Effective Thrombolysis Without Marked Plasminemia After Bolus Intravenous Administration of Vampire Bat Salivary Plasminogen Activator in Rabbits

Stephen J. Gardell, PhD; Denise R. Ramjit, BS; Inez I. Stabilito, BS; Tsuneo Fujita, MS; Joseph J. Lynch, PhD; Gregory C. Cuca, MS; Deepak Jain, PhD; Shiping Wang, MS; Jwu-sheng Tung, PhD; George E. Mark, PhD; and Ronald J. Shebuski, PhD

Background. The use of recombinant tissue-type plasminogen activator (t-PA) in thrombolytic therapy is frequently associated with significant fibrinogenolysis. In contrast, recombinant vampire bat salivary plasminogen activator (Bat-PA) displays strict fibrin specificity, an attribute that could be desirable in a fibrinolytic agent.

Methods and Results. The efficacy and fibrin selectivity of Bat-PA was evaluated and compared with that of t-PA using a rabbit model of femoral arterial thrombosis. Administration of 8.1, 14, and 42 nmol Bat-PA/kg by bolus intravenous injection restored flow in 50%, 75%, and 80% of the rabbits, respectively. The incidence of reperfusion after bolus intravenous injection of 14 and 42 nmol t-PA/kg was 15% and 78%, respectively. The maximal femoral artery reperfusion flows were equivalent after treatment with 42 nmol Bat-PA/kg or 42 nmol t-PA/kg, but the time to reach maximal flow for Bat-PA was approximately one half that of t-PA. Furthermore, the rapid restoration of flow by 42 nmol Bat-PA/kg, in contrast to equimolar t-PA, was accomplished without fibrinogenolysis and with only small decreases in the plasminogen and α2-antiplasmin levels. Equipotent doses of Bat-PA and t-PA both resulted in approximate 2.5-fold increases in the template bleeding times of aspirin-pretreated rabbits. The clearance of Bat-PA from rabbits exhibited biexponential elimination kinetics; approximately 80% was cleared by the relatively slow β phase (half-life of 17.1 minutes). Overall, Bat-PA was cleared approximately fourfold slower than t-PA.

Conclusions. Bolus intravenous administration of Bat-PA would facilitate prompt initiation of thrombolytic therapy, and the avoidance of plasminemia could result in fewer and less severe bleeding complications. (Circulation 1991;84:244–253)

The salutary effect of thrombolytic therapy depends on the timely administration of a fibrinolytic agent soon after the onset of the ischemic event. Hence, there has been interest in establishing therapeutic regimens for the facile and rapid introduction of plasminogen activators (PAs) by intravenous or intramuscular bolus administration.

From the Departments of Biological Chemistry (S.J.G.), Pharmacology (D.R.R., I.I.S., T.F., J.J.L., R.J.S.), and Cellular and Molecular Biology (G.C.C., D.J., S.W., J-S.T., G.E.M.), Merck Sharp & Dohme Research Laboratories, West Point, Pa., and Rahway, N.J.

Presented in part at the Tenth International Congress on Fibrinolysis, Indianapolis, Ind., August 1990.

Address for correspondence: Stephen J. Gardell, PhD, Department of Biological Chemistry, Merck Sharp & Dohme Research Laboratories, West Point, PA 19486.

Received November 21, 1990; revision accepted February 26, 1991.

Bolus intravenous administration of a PA not only hastens the initiation of therapy but may also improve efficacy, as suggested by the results of recent clinical studies indicating that rapidly achieving a high concentration of tissue-type plasminogen activator (t-PA) enhances thrombolysis. t-PA is not ideally suited for dosing by bolus intravenous administration because of its relatively rapid clearance. t-PA variants that are eliminated slower than the natural protein are currently being developed and may be eventually useful for this dosing schedule. An alternative strategy to circumvent the dosing restriction imposed by the rapid clearance of t-PA is to administer larger amounts; however, this option is generally avoided because of the fear of catastrophic hemorrhagic complications.

See p 436
The spontaneous, internal bleeding complications that can accompany thrombolytic therapy probably result from degradation of blood coagulation factors and vascular injury. Spontaneous hemorrhagic complications attendant to fibrinolytic therapy have been shown to be correlated with elevated fibrinogen degradation products\textsuperscript{11}–\textsuperscript{13} and prolonged template bleeding times.\textsuperscript{14} Interestingly, short courses of thrombolytic therapy appear to induce less serious bleeding than regimens involving prolonged administration of the fibrinolytic agent.\textsuperscript{15}

