Production of Monoclonal Antibodies to the High Fibrin-Affinity, Tissue-Type Plasminogen Activator of Human Plasma. Demonstration of Its Endothelial Origin by Immunolocalization

By Eduardo Angles-Cano, André Balaton, Bernard Le Bonniec, Elisabeth Genot, Jacques Elion, and Yvette Sultan

Monoclonal antibodies (MAbs) to vascular plasminogen activator (vPA), the tissue-type plasminogen activator (tPA) in human plasma, were produced to be used as probes for immunochemical analysis. Human tissue sections and one of these MAbs were used to demonstrate the endothelial origin of plasma-tPA by immunohistochemistry. To produce MAbs, mice were immunized with semipurified vPA isolated from postocclusion human venous blood. Primed spleen cells were fused with the mouse myeloma cell line NS-1. Screening for MAB-producing hybridomas was performed with postocclusion globulins as a source of antigen by means of a solid-phase fibrin-vPA immunoassay. The selective and high-affinity binding of vPA for fibrin ensures the specificity and sensitivity of this test. Thus, eight hybridomas secreting MAbs to vPA were selected, cloned, and established as permanent hybridoma cell lines. Immunohistochemical analysis of cryostat sections of human tissues was performed with EA-12D, a MAb having no inhibitory effect against vPA activity but binding to vPA with a high affinity. Thus, the only structures immunostained were endothelial cells of venules, capillaries, and arterioles. The EA-12D monoclonal localization of plasma vPA in the endothelial lining of blood vessels provides evidence that tPA in plasma originates from the vascular wall and validates its designation as vascular plasminogen activator, i.e., vPA. Also, our results are consistent with the fact that vPA in blood and tPA in tissues are immunologically identical and have a common endothelial origin.

© 1985 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Reagents

All the chemicals used were reagent grade and were obtained from either Prolabo, Paris, or Aldrich, Beerse, Belgium. The following products were obtained as indicated: bovine serum albumin (BSA) and Tween-20 from Serva, Heidelberg, FRG; glutaraldehyde 25% aqueous solution from TAAB Labs, Reading, UK; polyvinylchloride (PVC) U microtiter plates from Dynatech Labs, Alexandria, Va; synthetic substrate D-Val-Leu-Lys-pNA (S-2251) from KabiVitrum AB, Malmö, Sweden; an anti-mouse IgG horse radish peroxidase-linked sheep antibody from Nordic Immunology Labs, Tilburg, The Netherlands; aprotinin (Trasybol) from Boehringer Mannheim, FRG; Sepharose 6B, Sepharcl G-200, Sephadex G-25, Lysine-Sepharose 4B, and low-mol-wt markers from Pharmacia, Uppsala, Sweden; polyethylene glycol (PEG 4000) from British Drug House, Poole, England; Ultrogel AcA 44 and DEAE-Trisacryl from IBF, Villeneuve-La Garenne, France. Human urokinase (UK) was from Leo Pharmaceutical, Ballerup, Denmark, and Institut Chaoy, Paris (a kind gift from Dr F. Toulemonde).

From INSERM U152, Hôpital Cochin, the Département d’Anatomo Pathologique, Hôpital Saint Joseph, and the Département de Biochimie, Hôpital Bichat, Paris.

Supported by a grant from INSERM.


Address reprint requests to Dr E. Angles-Cano, INSERM U152, Prof Levy, Hôpital Cochin, 27 rue du Fg Saint Jacques, 75674 Paris Cedex 14, France.

© 1985 by Grune & Stratton, Inc.
Protein Purification

Fibrinogen grade L (KabiVitrum) was further purified as described previously. The final product was free of plasminogen, von Willebrand factor, factor XIII, and fibronectin as determined by an enzyme-linked immunosorbent assay specific for these proteins. No traces of PA activity were detected in a fibrin-agar plate made with the purified fibrinogen. Plasminogen (Pg) was purified from outdated human plasma by affinity chromatography on Lysine-Sepharose, followed by gel filtration on Ultrogel AcA 44 and ion exchange chromatography on DEAE-Trypsaryl. Goat anti-mouse immunoglobulin (Gam Ig) was purified from goat antisera by ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography on a mouse immunoglobulin immunoadsorbent. Labeling of Gam Ig was carried out using the iodogen reaction.

