Two monoclonal antibodies (mAbs), 10-2 and 10-5, both directed against recombinant hirudin variant 2-Lys47 (rHV2), were selected for their high affinity and epitopic specificities to develop a two-site immunoassay of rHV2. The mAb concentrations, incubation time, and temperature were optimized. The immunoassay has a detection limit for rHV2 of 45 ng/L in plasma and 30 ng/L in urine. The reactivity of the mAbs was tested against rHV2 and several forms of this protein truncated in the carboxyl terminus. The capture mAb 10-2 was found to be mainly directed against rHV2, whereas tracer mAb 10-5 was independent of the carboxyl-terminal region of the protein. This explains the high specificity of the immunoassay for the 65-amino acid form of hirudin.

Indexing Terms: anticoagulants/thrombin/immunoenzymometric assay/urine

Hirudin, the 65-amino-acid anticoagulant isolated from the leech Hirudo medicinalis, specifically inhibits α-thrombin, thus preventing fibrinogen conversion to fibrin and activation of factors V, VIII, XIII, and platelets. More than 10 natural variants of hirudin have been described (1); they differ in their amino acid sequences but have the same pharmacological properties. Molecular engineering techniques have now made it possible to obtain several recombinant variants of hirudin (2–5). Three are easily produced: rHV1, rHV2, and rHV3, corresponding to the natural variants HV1, HV2, and HV3 (6).

Hirudin acts as a potent anticoagulant by forming a stable noncovalent tight equimolar complex with thrombin (EC 3.4.21.5) with high specificity (7) and affinity (Kd = 2 × 10−14 mol/L) (8). The structure of natural and recombinant hirudin (variant rHV1) in solution, determined by nuclear magnetic resonance, exhibits a triple disulfide-bonded NH2-terminal core (amino acids 1–52) with an unstructured COOH-terminus (amino acids 53–65) (9, 10). Hirudin inactivates thrombin by binding to an extensive surface region of the enzyme, including the catalytic site, which interacts with the NH2-terminal region of hirudin, and the anion-binding exosite, which interacts with the last 10 COOH-terminal residues of hirudin, a region removed from the catalytic site and important for fibrinogen binding (11–13). Studies of the kinetic mechanisms of thrombin inhibition by hirudin indicate that the rate-limiting first step in the formation of the complex is dependent on ionic strength (14, 15); in a second step, hirudin is bound near the active site.

Several studies with rats and with human volunteers (16–19) have shown that administration of hirudin produces a clear anticoagulant effect, with no influence on cardiac or respiratory functions and no allergic or immune response. Moreover, hirudin has many advantages over heparin, the most commonly used anticoagulant (20–22): (a) no endogenous factors are involved in the anticoagulant action of recombinant hirudin; (b) no antagonist is necessary for inhibition of bleeding; and (c) hirudin inhibits thrombin in solution and binds to fibrin. All these unique properties of hirudin have promoted considerable interest in its potential use as an antithrombotic agent.

The first method described for quantifying recombinant hirudin was thrombin titration by hirudin (23, 24), also called the clotting assay. Another frequently used method is the amidolytic assay (25), in which the residual thrombin activity is spectrophotometrically determined after addition of a chromogenic peptide substrate. Several immunoassays for quantifying hirudin have been developed (26, 27): The most sensitive one allows the determination of hirudin at concentrations of 0.2–25 μg/L. Here, we describe the selection of monoclonal antibodies (mAbs) directed against the recombinant desulfathirudin variant 2-Lys47(rHV2) to develop a sensitive and specific immunoenzymometric assay of active hirudin in human plasma and urine.

Materials and Methods
Production, Purification, and Properties of mAbs

Five hybridomas secreting anti-hirudin mAbs were given to us by C. Roitsch (Transgène, Strasbourg, France). We expanded the hybridomas in RPMI medium (Boehringer Mannheim, Mannheim, Germany) supplemented with penicillin, streptomycin, fungizone, glutamine, and 150 mL/L fetal calf serum (Gibco BRL S.a.r.l., Cergy-Pontoise, France). We centrifuged the culture supernatant at 500g to remove cells and injected these cells into pristane-primed BALB/c mice. The ascitic fluid from the mice was centrifuged at 700g and filtered through a 0.22-μm (pore size) Millipore filter (Millipore, Guyancourt, France). All mAbs from the culture supernatant were concentrated 10-fold and then purified on a
Protein A–Sepharose CL4B column (Pharmacia, Uppsala, Sweden). After elution, each mAb was dialyzed against a 50 mmol/L phosphate buffer (PB), pH 7.2, and frozen at −80°C. The purity of the mAbs was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (28).