A PA that exhibits strict selectivity toward fibrin-bound plasminogen may cause fewer and less severe bleeding complications by avoiding the perilous consequences of plasminemia. Accordingly, t-PA was predicted to be a safer thrombolytic agent than streptokinase largely because of the relative preference of t-PA for fibrin-bound plasminogen.\textsuperscript{16} However, the results of numerous clinical investigations have shown that the use of t-PA is frequently associated with a significant, although variable, degree of fibrinogenolysis.\textsuperscript{12,17} The pronounced systemic effect of t-PA may arise from the ability of fibrinogen in a plasma milieu to serve as a cofactor for t-PA activity.\textsuperscript{18} In contrast to t-PA, the PA present in vampire bat saliva (Bat-PA) exhibits a strict requirement for a fibrin cofactor\textsuperscript{19} that is not satisfied by fibrinogen (P.W. Bergum and S.J. Gardell, manuscript in preparation). Furthermore, we have also shown that Bat-PA activity is quiescent in human plasma but levels of activity similar to that of t-PA are manifested in the presence of fibrin.\textsuperscript{20}

In the present study, a rabbit model of arterial thrombosis was used to initially evaluate the use of Bat-PA as a fibrinolytic agent. The efficacy and fibrin selectivity of Bat-PA were monitored as well as its pharmacokinetics and its effect on template bleeding times. Bat-PA exhibits remarkable selectivity toward fibrin-bound plasminogen in vivo despite a bolus intravenous injection dosing schedule. Hence, Bat-PA represents a unique opportunity to evaluate the importance of fibrin selectivity for the safe and efficacious implementation of thrombolytic therapy.

Methods

Plasminogen Activators

Recombinant t-PA (Activase), predominantly one-chain material, was obtained from Genentech, South San Francisco, Calif. t-PA was dissolved in buffer supplied by the manufacturer to a final concentration of 2 mg/ml (equivalent to 28 nmol/ml). Recombinant Bat-PA was produced by heterologous expression of the Bat-PA cDNA\textsuperscript{19} in an African Green Monkey kidney cell line (CV-1P) using a microcarrier bead process in spinner flasks (J.W. Tung et al, manuscript in preparation). Briefly, a vampire bat salivary gland cDNA library\textsuperscript{19} provided the template for polymerase chain reaction amplification of the cDNA encoding Bat-PA. The 5' oligonucleotide primer used for the polymerization chain reaction contributed the vertebrate consensus initiation flanking sequence, CCACC.\textsuperscript{21} Both the 5' and 3' oligonucleotide primers contained appended restriction endonuclease sites that enabled subcloning of the amplified product into the intermediate vector pSP73 (Promega, Madison, Wis.). One of these Bat-PA cDNA clones was chosen as the source of the insert DNA; it was sequence-verified and subsequently ligated into an HIV LTR promoter expression vector (pCD23). Calcium phosphate–mediated transfection of CV-1P cells constitutively expressing HIV-TAT led to the isolation of the clones stably expressing recombinant Bat-PA. The expressed protein was secreted into the conditioned media and purified by passage over an affinity column containing immobilized Erythrina trypsin inhibitor (Amer. Diagnostica, Inc., Green-which, Conn.).\textsuperscript{22} The purified Bat-PA preparation was dialyzed into a buffer containing 50 mM sodium acetate and 0.01% Triton X-100 at pH 5.0 (SAT buffer) and stored as a lyophilized powder at −70°C. Immediately before use, the Bat-PA was resuspended, and its concentration was determined using an extinction coefficient (1%, 1 cm, 280 nm) of 19.0. The extinction coefficient was derived by amino acid composition analysis and verified by active site titration using 4-methylumbelliferyl p-guanidinobenzoate.\textsuperscript{19} For comparative purposes, the PAs used in this study were administered on a molar basis rather than on a weight basis due to their dissimilar molecular mass values (Bat-PA and t-PA are approximately 50 and 70 kDa, respectively).

Rabbit Model of Peripheral Arterial Thrombosis

Male New Zealand White rabbits weighing 2.0–2.5 kg were anesthetized with sodium pentobarbital (35 mg/kg) administered via the marginal ear vein. The right carotid artery was cannulated for recording of arterial blood pressure with a Statham P50 transducer (Grass Instruments, Quincy, Mass.). A solution of sodium pentobarbital was infused continuously (6 mg/kg/hr in a volume of 0.6 ml/hr) through the carotid artery cannula to maintain the anesthetic level of the rabbit.

The establishment of an occlusive thrombus in the femoral artery was carried out essentially as described elsewhere.\textsuperscript{23} Briefly, the right femoral artery was isolated distal from the inguinal ligament and traumatized distal from the lateral circumflex artery by rubbing the artery on the jaw of a forceps. An electromagnetic flow probe (Carolina Medical Electronics, Inc., King, N.C.) was placed around the right femoral artery distal to the lateral circumflex artery to monitor femoral artery blood flow. The superficial epigastric artery was cannulated for induction of a thrombus. Thrombi were localized distal from the lateral circumflex artery with snares approximately 1 cm apart and induced by the sequential injection of 5 µl human thrombin (5 units), 5 µl of 0.25 M CaCl\textsubscript{2}, and 10–20 µl autologous arterial whole blood sufficient to distend the artery. After 30 minutes, the snares were released, and the flow was monitored for...
30 minutes to confirm that blood flow was completely obstructed by the thrombus.