Plasma-tPA Isolation

Plasma-tPA (ie, vPA) was separated from plasma obtained from healthy volunteers after a 15-minute period of venous occlusion, essentially as described previously. Briefly, plasma containing Trasylol 100 KIU/mL was first chromatographed using a Sepharose 6B column in 5 mmol/L phosphate buffer, pH 7.4, containing 145 mmol/L NaCl, 2 mmol/L EDTA, 0.01% Tween-20 and 5 KIU/mL Trasylol. This was followed by a second chromatographic step on Sephacryl S-200 superfine in 5 mmol/L phosphate buffer, pH 7.4, containing 1 mol/L NaCl and 0.01% Tween-20. In some experiments, Trasylol was omitted in plasma and in the low-ionic strength buffer. To detect tPA, fractions were tested spectrophotometrically using a solid-phase fibrin-tPA assay. Fractions containing tPA activity were pooled and lyophilized. The product was dissolved in 5 mL distilled water and was desalted on a Sephadex G-25 column. The protein solution was lyophilized and stored at -80°C until used.

Plasma-tPA Characterization

Sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analysis of this preparation was performed on 7.5% and 10% gels as described by Weber and Osborn. Slab-gel/fibrin-agar zymography was performed as follows. Unreduced samples (15 μL) were electrophoresed at 4 mA constant current in 4% stacking and 10% separating polyacrylamide slab gels (0.8 mm thick and 15 cm long), using the SDS-gel electrophoretic system of Laemmli. Mol wt standards were run under the same conditions on a portion of the gel that was removed and stained with 2.5% Coomassie blue. The remaining part of the gel was washed for one hour in 2.5% Triton X-100 to remove SDS and then washed in distilled water and placed on a fibrin-agar indicator gel prepared in a moist chamber (12 x 12 x 1.5 cm) as described previously. Zymograms were allowed to develop at 37°C for six to 48 hours and then photographed using dark-ground illumination.

The adsorption of plasma-tPA to solid-phase fibrin and its fibrin-dependent PA activity was determined spectrophotometrically by measuring plasmin generation. The procedure is indicated later (see Binding of vPA to solid-fibrin phase supports) except that 100 μL per well of either the vPA preparation or UK (1 IU/mL) was used. Supernates were not discarded, but tested apart for residual (unbound) PA activity by adding 100 μL of the substrate solution (Pg-SS) in reaction buffer containing 100 μg per milliliter of fibrin (freeze-dried clotted purified fibrinogen). The Δ A405 min⁻¹ of fibrin-bound and residual (unbound) PA was used to calculate the maximum percentage of binding for each activator sample. Parallel experiments were performed on plate supports containing BSA instead of fibrin.

The final product was tested for plasminogen and fibrinogen content on plasminogen-free fibrin-agar plates and with specific antisera to these proteins.

Immunization Procedure

To produce monoclonal antibodies, (BALB/c x C57Bl/6)F1, mice, 8 to 10 weeks old, were injected intraperitoneally (IP) with 0.2 mL of a 2.5 μg/mL solution of semi-purified vPA in complete Freund's adjuvant. Mice were boosted with 0.2 mL injection (IP) of the same solution in incomplete Freund's adjuvant three and six weeks later. Sera-antibody titers were determined by radioimmunoassay (RIA) using the solid-fibrin-phase immunoassay (SOFIA) described later and by neutralization of vPA activity using a fibrin-agar method. After three injections, binding titers between 10^5 and 10^6 and neutralization titers between 10^4 and 10^5 were obtained.

