Two-Site Direct Immunoassay Specific for Active Renin

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A sensitive immunoradiometric assay, without an enzymatic step and specific for active human renin, was developed with use of two monoclonal antibodies (MAbs). In this assay system, the first MAb was coupled to magnetic beads (Magnogel®); the second one, directed against the active form of the enzyme, was radiolabeled with 125I. The specificity of this assay was demonstrated in experiments measuring the active plasma renin concentration in the presence or absence of inactive renin. The assay, performed in two steps, was sensitive enough to detect 0.9 pg of renin per tube (3.5 ng/L). Intra- or interassay CVs were <10%. Concentrations of active plasma renin measured in normotensive subjects were between 7 and 40 ng/L.

Additional Keyphrases: immunoradiometric assay • monoclonal antibodies • hypertension

The development of angiotensin I radioimmunoassays to measure plasma renin activity by means of its ability to generate angiotensin I tremendously increased the practicability and the usefulness of renin assays (1, 2). However, all methods that measure renin by its enzymatic activity are limited by dependency on the amount of available angiotensinogen (a limiting step in the enzymatic reaction that may be modified in certain clinical situations (congestive heart failure, liver cirrhosis) (3, 4)), on the pH used for the in vitro incubation of plasma, and on the possible presence of activators or inhibitors in some plasma samples (5, 6). The potential variability of these factors explains why identical plasma samples, investigated in separate laboratories, generate quite different amounts of angiotensin I, even if the distribution into low-, normal-, and high-concentration renin groups is generally appropriate for a given laboratory (7).

All polyclonal antibodies so far obtained are unable to distinguish between active and inactive renin (8–10). Among the monoclonal antibodies available, some inhibit enzymatic activity but fail to specifically detect active renin in human plasma (11–13).

We have developed monoclonal antibodies against active human renin and identified one monoclonal antibody (MAb) that not only is a noncompetitive inhibitor of renin enzymatic activity but also is able to distinguish between active and inactive renin in blood (14).

This antibody has proved useful in setting up a one-step immunoassay of active renin (15–17). Here, we describe the development of a highly sensitive two-step immunoradiometric assay (IRMA) of renin, which measures only the active form of the enzyme and makes direct access to active renin measurement possible without an enzymatic step.

Materials and Methods

Materials

Human and monkey renin. International standard of human renin [lot 68/356, 0.1 international unit (IU) per vial] was kindly provided by the National Institute for Biological Standards and Control (Herts., UK). Monkey serum with a high concentration of active renin was obtained from baboons treated with furosemide (15 mg/kg of body wt. daily, intramuscularly, for 5 days). The animals were bled on the sixth day and the serum, collected at 4°C, was centrifuged, aliquoted, and stored at −80°C. The renin concentration in monkey serum was calibrated by reference to the international standard. These sera were used as working standards for the immunoradiometric assay (assuming the equivalence of 0.6 μg for 1 IU).

Monoclonal antibodies. Several MAbs directed against human renin were obtained by cell fusion. Immunization of mice, cell fusion, selection of hybridomas, mass production, purification of the immunoglobulins, and characterization of the antibodies have been previously reported (14). For the IRMA, we selected the MAbs 3E8 and 4G1, which recognize two different epitopes on the renin molecule (14). MAB 3E8 was coupled to Magnogel® magnetic beads (AcA 44; Industrie Biologique Française, Paris, France), and MAB 4G1 was labeled with 125I.

Another MAB (4C1) was raised against a synthetic peptide corresponding to a portion of the human renin prosegment (residues −19 to −7). This antibody recognizes inactive renin (prorenin) but does not recognize trypsin-activated renin (18). For control experiments, we used MAB 8A6, an antibody directed against human T-lymphocytes (CD7).

MABs 4C1 and 8A6 were coupled to CNBr-Sepharose 4B (Pharmacia, Uppsala, Sweden), as described (19), at 3.5 mg of IgG per milliliter of gel.

Procedures

Immobilization of MAB 3E8. MAB 3E8 was coupled to activated Magnogel by the glutaraldehyde method (20). The coupling efficiency was ~1 mg of antibody per
milliliter of Magnogel. For storage, we diluted the Magnogel to 20 mL/L in 0.2 mol/L imidazole·HCl buffer, pH 7.4, containing 2.5 g of gelatin and 1 g of NaN₃ per liter.

**Iodination of MAb 4G1.** Purified MAb 4G1 was labeled with ¹²⁵I by the Chloramidine T method at room temperature as previously described (21). After purification, the tracer was >95% precipitable by 200 g/L trichloroacetic acid reagent. The overall iodination yield was ~60% and the average specific activity of labeled MAb 4G1 was 925 MBq/g. The labeled MAb could be stored without loss of activity for 2 months at 4 °C in 0.05 mol/L Tris·HCl buffer, pH 7.4, containing 100 mL of horse serum and 1 g of NaN₃ per liter.