Rabbits were randomized into seven treatment groups: 1) saline, n=12; 2) 14 nmol/kg t-PA, n=13; 3) 42 nmol/kg t-PA, n=18; 4) 4.7 nmol/kg Bat-PA, n=7; 5) 8.1 nmol/kg Bat-PA, n=6; 6) 14.0 nmol/kg Bat-PA, n=8; and 7) 42 nmol/kg Bat-PA, n=10. PAs were administered as bolus injections via the marginal ear vein at 60 minutes after the initiation of thrombus induction (30 minutes after removal of the femoral artery snares, as described above). Reperfusion, or restoration of femoral arterial blood flow, was defined as measurable flow that persisted for more than 3 minutes. At 120 minutes after the administration of PAs, the residual thrombus within the femoral artery was excised and blotted, and wet weight was determined. Data are expressed as mean±SEM. Among-group comparisons of incidences of reperfusion and residual thrombus mass were conducted using Fisher’s exact probability test or analysis of variance, followed by Dunnett’s test for multiple group comparisons, where appropriate.

Blood for the measurement of plasma fibrinogen concentrations and \(\alpha\)-antiplasmin and plasminogen activities was drawn from the right carotid artery cannula into EDTA-precocated tubes containing 5 \(\mu\)l of 1 \(\mu\)M D-Phe-Pro-Arg-CH\(_2\)Cl/ml blood (to prevent fibrinogen degradation) before (time 0) and at 30-minute intervals up to 2 hours after the administration of the PA. The samples were centrifuged at 4°C at 1,500g for 15 minutes, and the platelet-free plasma was stored in liquid nitrogen until assayed.

**Rabbit Bleeding Time Model**

Template bleeding times were measured in aspirin (ASA)-pretreated animals essentially as described elsewhere. Male New Zealand White rabbits (2–2.5 kg) were anesthetized with sodium pentobarbital (35 mg/kg) administered via the marginal ear vein. The right carotid artery was cannulated for recording the arterial blood pressure and for continuous intravenous infusion of sodium pentobarbital, as described previously. The carotid artery was also cannulated to withdraw blood samples for the determination of platelet counts. The lateral and medial aspects of both upper hind legs of the rabbits were shaved, and a depilatory agent (Nair, Carter Products, New York) was applied to prepare the skin for the determination of template bleeding times. Bleeding times were performed using a spring-loaded Simplate bleeding time device (Organon Teknika, Durham, N.C.). Uniform incisions were made with the Simplate device while avoiding superficial veins. Blood was blotted from the incision with filter paper every 30 seconds; care was taken to avoid contact with the incision. The bleeding time was determined by measuring the time from incision until blood no longer stained the filter paper.

Rabbits were administered ASA (15 mg/kg) via the marginal ear vein 60 minutes before the administration of test agent. Rabbits were randomized into four treatment groups: 1) ASA+SAT buffer (Bat-PA vehicle), n=5; 2) ASA+42 nmol/kg t-PA, n=5; 3) ASA+14 nmol/kg Bat-PA, n=5; and 4) ASA+42 nmol/kg Bat-PA, n=5. PAs or SAT were administered as bolus injections via the marginal ear vein (at time designated as 0 minutes). Template bleeding times were determined 5 minutes before ASA administration (control), 5 minutes before the administration of PA or SAT (post-ASA), and at 5, 15, 30, 60, 90, 120, and 180 minutes after administration of PA or SAT. Data are expressed as mean±SEM. Comparison of the control and post-ASA template bleeding times (n=20) was made by Student’s paired t test. Within-group comparisons of post-ASA bleeding times to post-ASA values were made using a one-way repeated-measures analysis of variance, followed by a Dunnett’s test for multiple comparisons to the corresponding post-ASA group. Blood was drawn from the carotid artery and anticoagulated with 3.8% trisodium citrate before ASA administration (control), immediately before PA or SAT administration (post-ASA), and at 15 and 60 minutes after PA or SAT administration. Whole-blood platelet counts were determined using a System 9000 cell counter (Baker Instrument Corp., Allentown, Pa.).