Cell Fusion and Cultures

Two mice with the highest binding and neutralizing titers were selected for fusion and were given an IP boost of 5 μg of semipurified vPA in saline. Three days later the spleen was aseptically removed, single cell suspensions were obtained and mixed with the P3-NS1-1-Ag4/1 hyposalmine-guaine phosphoribosyltransferase-deficient, immunoglobulin-negative secreting plasmacytoma line (NS-1, a gift of Dr Philip Avner, Institut Pasteur, Paris). The procedure reported by Fazekas de St Groth and Scheidegger for obtaining optimal number of hybrids was followed. Briefly, 5 x 10^5 NS-1 cells cultivated in complete Dulbecco's modified Eagle's medium (DMEM containing 10% fetal calf serum, 1% 100 mmol/L sodium pyruvate, 1% 200 mmol/L L-glutamine, 1% d-glucose, and 0.05% 0.1 mol/L 2-mercaptoethanol) were fused with 3 x 10^5 spleen cells by using 50% PEG 4000. Immediately after fusion, cells were pelleted, washed, and resuspended in HAT-containing medium (complete DMEM supplemented with hypoxanthine, aminopterin, and thymidine). To achieve an initial cloning stage, hybrids were seeded at low density in five 96-well Linbro plates containing 3 x 10^4 peritoneal-macrophages per well as feeders. Cultures were refed at day 5 and then twice weekly. Hybridomas grew in 70% of the wells and were screened for production of vPA-directed antibodies as described later, ten to 25 days after fusion.

Screening for vPA-Specific Antibodies

Mice antisera and hybridoma culture supernates were screened for antibodies to vPA by means of the SOFIA radioimmunoassay. This is a highly specific screening assay based on a unique property of tPA, i.e., high fibrin affinity, which ensures its specific and selective binding to fibrin. A solid-fibrin phase able to separate tPA from the bulk of proteins present in biological fluids is feasible. It has already been used as an RIA support for the selection of porcine tPA-specific hybridomas and therefore may be used for selection of hybridomas to vPA even if this antigen is available only as a minor component in the complex plasma mixture. A concise description of the procedure and modifications indicating optimal conditions for the selection of MAbs to vPA is given later.

Construction of the solid-fibrin-phase support (PVG-Fg-Fn). All procedures were performed in PVC 96-well U-microtiter plates using a multichannel pipetor. PVC plates were first treated with 2.5% polyglyutaraldehyde in 0.1 mol/L bicarbonate buffer (pH 9.5) for two hours at 22°C (PVG). Then, fibrinogen (0.29 μmol/L in 0.1 mol/L bicarbonate buffer, pH 8.5, 1 mmol/L CaCl₂) was covalently fixed (PVG-Fg) and a fibrin network (PVG-Fg-Fn) was generated by thrombin treatment (1 NIHU/mL in 0.05 mol/L phosphate buffer, pH 7.4, 0.08 mol/L NaCl, 2 mmol/L CaCl₂). Fibrin-bound thrombin was eluted with high-ionic strength buffer (5

From www.bloodjournal.org by guest on October 22, 2017. For personal use only.
mmol/L phosphate, pH 7.4, 0.05 mol/L NaCl, 8 mmol/L CaCl₂, 0.05% Tween-20).

**Binding of vPA to solid-fibrin-phase support.** Euglobulins precipitated from postocclusion venous plasma at low ionic strength (1 = 0.002) at pH 5.9 were used as the source of vPA. Euglobulins were diluted in binding buffer (0.05 mol/L phosphate buffer, pH 6.8, 0.08 mol/L NaCl, 0.4% BSA, 0.05% Tween-20, 1 mmol/L EDTA) to obtain 0.5 IU/mL, and 100 µL per well was added to a PVC-Fg—Fn support, sealed, and incubated for one hour at 37 °C. Unbound proteins were discarded, and nonspecific binding was eliminated by washing four times with the same buffer. A PVC-Fg—Fn-vPA support was thus obtained. The specific binding of vPA to the PVC-Fg—Fn support was verified spectrophotometrically, measuring plasmin generation with the SOFIA-IPA activity assay (to be described elsewhere). Briefly, 100 µL per well of substrate solution—0.2 mmol/L Glu-Pg and 1 mmol/L S-2251 (Pg-SS) in reaction buffer (0.05 mol/L phosphate buffer, pH 7.4, 0.08 mol/L NaCl, 0.1% BSA)—was added, sealed, and incubated at 37 °C for one hour. Absorbance at 405 nm was determined at 15-minute intervals in a Dynatech MR580 microplate reader.