The immunoglobulin class and subclass of the mAbs were determined by an ELISA technique. They were all IgG1; mAbs 10-5 and 10-6 have a λ-light chain; mAbs 10-2, 10-3, and 10-9, a κ type.

The affinity constant of each mAb was determined by the method of Friguet et al. (29). The five mAbs had affinity constants in the range of $2.2 \times 10^{-9}$ to $2.8 \times 10^{-8}$ mol/L.

Hirudin and COOH-Truncated Forms

rHV2 and the COOH-terminal truncated derivatives of recombinant hirudin (H54, H57, H61, H62, H63, H64) were prepared and characterized as previously reported (30).

Protein Labeling

The mAbs were labeled with horseradish peroxidase (HRPO; EC 1.11.1.7; Boehringer Mannheim) (31). rHV2 was radioiodinated by the Chloramine T method as described by Tuong et al. (32). We allowed 0.2 mCi of Na$_{125}$I (Amersham, Les Ulis, France) to react with 100 μg of rHV2. Free iodide was separated by gel filtration on a prepacked Sephadex G25 cartridge (PD 10) from Pharmacia.

ELISAs

All the enzyme immunoassays were performed in 96-well Nunc Immunoplates (Nunc, Roskilde, Denmark).

Direct ELISA. Briefly, 100 μL of rHV2 at a concentration of 0.2 mg/L in coating buffer (phosphate-buffered saline (PBS), pH 7.4) was coated onto microtiter plates by incubation overnight at 4°C. The plates were washed three times with PBS containing 1 mL/L Tween 20 (PB-T). Different dilutions of each HRPO-labeled mAb in PBS-T (each 100 μL) were incubated for 2 h at 37°C. After three washings, the antigen–antibody reaction was detected by adding 100 μL of substrate [4 g/L o-phenylenediamine (Sigma Chemical Co., St. Louis, MO) in 0.1 mol/L citrate buffer, pH 5, containing 0.03% hydrogen peroxide]. After incubation for 15 min at room temperature in the dark, the absorbance was measured at 450 nm with an automated microtiter plate reader (Molecular Devices, Menlo Park, CA).

Two-site ELISA. Microtiter plates were coated with 100 μL of capture mAb diluted in 0.1 mol/L PB, pH 7.4, to the optimal concentration determined as described below. After 18 h at 4°C, the plates were washed three times with a 100 mmol/L PB containing 1 mL/L Tween 20 (PB-T). To saturate free sites, a 1 g/L solution of bovine serum albumin in PB was added to the wells and the plates were incubated for 90 min at 37°C.

One-step procedure: Add 50 μL of rHV2 diluted in human plasma or urine to the mAb-coated microtiter plates and incubate simultaneously with 50 μL of an optimal concentration of HRPO-labeled mAb diluted in PB-T for a fixed time and temperature. Discard the unbound fraction, and wash the microtiter plates three times with PB-T. To detect bound HRPO-labeled mAb, add 100 μL of substrate solution, incubate for 15 min at room temperature in the dark, and measure the absorbance at 450 nm. (In some experiments we stopped the enzyme reaction by adding 50 μL of 2 mol/L H$_2$SO$_4$; the absorbance was then measured at 490 nm.)

Two-step procedure: Add 100 μL of various concentrations of rHV2 in human plasma (same final concentration as for the one-step procedure) to the mAb-coated plates and incubate for 2 h at 37°C. Wash the plates three times in PB-T and then incubate for 1 h at 37°C with 100 μL of HRPO-labeled mAb diluted in PB-T to the optimal concentration. Detection and quantification were as described above.

**Determination of optimal capture-antibody concentration.** Microtiter plates were coated with capture mAb 10-2 diluted in PB to give a concentration range of 2.5–20 mg/L; then 100 μL of three concentrations of rHV2 diluted in pure human plasma was added simultaneously with a fixed concentration of HRPO-labeled mAb 10-5. After incubation for 1 h at 37°C, the microtiter plates were washed and the immunological reaction was quantified as described above.

**Determination of optimal HRPO-conjugated second mAb concentration.** Microtiter plates were coated with 5 mg/L capture mAb 10-2 in PB; then rHV2 at a concentration range of 1 ng/L–10 μg/L in human plasma was added. Finally, various concentrations of mAb 10-5 were added to the plates, and the experimental procedure described above was followed.

**Determination of optimal incubation time and temperature.** Microtiter plates were coated with 5 mg/L capture mAb 10-2 diluted in PB; then 50 μL of three concentrations (0.1, 1, and 10 μg/L) of rHV2 in human plasma and 50 μL of 0.5 mg/L HRPO-labeled mAb 10-5 were incubated for 15, 30, or 60 min at 37°C, 3 or 4 h at room temperature, or 18 h at 4°C. The plates were washed and the assay was then performed as described above.