**Clearance of Bat-PA or t-PA in Rabbits**

Bat-PA or t-PA (2.2 nmol/kg) was administered to rabbits by bolus intravenous injection via the marginal ear vein. Blood samples (0.9 ml) were collected at various times through an indwelling catheter in the carotid artery into a syringe containing 0.1 ml of 3.8% trisodium citrate. Samples (0.5 ml) were acidified by the addition of 0.25 ml of 1 M acetic acid at pH 4.0 and centrifuged. The plasma was removed immediately and stored at −80°C until assayed for residual PA activity. PA activities were converted to functional molarities using standard activity curves generated with known concentrations of Bat-PA or t-PA. The elimination rates of Bat-PA or t-PA after their administration to rabbits were analyzed with an iterative nonlinear regression computer program that fitted the data to monoexponential and multiexponential decay functions (GraphPad Inplot, GraphPAD Software, San Diego). Pharmacokinetic parameters were calculated from the derived coefficients and exponents using standard formulas for the disposition of compounds from plasma after bolus injection.

**Analytical Methods**

**Plasminogen activator assay.** Fibrinogen, Glu-plasminogen, hirudin, Spectrozyme Pl, and two-chain t-PA activity standard were from American Diagnostica Inc., Greenwich, Conn., and thrombin was from Sigma Chemical Co., St. Louis. Solutions (190 \(\mu\)l) placed in the wells of a microtitration plate containing fibrinogen (0.132 mg/ml), Glu-plasminogen (0.66 \(\mu\)M), and thrombin (0.26 units/ml) in 10 mM HEPEs, 150 mM NaCl, and 0.24 mM Triton X-100 at pH 7.5 were incubated at 37°C for 30 minutes. Ten-microliters hirudin (200 units/ml) was added,
TABLE 1. Recanalization of Occluded Femoral Artery in Rabbits After Intravenous Bolus Administration of Tissue-Type Plasminogen Activator, Vampire Bat Salivary Plasminogen Activator, or Saline Control

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Incidence of reperfusion</th>
<th>Median time to reperfusion (min)</th>
<th>Residual thrombus mass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ratio %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>0/12 0</td>
<td>&gt;120</td>
<td>9.6±0.8</td>
</tr>
<tr>
<td>t-PA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 nmol/kg</td>
<td>2/13 15</td>
<td>&gt;120</td>
<td>8.2±0.7</td>
</tr>
<tr>
<td>42 nmol/kg</td>
<td>14/18 78</td>
<td>45</td>
<td>4.7±0.5†</td>
</tr>
<tr>
<td>Bat-PA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.7 nmol/kg</td>
<td>0/7 0</td>
<td>&gt;120</td>
<td>8.6±1.7</td>
</tr>
<tr>
<td>8.1 nmol/kg</td>
<td>3/6 50‡</td>
<td>109</td>
<td>6.3±1.6</td>
</tr>
<tr>
<td>14.0 nmol/kg</td>
<td>6/8 75*</td>
<td>35</td>
<td>6.5±2.0</td>
</tr>
<tr>
<td>42.0 nmol/kg</td>
<td>8/10 80*</td>
<td>26</td>
<td>2.6±0.4†</td>
</tr>
</tbody>
</table>

Residual thrombus mass values are mean±SEM. t-PA, tissue-type plasminogen activator; Bat-PA, vampire bat salivary plasminogen activator.

* p<0.01 vs. saline group by Fisher’s exact test; † p<0.01 vs. saline group by one-way analysis of variance, followed by Dunnett’s multiple comparison test; ‡ p<0.05 vs. saline group by Fisher’s exact test.

and the clots were incubated for an additional 30 minutes at 37°C. Thirty microliters of 3.33 mM Spectrozyme Pl and 20 μl PA were added, and the release of p-nitroaniline from the chromogenic substrate was measured spectrophotometrically using a Thermomax microplate reader (Molecular Devices Corp., Menlo Park, Calif.). The activities (IU/ml) were derived from a standard curve generated with a two-chain t-PA activity standard.

Miscellaneous ex vivo assays. Plasminogen levels were measured using the chromogenic substrate Spectrozyme Pl and urokinase (Calbiochem, Behring Diagnostics, San Diego). Plasma for this assay was acidified and neutralized to destroy plasmin inhibitors before testing. The activity of α2-antiplasmin was determined by a modification of the Coatest Antiplasmin method (KabiVitrum AB, Stockholm) using the chromogenic substrate S-2251. The results for plasminogen and α2-antiplasmin are expressed as a percentage of activity relative to control (pretreatment) activity. The fibrinogen concentration was determined by the Clauss thrombin-clotting method using the Electra 800 automated clot timer (Medical Laboratory Automation, Mt. Vernon, N.Y.) and commercially available reagents (American Dade, Aquada, Puerto Rico). Among-group comparisons of fibrinogen, plasminogen, and α2-antiplasmin values were performed by analysis of variance, followed by Dunnett’s test for multiple comparisons.