**Immunosassay procedure.** To a PVC-Fg—Fn-vPA support, dilutions of mice sera in phosphate-buffered saline (PBS) (10 mmol/L Na₂HPO₄, 5 mmol/L KH₂PO₄, 5 mmol/L KCl, 137 mmol/L NaCl, pH 7.3) containing 0.1% BSA, 0.05% Tween-20 (PBSA-Tween) or hybridoma culture supernates (50 µL per well) were added and incubated at 37°C. After one hour, the plate was washed three times with PBSA-Tween and 50 µL per well of 125I-labeled affinity-purified Gamlg (~50,000 cpm) was added, incubated for one hour at 37 °C, and washed with the same buffer. The wells were then cut out, and the bound radioactivity counted in a γ-counter. Sera from splenocyte donor mice diluted 10⁻³ and undiluted culture media from NS-1 were used as positive and negative controls, respectively. The background binding with the latter was less than 1% of the input radioactivity, and the positive wells showed binding of fivefold to tenfold over background.

**Cloning and Antibody Production**

All positive hybridomas were further grown and then subcloned by limiting dilution in 96-well culture plates on peritoneal-macrophages monolayers. Cloned hybrids were grown in culture and developed as ascites by injecting (IP) 10⁹ hybridoma cells into congenic mice that had received (IP) 0.2 mL of pristane (2, 6, 10, 14 tetramethyl pentadecane, Aldrich, Beerse, Belgium) two weeks earlier.

**Purification and Isotype Characterization of MABs**

Antibodies in supernates were purified by 45% ammonium sulfate precipitation and affinity chromatography on a goat anti-mouse Ig immunoabsorbent. Ascitic fluids were collected in tubes containing Trasylol 10 KIU/mL. Fibrin clots were eliminated, the serum precipitations and ion exchange chromatography on DEAE-Tryptaeryl. The IgG concentration in the purified preparations was determined using rabbit anti-mouse Ig antibodies EA-γ 2H, EA-δ 12C, EA-δ 12D, and EA-ε 5B were conducted to measure their abilities to bind to a constant amount of vPA immobilized on PVC-Fg—Fn supports. The conditions were the same as screening assays except that fibrin-bearing wells, prepared as described in the text, were treated for 18 hours at 4 °C with postocclusion euglobulins adjusted to 0.5 IU/mL of vPA activity, and then increasing concentrations of MABs were incubated for 24 hours at 4 °C. BSA instead of euglobulins was used to estimate nonspecific binding. Specifically bound antibodies were detected by treatment with 125I-Gamlg (100,000 cpm) for 16 hours at 4 °C. Data were analyzed by using a computerized approach involving an exact mathematical model for a single ligand binding to one class of specific sites and appropriate statistical treatment. Curve fitting for 22 experimental points was determined by fifth-order polynomial approximation using nonlinear least squares analysis of data in their original coordinate system, ie, bound ligand v total concentration. The apparent affinity constants (Kapp) were estimated from these curves by determining the abscissa of the point where the derivative of the calculated curve is maximal. This was obtained by computerized approximation of the derivative vY at the point Xn as follows

\[ Y' = \frac{v + 1}{v} - \frac{Yn + 1}{Xn + 1} \]

where Xn are the approximated abscissas (steps of 10⁻³ scale units, ie, mmol/L) and Yn the corresponding ordinates.

The analytical method makes use of a computer program written in the Applesoft version of BASIC. It was developed by one of us (B.L.B., to be published elsewhere) for an Apple II microcomputer.

**Specificity of Monoclonal Antibodies**

A spectrophotometric assay performed on PVC-Gamlg—coated plates was used to test the specificity of hybridoma antibodies, as described previously. Briefly, 100 µL per well of affinity-purified Gamlg (1 µg/mL) was added to a glutaraldehyde-activated PVC plate and incubated at 4 °C for 18 hours. After washing, remaining free aldehyde groups were saturated with PBSA-Tween buffer. Then, 100 µL per well of tenfold concentrated positive-hybridoma culture supernates was added and incubated for 18 hours at 4 °C. After washing with PBSA-Tween buffer, 100 µL per well undiluted euglobulins from postocclusion plasma (5 IU/mL of vPA activity) was added and incubated overnight at 4 °C. After extensive washing with PBSA-Tween buffer, bound vPA was estimated as follows: 100 µL per well of Pg-SS reagent solution in reaction buffer containing 0.1 mg/mL of fibrin was added, sealed, and incubated at 37 °C. After four hours, the absorbance at 405 nm was determined as described earlier.