**Competitive RIA.**

Binding of the mAbs to iodinated rHV2 was determined by RIA in liquid phase. Briefly, 100 μL of mAb diluted in PB-T containing 1 g/L bovine serum albumin was incubated with 50 μL of iodinated rHV2 and 50 μL of either rHV2 or one of the COOH-terminal truncated forms of hirudin for 18 h at 4°C. Immune complexes were coprecipitated by adding 1 mg of bovine gamma globulin in 100 μL of PB-T and 1 mL of 0.1 mol/L PB containing 200 g/L polyethylene glycol 6000. After incubation for 1 h at room temperature and centrifugation, the supernates were discarded by aspiration, and the residues were counted in a gamma scintillation counter.

**Amidolytic Assay of Thrombin Activity.**

The chromogenic substrate S-2238 (D-Phe-L-Pip-L-Arg-pNA) was purchased from Kabi Vitrum (Stock-
holm, Sweden). Human α-thrombin (3000 NIH units/mg) was obtained from J. P. Cazenave (CRTS, Strasbourg, France). All other chemicals were purchased from Prolabo (Paris, France). Thrombin-catalyzed hydrolysis of the chromogenic substrate (S-2238) was monitored in a microtiter plate and the absorbance at 405 nm was determined by an automatic microtiter plate reader (Titer-tek; Flow Labs., Paris, France). The enzymatic reactions were performed in a final volume of 150 μL at 37°C in 50 mmol/L Tris-HCl buffer, pH 7.8, containing 0.1 mol/L NaCl and 1 g/L polyethylene glycol 6000. Hydrolysis was initiated by adding the chromogenic substrate (2 mmol/L) dissolved in 0.1 mol/L Tris-HCl buffer, pH 7.8, to a preincubated solution of enzyme (0.05 NIH units/well) and various concentrations of rHV2 or of the C-truncated forms dissolved in the same buffer. Color development was allowed to proceed for 4 min; the reaction was stopped by adding 50 μL of an aqueous solution of acetic acid (50:50, by vol) to each well. Absorbance values were read at 405 nm and analyzed by a nonlinear regression program. Specific activity of the test samples was calculated by the method of Degryse (33).

Results

Antigen Mapping

Epitopic specificity of the five mAbs was determined by the one-step and the two-step ELISA procedures, testing all combinations of the five mAbs. One mAb was coated onto the plate and the same or another HRPO-labeled mAb was used as tracer. Direct ELISA showed that labeling the mAbs with HRPO did not affect their activity. The results of the one-step assay are shown in Fig. 1. Two immunological domains were defined on the antigen surface: The first one reacted with mAb 10-5, 10-6, and 10-9 (group 1); the second one with 10-2 and 10-3 (group 2). The one-step ELISA sandwich proved to be 10-fold more sensitive than the two-step assay (data not shown).

Hirudin 65 (rHV2) and six hirudin derivatives processed at the COOH terminus (H64, H63, H62, H61, H57, H54) were used to probe the specificity of the two mAbs by liquid-phase RIA. MAb 10-2 reacted strongly with H65 and only weakly with H64 (10% cross-reactivity); this mAb did not recognize any of the other forms with truncated COOH termini (Fig. 2A). In contrast, mAb 10-5 recognized the COOH-terminal truncated molecules up to the shortest fragment tested, H54 (Fig. 2B).

Optimization of the One-Step Antigen-Capture ELISA

HRPO-labeled 10-5 and rHV2 were added simultaneously to microtiter plates coated with different concentrations of capture mAb 10-2. The optimal concentration of mAb 10-2 to detect rHV2 in plasma was 5 mg/L (Fig. 3A). Various concentrations of diluted HRPO-labeled mAb 10-5 in PB were then tested; the lowest concentration that gave the highest significant ELISA signal was 0.5 mg/L (corresponding to a dilution of 1000-fold), with 5 mg/L 10-2 as capture mAb (Fig. 3B).

rHV2 and tracer mAb were added to the antibody-coated microtiter plate and incubated under the following conditions: 3 or 4 h at room temperature; 15, 30, or 60 min at 37°C; or 18 h at 4°C. The best experimental conditions for the antigen–antibody reaction for a sensitive immunoassay of rHV2 were 30 min at 37°C (Fig. 4).

Detection Limit

Figure 5 illustrates calibration curves for the one-step immunoassay, covering concentration ranges of rHV2 of 0–1200 ng/L in urine and 0–1000 ng/L in plasma. The curves are linear up to the highest concentration assayed.
Fig. 3. Determination of the optimal concentrations of capture (A) and labeled (B) antibody in the two-site immunoassay. (A) Capture antibody 10-2: Microtiter plates were coated with various concentrations of capture antibody; two rHV2 concentrations were tested with a 4000-fold-diluted labeled antibody 10-5. (B) Labeled antibody 10-5: The optimal dilution of mAb 10-5 was determined by assaying three concentrations of rHV2 in wells coated with 5 mg/L mAb 10-2.