Results

Efficacy of Bat-PA In Vivo

The incidence and median time to reperfusion as well as residual thrombus wet weights after intravenous bolus administration of t-PA and Bat-PA to the rabbits are summarized in Table 1. No spontaneous reperfusion was observed in 12 saline-treated control rabbits. t-PA at an intravenous bolus dose of 1 mg/kg (or 14 nmol/kg) was essentially ineffective, eliciting restoration of blood flow in only two of 13 rabbits. However, the administration of t-PA at 42 nmol/kg resulted in successful reperfusion in 14 of 18 (78%) rabbits, with a median time to reperfusion of 45 minutes. Residual thrombus mass, determined at the termination of the study, was reduced significantly by the intravenous administration of t-PA (42 nmol/kg) compared with saline control (4.7±0.5 versus 9.6±0.8 mg, respectively, p<0.01). The intravenous administration of 8.1, 14, and 42 nmol Bat-PA/kg resulted in dose-dependent increases in reperfusion efficacy (successful reperusions: three of six rabbits [50%], six of eight rabbits [75%], and eight of 10 rabbits [80%], respectively), with median reperfusion times of 109, 35, and 26 minutes, respectively. Residual thrombus mass was reduced significantly by the intravenous administration of 42 nmol Bat-PA/kg com-

![Figure 1. Graph showing restoration of blood flow in the occluded femoral artery. Rabbits received 42 nmol/kg tissue-type plasminogen activator (●), 14 nmol/kg vampire bat salivary plasminogen activator (□), or 42 nmol/kg vampire bat salivary plasminogen activator (■) by intravenous bolus injection.](image-url)
pared with saline controls (2.6±0.4 versus 9.6±0.8 mg, respectively, p<0.01).

Figure 1 depicts femoral arterial reperfusion blood flows in rabbits treated intravenously with 42 nmol t-PA/kg, 14 nmol Bat-PA/kg, and 42 nmol Bat-PA/kg. Early femoral artery reperfusion blood flow profiles were similar for the 42 nmol t-PA/kg and 14 nmol Bat-PA/kg. The maximal femoral artery reperfusion blood flows achieved tended to be greater in rabbits treated with t-PA at 42 nmol/kg relative to those treated with a threefold lower dose of Bat-PA (14 nmol/kg), although the observed difference was not statistically significant. The administration of the higher dose of Bat-PA (42 nmol/kg) resulted in an earlier reestablishment of femoral artery reperfusion blood flow compared with 14 nmol Bat-PA/kg and 42 nmol t-PA/kg. This difference reflected the shorter median reperfusion time for 42 nmol Bat-PA/kg (26 minutes) compared with those of 14 nmol/Bat-PA/kg (35 minutes) and 42 nmol t-PA/kg (45 minutes) (Table 1). The maximal femoral artery reperfusion flows were equivalent in the preparations treated with t-PA or Bat-PA at 42 nmol/kg.

Fibrin Selectivity of Bat-PA In Vivo

Figure 2 shows the effects of the intravenous bolus administration of saline, Bat-PA (42 nmol/kg), or t-PA (42 nmol/kg) on plasma levels of clottable fibrinogen. During the course of the experimental protocol, plasma fibrinogen concentrations decreased maximally by approximately 6% (from 225.6±11.7 to 212.1±9.9 mg/dl) from control values in saline-treated rabbits. Plasma fibrinogen concentrations decreased maximally by approximately 14% and 69% from control values in rabbits treated with Bat-PA (from 238.3±17.8 to 204.9±11.2 mg/dl) and t-PA (from 231.6±6.1 to 71.2±13.1 mg/dl), respectively (Figure 2). The fibrinogen concentrations for each sampling time after the administration of t-PA, but not Bat-PA, were significantly less (p<0.01) compared with the corresponding values for the saline-treated rabbits.

Figure 3 shows the accompanying effects of saline, Bat-PA, or t-PA administration on the plasma levels of functional plasminogen and α2-antiplasmin. Plasminogen and α2-antiplasmin activities were expressed as a percentage of control, preadministration values (Figure 3). During the course of the experimental protocol, the plasma plasminogen activities were reduced to the following minimal values: 95.1±1.6% for the saline treatment group, 81.0±2.6% for the Bat-PA treatment group, and 26.5±2.6% for the t-PA treatment group (Figure 3, left panel). The minimal values for the plasma α2-antiplasmin activities were: 86.2±5.4% for the saline treatment group, 73.7±3.3% for the Bat-PA treatment group, and 35.2±3.1% for the t-PA treatment group (Figure 3, right panel). The reductions in plasminogen and α2-antiplasmin after the administration of t-PA were

FIGURE 2. Graph showing effect of vampire bat salivary plasminogen activator and tissue-type plasminogen activator administration on the plasma level of clottable fibrinogen. Rabbits containing an occluded femoral artery received 42 nmol/kg tissue-type plasminogen activator (●, n=18) or vampire bat salivary plasminogen activator (■, n=10) by bolus intravenous injection. Control rabbits received saline (▲, n=12). *p<0.01 vs. the corresponding saline-treatment sampling times.