**Immunoprecipitation of vPA**

To further characterize the specificity of EA-δ 12D for vPA, PBSA or purified MAb (0.1 mg/mL) was added to an equal volume (100 µL) of postocclusion venous plasma diluted to 20%, 10%, and 5% of original with PBSA. After 30 minutes' incubation at 37 °C, 10 µL of a 1:2 diluted mouse serum, followed by 40 µL of Gamlg undiluted serum was added and incubated at room temperature. Thirty minutes later the complexes were precipitated by centrifugation. Supernates were tested for remaining vPA activity as indicated under "Binding of vPA to Solid-Fibrin-phase supports," except that absorbance readings were performed after two hours' incubation at 37 °C.

**Immunohistochemical Method**

Cryostat sections (5 µm) were air-dried for ten minutes and fixed for 15 minutes in acetone at 4 °C. EA-δ 12D diluted 1:25 was overlaid on the tissue and incubated for six hours at 4 °C. After one
wash in PBS (ten minutes), a rabbit antibody to mouse IgG conjugated to peroxidase (Nordic) and diluted 1:50 was then incubated with the tissue for 30 minutes at room temperature. Sections were washed in PBS (ten minutes). Immunostaining was performed by adding a TBS solution containing 0.7 mg/mL 3,3'-diaminobenzidine ( Sigma Chemical Co., St Louis) and 0.03% hydrogen peroxide. After incubation for ten minutes in the dark, at room temperature, the sections were immersed in PBS for ten minutes. A minimal counterstaining with methylgreen was performed. Normal mouse serum, EA-12D preabsorbed with vPA as previously described, and rabbit anti-fibrinogen (Nordic) (to exclude passive absorption of plasma constituents by endothelial cells) provided negative controls.

RESULTS

Plasma-tPA (or vPA) antigenic preparation. The plasma-tPA antigen used for immunization was separated from plasma obtained from human volunteers whose postocclusion venous blood had 8 to 10 IU/mL of vPA activity. The preparation consisted in a protein fraction (40 to 60 mg) with vPA activity isolated from plasma (120 mL total volume) by gel filtration chromatography with low- and high-ionic strength buffers in the presence or absence of Trasybol. Elution profiles for both chromatographic steps are shown in Fig 1. After SDS-PAGE of the product prepared in the presence of Trasylol, one major protein band of M₇, 71,000 under reduced or unreduced conditions was obtained (Fig 2). However, when the inhibitor was omitted, a mixture of the one-chain and the two-chain forms was observed on reduced gels (Fig 3), as previously reported for human melanoma and uterine tPA. The slab-gel/fibrin-agar zymographic analysis of the same preparation revealed only one PA activity zone of M₇, 70,000 (Fig 4). The prolonged incubation at 37 °C (up to 48 hours) of the zymograms failed to reveal any other lytic zone. In addition, no traces of plasmin(ogen) or fibrinogen could be demonstrated in the final product by immunologic or functional analysis. However, minor contaminants were observed in some preparations after SDS-PAGE in 7.5% gels and the final specific activity (expressed in UK equivalent units by comparison of their fibrinolytic activity on fibrin-agar plates) was only 18,000 to 27,000 IU/mg. Because purified tPA has a specific activity of more than

![Fig 1](image1.png)

**Fig 1.** Gel filtration of human postocclusion venous plasma. The figure indicates both the protein elution profile (---) and the vPA activity (-----) on Sepharose 6B (A) and Sephacryl S-200 (B). Fractions containing vPA activity in the void volume (A) and after the second protein peak (B) were pooled and lyophilized for further processing as indicated in Materials and Methods.

![Fig 2](image2.png)

**Fig 2.** Sodium dodecyl sulfate-polyacrylamide gel (10%) electrophoresis of vascular plasminogen activator (b, c) from postocclusion venous plasma isolated in the presence of Trasylol. Reduced gels (a, b), unreduced gels (c, d). Low-mol-wt markers (a, d): phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α lacto-albumin. Vascular plasminogen activator indicated by arrows.

