.40</td>
</tr>
</tbody>
</table>

The data represent mean ± SEM of three experiments.

\(\ast C_{p0}\) represents the t-PA-related antigen concentration in a blood sample taken 30 seconds after bolus injection.

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**Fig 10.** Plasma disappearance rate of t-PA-related antigen after intravenous bolus injection of 0.1 mg/kg single-chain (•) rt-PA del(K296-G302); (■) wild-type rt-PA; or (▼) Actilyse in hamsters. The results, expressed in percent of the value of the first sample, are mean ± SEM of three experiments.
del(K296-G302), confirming the resistance of the mutant to PAI-1 in a plasma milieu.

In a hamster pulmonary embolism model, the thrombolytic potency (clot lysis per unit dose) and the specific thrombolytic activity (clot lysis per unit steady state plasma level of antigen) were not significantly different from those of wild-type rt-PA. As observed previously in this model, our homemade wild-type rt-PA is about twofold less active than Actilyse; therefore, the results obtained with the mutant are compared with those obtained with wild-type rt-PA that is produced in the same expression system. In hamsters supplemented with PAI-1 by bolus injection of 1 mg/kg reactivated rPAI-1 immediately before bolus injection of rt-PA del(K296-G302) or Actilyse, the thrombolytic potency of 1 mg/kg Actilyse was virtually abolished, whereas 1 mg/kg rt-PA del(K296-G302) retained approximately half of its thrombolytic potency.

These results suggest that, in the presence of high PAI-1 levels, the thrombolytic potency of rt-PA del(K296-G302) is superior to that of wild-type rt-PA, whereas in the absence of PAI-1 both are equipotent. Whether the clinical use of such PAI-1-resistant rt-PA mutants would be associated with less PAI-1-mediated reocclusion after thrombolysis and/or with a better thrombolytic potency towards platelet-rich blood clots (with high PAI-1 levels) remains to be investigated.

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PAI-1 RESISTANT t-PA

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Biochemical and biologic properties of rt-PA del (K296-G302), a recombinant human tissue-type plasminogen activator deletion mutant resistant to plasminogen activator inhibitor-1

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