FIGURE 3. Graphs showing effect of vampire bat salivary plasminogen activator and tissue-type plasminogen activator administration on the plasma levels of plasminogen (left panel) and α2-antiplasmin (right panel). Rabbits containing an occluded femoral artery received 42 nmol/kg tissue-type plasminogen activator (●, n=18) or vampire bat salivary plasminogen activator (■, n=10) by bolus intravenous injection. Control rabbits (▲, n=12) received saline. *p<0.05 and *p<0.01 vs. corresponding saline-treatment sampling times.
Statistically different ($p<0.01$) from each of the corresponding sampling times in the saline-treated rabbits. In contrast, none of the $\alpha_2$-antiplasmin and only one of the plasminogen determinations from the Bat-PA treatment group (60-minute sampling time) were significantly less ($p<0.05$) than the corresponding samples from the saline-treated rabbits.

**Pharmacokinetics ofBat-PA and t-PA in Rabbits**

The disappearance rates of Bat-PA or t-PA administered to rabbits by bolus intravenous injection were monitored by measuring PA activities in plasma. The activity determinations were converted to molarities of active PA (nM) using standard curves generated with known concentrations of Bat-PA or t-PA. The clearance of Bat-PA was best described by a biphasic elimination profile that exhibited a dominant $\beta$ phase (Figure 4, left panel). The elimination profile of t-PA from rabbits is much steeper than that of Bat-PA and can adequately be described as a monophasic profile (Figure 4, right panel). Table 2 summarizes the pharmacokinetic parameters deduced from multiple experiments ($n=4$) using Bat-PA or t-PA. The estimated plasma concentrations at time 0 for Bat-PA (25.3 nM) and t-PA (32.8 nM) were 58% and 75%, respectively, of the theoretical values, assuming uniform distribution into a plasma volume of 50 ml/kg. The $\alpha$ and $\beta$ phases for Bat-PA elimination displayed half-lives of 1.5 and 17.1 minutes, respectively. Interestingly, approximately 80% of the Bat-PA is cleared by the relatively slow $\beta$ elimination phase. The overall clearance rate for Bat-PA is approximately 11 ml·min$^{-1}$·kg$^{-1}$. The half-life for the elimination of t-PA was 1.1 minutes, whereas the clearance rate was approximately 44 ml·min$^{-1}$·kg$^{-1}$. Hence, the clearance rate of Bat-PA from plasma after bolus intravenous administration to rabbits was approximately fourfold less than that of t-PA.

**Effect of Bat-PA on Bleeding Times in Aspirin-Pretreated Rabbits**

The control template bleeding times performed on 20 rabbits consolidated from the four experimental groups was 1.8±0.1 minutes (Figure 5). One hour after bolus intravenous injection of ASA, the consolidated group displayed template bleeding times that were increased slightly but significantly to 2.4±0.2 minutes ($p<0.02$). The administration of SAT buffer (Bat-PA vehicle) to ASA-pretreated rabbits did not result in a further elevation of the template bleeding times. After the administration of Bat-PA (14 nmol/kg) to ASA-pretreated rabbits ($n=5$), the template bleeding times increased significantly, from 1.9±0.2 to 5.2±1.4 ($p<0.01$) and 4.2±0.6 ($p<0.05$) minutes at 15 and 30 minutes, respectively. The administration of t-PA (42 nmol/kg) to ASA-pretreated rabbits ($n=5$) caused significant elevations in the template
bleeding times from post-ASA values, from 1.9±0.2 to 5.2±0.4 (p<0.01), 5.1±0.7 (p<0.01), and 3.9±0.6 (p<0.05) minutes at 15, 30, and 90 minutes, respectively. The 14 and 42 nmol/kg doses of Bat-PA and t-PA, respectively, were compared because of their equivalence at restoring flow in the rabbit femoral arterial thrombosis model (Table 1).

A dose of Bat-PA (42 nmol/kg) equimolar to that of t-PA was also evaluated for its effect on the template bleeding time. In this high-dose Bat-PA group (n=5), there was a marked elevation in template bleeding times earlier in the protocol compared with those in the low-dose Bat-PA and t-PA treatment groups. Five minutes after the administration of 42 nmol/kg Bat-PA, the template bleeding time increased from 3.1±0.3 to 7.4±0.8 minutes (p<0.01). The corresponding values at 5 minutes for the low-dose Bat-PA and t-PA treatment groups were 3.2±0.6 (p=NS versus post-ASA) and 4.1±0.6 (p=NS versus post-ASA) minutes, respectively. After this early time point, the profiles for elevation in template bleeding times were similar for 42 nmol/kg Bat-PA, 14 nmol/kg Bat-PA, and 42 nmol/kg t-PA. Furthermore, in each instance, the template bleeding time values approached post-PA treatment values by 60 minutes after PA administration.