![Fig 3](image3.png)

**Fig 3.** Sodium dodecyl sulfate-polyacrylamide gel (7.5%) electrophoresis of vascular plasminogen activator from postocclusion venous blood isolated in the absence of Trasylol. The mixture of one-chain and two-chain forms of vPA is observed in reduced gels (b, c) but is not evident in the unreduced gel (a). The positions of mol wt markers are indicated.
were electrophoresed in a slab polyacrylamide gel under nonreducing conditions and the gel was placed on fibrin-agar underlays, incubated at 37 °C for 18 hours, and then photographed using dark-ground illumination. The M of proteins markers (lane a: ovotransferrin, bovine serum albumin, ovalbumin and carbonic anhydrase) are indicated at the left. The apparent mol wt of PAs obtained from postocclusion plasma is a semipurified protein fraction with a high content of a M, 71,000 protein having fibbin-dependent PA activity, ie, vPA. Therefore, the product obtained in this manner is a convenient preparation to be used as antigen for the production of monoclonal antibodies to human vPA.

Fusion experiments. Eight MAbs reacting with vPA were obtained from one immunized spleen-myeloma NS1 cell fusion. More than 300 wells were screened by indirect measurement of antibody binding to PVG-Fg—Fn-vPA supports. Twenty-six microcultures initially displayed positive, but only eight stable cultures with the highest vPA-binding activities survived. Three of these arose as single clones directly from primary cultures. However, all stable cultures were subcloned to ensure monoclonality.

Isotype and inhibition studies. The isotype analysis of culture supernates revealed that all hybridomas produced IgG antibodies (Table 2). This provided further evidence for monoclonality. Three MAbs were found to inhibit the enzymatic activity (Table 2) and were therefore considered as antibodies directed to the active site or related regions.

Apparent affinity constants (Kapp) of MAbs. The apparent affinity constants of MAbs to vPA were determined by measuring the binding of increasing amounts of MAbs to a limited constant amount of vPA by means of the SOFIA method. A computerized approach was used to analyze the data. Thus, maximally efficient estimates of the parameters were obtained. Curve fitting and apparent affinity constants estimates were obtained by analyzing raw data in their original coordinate system, ie, bound ligand v total concentration. Computer-generated graphic representation of the data with the fitting curves provide a visual check on the validity of the data analysis. The graphic display of the fitting curves for four MAbs is shown in Fig 5. The inset shows the determination of Kapp. The apparent affinities of EA-γ 2H, EA-δ 12C, EA-δ 12D, and EA-ε 5B are shown in Table 2.

Specificity of MAbs. The specificity of MAbs to vPA was controlled as follows:

1. Using solid-phase fibrin plates made with purified fibrinogen and subsequently "coated" with proteins other than vPA such as Glu-Pg, Lys-Pg, plasmin, BSA, and

Table 2. Characterization and Reactivity Pattern of MAbs to vPA

<table>
<thead>
<tr>
<th>MAb</th>
<th>Immunoglobulin</th>
<th>Kapp mol/L x 10^6</th>
<th>vPA Binding by MAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA-ε 1D</td>
<td>IgG1</td>
<td>ND</td>
<td>0.350</td>
</tr>
<tr>
<td>EA-δ 1F</td>
<td>IgG3</td>
<td>ND</td>
<td>0.410</td>
</tr>
<tr>
<td>EA-β 4B</td>
<td>IgG2b</td>
<td>ND</td>
<td>0.055</td>
</tr>
<tr>
<td>EA-γ 2H</td>
<td>IgG1</td>
<td>50.9</td>
<td>0.050 +</td>
</tr>
<tr>
<td>EA-ε 12C</td>
<td>IgG2a</td>
<td>12.3</td>
<td>0.320</td>
</tr>
<tr>
<td>EA-δ 12D</td>
<td>IgG1</td>
<td>6.0</td>
<td>0.430</td>
</tr>
<tr>
<td>EA-ε 5B</td>
<td>IgG2a</td>
<td>10.4</td>
<td>0.040</td>
</tr>
<tr>
<td>EA-γ 7D</td>
<td>IgG2b</td>
<td>ND</td>
<td>0.510</td>
</tr>
<tr>
<td>NS-1</td>
<td>—</td>
<td>0.045</td>
<td></td>
</tr>
</tbody>
</table>