The limit of detection of rHV2 was 45 ng/L in plasma and 30 ng/L in urine, corresponding to 2 SD of the mean of 20 normal plasma and urine samples, analyzed in one experiment.

To determine the intraassay CV, we measured in the same assay 10 replicates of three rHV2 samples in plasma (1510, 740, and 280 ng/L) and urine (1570, 790, and 410 ng/L). To determine the interassay CV, we measured three rHV2 samples in plasma (mean 1530, 780, and 350 ng/L) and urine (mean 1650, 830, and 420 ng/L) on five different days. All the CVs were <9%.

Fig. 4. Effect of incubation time and temperature on detection of rHV2 by the one-step antigen-capture immunoassay. Tracer mAb 10-5 and various concentrations of rHV2 were incubated simultaneously with the capture mAb 10-2 for different times at 4°C, room temperature (RT), or 37°C.

Fig. 5. Calibration curves for rHV2 in urine (A) and plasma (B) for the optimized immunometric assay (one-step procedure, 30 min at 37°C).

Analytical recovery of rHV2 added to plasma and urine ranged from 94.0% to 101.7% and from 91.6% to 93.0%, respectively (Table 1).

Specificity of the Antigen-Capture ELISA

rHV2 and six forms of the protein truncated at the COOH terminus were used to determine the specificity of the antigen-capture ELISA. The 65-amino acid form was the main protein detected, reflecting the specificity of capture mAb 10-2 (Fig. 6).

Inhibition of Thrombin Activity

Using a chromogenic assay of thrombin activity, we tested the antithrombin effects of rHV2 and six COOH-terminal truncated forms (Table 2). rHV2 and the H64 form were the strongest inhibitors of the amidolytic
Table 1. Analytical recovery of rHV2.

<table>
<thead>
<tr>
<th>Added, ng/L</th>
<th>Plasma Recovered, ng/L</th>
<th>Urine Recovered, ng/L</th>
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<td>990</td>
<td>960</td>
<td>96.0</td>
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Original plasma and urine samples contained rHV2 at 500 ng/L. Recovery was calculated for the one-step immunoassay.

Fig. 6. Reactivity of rHV2 and six COOH-truncated forms in the two-site immunoassay.

reaction; however, the inhibitory activity of H63, H62, and H61 was not negligible (67%, 48%, and 44%, respectively).

Discussion

This highly sensitive two-site immunoassay for recombinant hirudin, which can be performed within 1 h, involves the simultaneous addition of test sample and tracer mAb 10-5 labeled with HRPO to microtiter plates coated with capture mAb 10-2. No apparent "hook" effect due to antigen excess was observed when the hirudin standard was tested at concentrations of 10-1000 ng/L in plasma or urine. When we introduced graded doses of precomplexed hirudin and thrombin as well as free hirudin into our two-site immunoassay, the signal obtained for the complex was identical to that of the background value (data not shown), indicating that only free hirudin is detected in our immunoassay. This is not surprising, because the capture mAb 10-2 is directed against the COOH-terminal region of hirudin, one of the sites of interaction with thrombin. This antigen-capture ELISA is far more sensitive than the clotting assay, amidolytic assays, physicochemical techniques, or other immunoassays that detect hirudin at microgram concentrations per liter (34).

The one-step procedure was 10-fold more sensitive than a two-step protocol (data not shown), probably because of cooperative binding between the antibodies and rHV2. Using COOH-truncated forms of hirudin, we showed that the new immunoassay is specific for the 65-amino acid form. This restricted specificity can be explained by the fact that recognition of rHV2 by the capture antibody mAb 10-2 is highly dependent on the last two amino acids at the COOH-terminus. In contrast, tracer mAb 10-5 is able to recognize the 1-54 C-truncated form of rHV2. Schleppi et al. (35) also produced an anti-hirudin mAb that is not directed against the COOH terminus; their mAb binds to region 41-47 of rHV2.

Evaluation of the biological activity of the truncated forms by the amidolytic assay indicated that the 64-amino acid form also was almost as active as rHV2, and that the 63-, 62-, and 61-amino acid forms had significant thrombin inhibitory activity. The reduced specificity of the amidolytic assay is in sharp contrast with the high specificity of the two-site immunoassay for rHV2. The restrictive nature of our immunoassay should not be a drawback for its future use, since a metabolic study on recombinant hirudin showed that intact rHV2 was the major form in dog urine and that H61, H62, H63, and H64 were only minor metabolites after intravenous bolus administration of this very promising pharmaceutical agent for thrombosis (36).

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References