![Bar graph showing prolongation of template bleeding times in rabbits pretreated with aspirin (ASA) and administered vampire bat salivary plasminogen activator (Bat-PA) or tissue-type plasminogen activator (t-PA).](http://circ.ahajournals.org/content/84/5/5.F)

**FIGURE 5.** Bar graph showing prolongation of template bleeding times in rabbits pretreated with aspirin (ASA) and administered vampire bat salivary plasminogen activator (Bat-PA) or tissue-type plasminogen activator (t-PA). Rabbits were administered 15 mg/kg ASA by bolus intravenous injection. After 1 hour, 14 nmol/kg Bat-PA, 42 nmol/kg Bat-PA, or 42 nmol/kg t-PA was also given by bolus intravenous administration. Template bleeding times were determined as detailed in “Methods.” Shown are the serial bleeding times (mean±SEM) assayed at the following times: pretreatment (Control), 60 minutes after ASA administration (Post-ASA), and at increasing times after plasminogen activator administration (Post-PA). *p<0.05 and **p<0.01 vs. corresponding ASA (c) sampling times.

### TABLE 3. Whole-Blood Platelet Counts in Rabbit Bleeding Time Model

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sampling times (platelet counts/µL×10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>SAT buffer</td>
<td>444±79</td>
</tr>
<tr>
<td>t-PA (42 nmol/kg)</td>
<td>332±64</td>
</tr>
<tr>
<td>Bat-PA (14 nmol/kg)</td>
<td>422±83</td>
</tr>
<tr>
<td>Bat-PA (42 nmol/kg)</td>
<td>422±35</td>
</tr>
</tbody>
</table>

Values are mean±SEM; n=5 in each treatment group. Blood was withdrawn and platelet counts were determined at: A, control (5 minutes before aspirin administration); B, 60 minutes after aspirin and 5 minutes before plasminogen activator administration; C, 15 minutes after plasminogen activator administration; D, 60 minutes after plasminogen activator administration; SAT buffer, 50 mM sodium acetate and 0.01% Triton X-100, pH 5.0; t-PA, tissue-type plasminogen activator; Bat-PA, vampire bat salivary plasminogen activator.
Table 3 summarizes whole-blood platelet counts determined in SAT buffer, t-PA, and Bat-PA treatment groups in the template bleeding time evaluation. No significant differences in platelet counts were noted during the course of the protocol within any of the treatment groups.

Discussion

Our studies demonstrate that bolus intravenous administration of Bat-PA will rapidly restore blood flow in an experimentally thrombosed peripheral artery without precipitating severe plasminemia. Bat-PA was significantly more fibrin specific than t-PA and equal to or superior in thrombolytic potency to t-PA with respect to absolute incidence of reperfusion, magnitude of reperfusion blood flow, and reduction in residual thrombus mass. The bolus intravenous dosing schedule was chosen because it would facilitate prompt initiation of thrombolytic therapy and, as pointed out by others for t-PA, may have beneficial consequences on efficacy. Additional studies are required to determine whether the remarkable fibrin selectivity of Bat-PA would result in less frequent and less severe bleeding complications than those encountered after fibrinolytic therapy with t-PA or streptokinase.

The causal relation between PA-mediated generation of the lytic state and bleeding complications is controversial. Although t-PA causes a milder fibrinogenolytic state than does streptokinase, the use of t-PA as a fibrinolytic agent does not consistently result in a decreased incidence of serious bleeding complications. A correlation between fibrinogen degradation and serious bleeding complications has been noted, but in other studies, the correlation is weak. Hence, a fibrinolytic agent that is more fibrin specific than t-PA, such as Bat-PA, may not be safer than either t-PA or streptokinase. However, the importance of fibrin specificity has not been convincingly assessed, because the most fibrin-specific agent heretofore available, t-PA, is only fibrin specific relative to streptokinase, and the use of t-PA for thrombolytic therapy frequently results in appreciable activation of circulating plasminogen.