Isotype was determined in a double-immunodiffusion system using specific rabbit antisera to mouse Ig subclasses. Binding and inhibition of vPA activity by MAbs was detected spectrophotometrically using postocclusion egulobulins, Pg, S-2251, and fibrin as indicated in Materials and Methods. The apparent affinity constant (Kapp) of MAbs was determined by computerized analysis of data from ligand binding experiments as described in Materials and Methods. ND, not done.
gelatin. A convenient dilution of MAb was then used and bound antibody was released with $^{125}$I-Gamlg. No significant binding of any MAbs was observed in the absence of vPA.

2. Using PVC-Gamlg plates and a spectrophotometric assay as described in Materials and Methods. MAbs were specifically bound to the plate and then vPA from postoclusion euglobulins was allowed to bind. This was shown spectrophotometrically by the increase in absorbance observed in the presence of fibrin, plasminogen, and S-2251. Absorbance readings were similar to the blank for three of these MAbs and were considered inhibitory antibodies (Table 2).

3. By immunoprecipitation of vPA with EA-δ 12D monoclonal antibody. EA-δ 12D, a single IgG1 subclass MAb having no PA-inhibitory activity, was added to vPA-rich plasma and was able to precipitate tPA activity after the addition of anti-mouse Ig (Fig 6). Moreover, vPA could be separated from plasma by EA-δ 12D-Sepharose chromatography and subsequently eluted without losing its PA activity. These results indicate that EA-δ 12D recognizes the protein support of vPA activity in human plasma.

**DISCUSSION**

Using a semipurified vPA preparation obtained from postoclusion human venous blood, we have derived eight monoclonal antibodies that are specific for tPA present either in plasma (vPA) or in tissues (tPA). To our knowledge, production of MAbs and specific antisera to plasma-tPA has not been previously reported, probably owing to difficulties in obtaining homogenous vPA preparations in sufficient quantities. Here, we present data showing that a plasma protein fraction containing high amounts of M, 71,000 high fibrin-affinity tPA can be separated from human postoclusion venous blood. The availability of such a

**Immunohistochemical studies.** Experiments reported here were performed with EA-δ 12D MAb obtained from culture supernatants or ascitic fluid. Different samples of human tissues were studied. The results obtained in human ovaries are shown in Fig 7. The characteristic dark reddish-brown positive reaction was contrasted with that of methyl-green counterstaining. Thus far, the only structures immunostained were endothelial cells of venules, capillaries, and arterioles. Controls were negative.
MONOCLONAL ANTIBODIES TO HUMAN vPA

semipurified preparation and the need for monospecific reagents to vPA prompted us to use monoclonal antibody technology. By using this technique and coupling it to an original screening assay linked to an intrinsic property of tPA, ie, high fibrin-affinity, we have overcome the problems of specific antibody production to an enzyme that is difficult to purify.

As a consequence of being monospecific and reactive with a single determinant of the enzyme, MAbs to vPA can be used for immunohistochemical studies, immunopurification, epitope mapping, as well as probes in molecular biology and clinical medicine. These types of studies that are necessary to the understanding of the structure-function relationship of vPA rely on the demonstration that the antibodies are indeed specific. This essential feature of the antibodies reported here has been assessed by several independent criteria. First, although Pro-UK is a known plasma constituent that may bind to fibrin, we were unable to demonstrate its presence in our semipurified vPA preparation. Also no inhibitory effect of polyclonal anti-UK antibodies on the PA activity of euglobulins adsorbed to PVG-Fg---Fn supports could be demonstrated. Therefore, the immunization of mice with the plasma protein fraction containing only the M, 71,000 high fibrin-affinity PA coupled to a screening method based on the selective binding of vPA to fibrin ensures the production of MAbs specifically directed to epitopes of vPA. Second, the specificity and sensitivity of the SOFIA as a reliable method for the detection of hybridoma producing MAbs to tPA has been demonstrated previously. Antibodies thus selected do not bind to fibrin, coagulation factors, plasminogen, or plasmin, but recognize vPA bound to fibrin. Third, immunoadsorption experiments performed with MAbs fixed to a solid-phase support (PVC-Gamlg) showed that these antibodies were able to bind vPA present in postocclusion plasma. Also, indirect immunoprecipitation studies with EA-5 12D showed that this antibody was able to bind and precipitate vPA from plasma.