A significant correlation between template bleeding time prolongation and incidence of spontaneous bleeding during thrombolytic therapy with t-PA was recently reported. If this relation is valid, then the similar protracted bleeding times exhibited by ASA-treated rabbits that received t-PA or Bat-PA suggests that the use of either of these PAs for thrombolytic therapy would yield similar frequencies of spontaneous bleeding complications. However, there are many examples in which prolonged bleeding times are not associated with spontaneous, unprovoked bleeding. For example, patients stricken with Glanzmann's thrombasthenia have elevated template bleeding times but generally bleed only as a result of physiological or pathological conditions that cause bleeding in normal subjects. An early fibrinolytic manufacturer studied the clearance of Bat-PA after bolus injection in rabbits is best described by a biphasic elimination profile. Interestingly, the β elimination phase is dominant and accounts for approximately 80% of the Bat-PA clearance. In contrast, the elimination of t-PA activity after bolus administration in rabbits bests fits a monoexponential function, as has been reported by others. Biphasic elimination profiles for t-PA activity in rabbits have also been described, but in these instances, in contrast to Bat-PA, the rapid α phase dominates the pharmacokinetic profile. The disparate pharmacokinetic behavior of Bat-PA and t-PA gives rise to an approximately fourfold difference in their respective clearance rates. The basis for the delayed elimination of Bat-PA is unclear at this time. It was established that elimination of t-PA from plasma is mediated mainly by the liver and that the rate of clearance is influenced by the nature of the oligosaccharide chains as well as polypeptide structures resident in the fibronectin fingerlike and epidermal growth factor–like domains. Each of these structural determinants of t-PA clearance may differ in the case of Bat-PA. Regardless, it is likely that the slower clearance rate of Bat-PA relative to t-PA contributes decisively to the apparent greater efficacy of Bat-PA as assessed by reperfusion incidence in our rabbit model of peripheral arterial thrombosis.

The fibrin selectivity exhibited by Bat-PA can also be responsible in part for the observed difference in potency according to the “plasminogen steal” hypothesis forwarded by Sobel et al. According to this hypothesis, a fibrin-selective plasminogen activator would not consume circulating plasminogen, and consequently, plasminogen bound to the occlusive thrombus would not be depleted because of the reestablishment of equilibrium between these two pools of plasminogen. As a result, plasminogen remains sequestered with the thrombus, where it is available for activation to form plasmin and result in sustained intense fibrinolysis.

The recanalization caused by t-PA, especially at the 42 nmol/kg dose, may be due, in large part, to the establishment of a systemic lytic state. Our comparison of Bat-PA with t-PA with regard to efficacy does
not distinguish between the lytic effects of plasmin formed on the clot or in circulation. Hence, the comparable efficacies of t-PA and Bat-PA at the higher doses probably reflect a lytic mechanism in the case of t-PA that may be undesirable in light of safety considerations.

Erythrocyte-rich thrombi such as those formed in our rabbit model are more susceptible to t-PA-mediated lysis than the platelet-rich variety that more closely mimics those found in occluded coronary vessels. The potency exhibited by Bat-PA in the present study might likewise be diminished when one attempts to lyse platelet-rich thrombi. Nevertheless, the extraordinary fibrin selectivity of Bat-PA and the potency of Bat-PA relative to t-PA would probably not change if thrombi enriched with platelets were used. Experiments are currently under way using other animal models of thrombotic complications to evaluate the consequences of a large contingent of platelets within thrombi on the pharmacologic profile of Bat-PA.

Aside from sparking teleological discussions regarding the machinations of a feeding vampire bat, the remarkable fibrin specificity of Bat-PA may be a serendipitous means of attaining a frequently cited therapeutic goal: localizing the activity of a fibrinolytic agent to the vicinity of a clot, thereby avoiding the activation of circulating plasminogen. The potential merits of this goal with regard to safety and efficacy of thrombolytic therapy have not yet been convincingly evaluated, because effective doses of the purported fibrin-specific plasminogen activators result in frequent and sometimes severe episodes of plasminemia. Hence, Bat-PA and its strict selectivity toward fibrin-bound plasminogen should serve to critically assess the possible advantages of a fibrin-specific plasminogen activator for the treatment of thrombotic complications.

Acknowledgments

We thank Dr. P. Friedman for encouragement and helpful discussions. Drs. M. Silberklang and R. Ellis for their participation in the heterologous expression of Bat-PA, Dr. C. Reilly for critical review of the manuscript, and Ms. D. Bonenberger for her assistance in the preparation of the manuscript.

References

20. Gardell SJ, Hare TR, Bergum PW, Cuca GC, O'Neill-Palladino L, Zavodny SM: Vampire bat salivary plasminogen
activator is quiescent in human plasma in the absence of fibrin. 
*Blood* 1990;76:2560–2564
32. Magnani B, for the PAIMS investigators: Plasminogen activator Italian multicenter study (PAIMS): Comparison of intravenous recombinant single-chain human tissue-type plasmino-
Effective thrombolysis without marked plasminemia after bolus intravenous administration of vampire bat salivary plasminogen activator in rabbits.

S J Gardell, D R Ramjit, I I Stabilito, T Fujita, J J Lynch, G C Cuca, D Jain, S P Wang, J S Tung and G E Mark

Circulation. 1991;84:244-253
doi: 10.1161/01.CIR.84.1.244

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/84/1/244

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/