We conclude from these data that MAbs produced in this manner are monospecific for vPA. Because of the characteristics of our screening test, it is possible that we selected preferentially MAbs to the specific conformation of vPA bound to fibrin. On the other hand, it is probable that we have missed MAbs to vPA epitopes masked by the vPA-fibrin interaction. These considerations have to be taken into account with regard to the suitability of our MAbs for some specific applications.

In addition to specificity, the affinity of MAbs for vPA is another essential feature in assessing their usefulness for a given application. The classic Scatchard analysis to estimate antibody affinity requires large amounts of highly purified labeled antigen. Because this requirement was not met for vPA, the affinities of MAbs reported here were estimated by nonlinear computerized analysis of data obtained by direct binding of MAbs to fibrin-immobilized vPA. This system allows apparent affinity determination despite the unavailability of a highly purified preparation of vPA. In fact, the SOFIA method involves a fibrin-affinity vPA separation step that is applied advantageously here and also provides the basis of a spectrophotometric method for the specific assay of tPA activity present in any biological fluid. Ka values found by using this approach varied from 6 to 51 x 10⁹ mol/L⁻¹.

Once the specificity and affinity of MAbs were established, it was of interest to study their usefulness in immunochromatographic analysis. In the present work, a high-affinity MAb was used to study human tissue sections in order to determine the precise cell localization of vPA. Although a vascular origin has been suspected and its synthesis by endothelial cells in culture has been demonstrated, definitive proof has not been forwarded. Indeed, the release of vascular PA into the circulating blood has not been proven, although indirect evidence suggests an immunologic relationship between uterine tPA and vPA in blood.

Our immunohistologic studies with EA-5 12D, a monoclonal antibody specific to vPA, indicate clearly that vPA liberated into the blood after venous occlusion and tPA present in tissues are primarily localized in the endothelial cell lining of blood vessels. In human kidney sections, in addition to blood vessels, vPA was localized also in the glomerular floculus. Passive adsorption of vPA by endothelial cells seems unlikely because one could expect a positive staining for major plasma constituents, eg, fibrinogen, and this was not the case. Therefore, we provide new evidence for the endothelial cell origin of tPA in plasma and for the identity between the high fibrin-affinity PA present in tissues (tPA) and that present in plasma (vPA). In addition, our findings validate its designation as vascular plasminogen activator, ie, vPA. A close relationship between the physiopathology of the vascular endothelium and the development of thrombosis due to defective plasminogen activation can now be firmly established.

ACKNOWLEDGMENT

We gratefully acknowledge Dr Constantin Mihaesco for helpful advice. We thank Dr Phillip Gambel for critical reading of the manuscript, Dr Marc Montagner for helpful discussion with the mathematical model, and Dominique Hirtzlin and Jocelyne Debie for secretarial assistance.

REFERENCES

1. Dastre A: Fibrinolyse dans le sang. Arch Physiol Normal Pathol 5:661, 1893
7. Camiolo SM, Thorsen S, Astrup T: Fibrinogenolysis and fibrinolysis with tissue plasminogen activator urokinase activated


17. Kok P: Separation of plasminogen activators from human plasma and a comparison with activators from human uterine tissue and urine. Thromb Haemost 41:734, 1979


24. Fraker PM, Speck JC Jr: Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1, 3, 4, 6-tetrachloro-3a, 6a-diphenylglycoluril. Biochem Biophys Res Commun 80:849, 1978


Production of monoclonal antibodies to the high fibrin-affinity, tissue-type plasminogen activator of human plasma. Demonstration of its endothelial origin by immunolocalization

E Angles-Cano, A Balaton, B Le Bonniec, E Genot, J Elion and Y Sultan