**Immunoradiometric assay.** These assays were carried out in disposable polystyrene test tubes on special racks (Diagnostics Pasteur, Marnes-Le Coquette, France; no. 97005). The one-step procedure was performed as previously described (15, 17). The two-step procedure was performed as follows: 250 μL of renin standard solution in 0.2 mol/L imidazole·HCl buffer, pH 7.4, containing 100 mL of horse serum and 1 g of NaN₃ per liter (assay buffer) or 250 μL of undiluted plasma sample was added to 250 μL of Magnogel-bound MAb SE8 suspension. The assay was performed in duplicate and the nonspecific binding was measured by replacing the plasma with 250 μL of assay buffer. Tubes were incubated for 2 h at 18–25 °C on a horizontal shaker (Diagnostics Pasteur; no. 97000). The beads were then washed on the magnetic rack with 2 mL of 0.2 mol/L imidazole·HCl buffer, pH 7.4; 250 μL of labeled MAb 4G1 (100 000 counts/min) was added and the samples were incubated with shaking for 3 h at 18–25 °C. Finally, the beads were washed three times with imidazole buffer, and the bound radioactivity was measured with a gamma counter.

**Specificity experiments.** We incubated 12 mL of a high-renin human plasma pool (460 ng/L) with 3 mL of each gel (anti-renin prosegment MAb 4C1–Sepharose 4B or MAb 8A6–Sepharose 4B) for 18 h at 4 °C. After incubation, the active renin and the renin activated in the supernate of both affinity gels were measured by the renin IRMA.

**Trypsin activation.** Trypsin activation was performed according to Sealey et al. (22); we added trypsin (Sigma Chemical Co., St. Louis, MO; cat. no. T-8253) to plasma samples to give a final concentration of 1 g/L and incubated the mixture for 1 h at −4 °C before performing the immunoassay.

**Human Sera**

We investigated 100 normotensive subjects (50 men and 50 women, ages 20 to 60 years), with normal sodium diet, in supine (>1 h) and upright (>1 h) positions. Blood was collected at room temperature into EDTA; after centrifugation, the plasma was divided into 1-mL samples for storage without delay.

**Results**

**Standard curve.** As shown in Figure 1, the amount of radioactive MAb 4G1 trapped on the Magnogel was directly proportional to the amount of renin. The signal obtained (counts/minute) varied linearly with renin concentrations between 7 and 270 ng/L.

**Kinetics of the assay.** Results of the two-step procedure were compared with those of the one-step method performed under the conditions initially used (15–17). The correlation between the two procedures was highly significant (r = 0.98, n = 63). As shown in Figure 1, the slope of the standard curve was greater in the two-step procedure than in the one-step procedure. The kinetics of the reactions were evaluated for renin standards at 15, 90, and 200 ng/L (Figure 2) and for human samples (results not shown). Equilibrium for the reaction between the immobilized antibody and renin was obtained after a 2-h incubation, and after a 3-h incubation for the reaction between immobilized renin and iodinated antibody.

**Sensitivity.** The limit of detection of the renin IRMA, defined as 2 SD from the zero point of the standard curve, was 3.5 ng/L (Figure 1).

**Reproducibility.** To determine the intra-assay CV of the renin IRMA, we measured 20 replicates of three renin samples in the same assay. Other renin samples were measured 45 times on 45 different days to determine the interassay CV. All CVs were <10% (Table 1).

**Accuracy.** Accuracy was estimated by analytical recovery studies and dilution assays. Recovery experiments were performed by adding five different amounts of human renin (international standard) to plasma samples and assaying them with the IRMA (Table 2). Human plasma diluted 2-, 4-, 8-, and 16-fold gave a
Fig. 2. Kinetics of the reactions in the renin IRMA. The kinetics of the first or the second incubation were studied for three concentrations of renin: 200, 90, and 15 ng/L. A fixed time (2 h) was chosen for one incubation and the other incubation was varied from 15 min to 24 h.

**Table 1. Imprecision Studies**

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Intra-assay (n = 20)</th>
<th>Interassay (n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD, ng/L</td>
<td>CV, %</td>
</tr>
<tr>
<td>1</td>
<td>13.3 ± 0.8</td>
<td>6.0</td>
</tr>
<tr>
<td>2</td>
<td>79.0 ± 2.8</td>
<td>3.6</td>
</tr>
<tr>
<td>3</td>
<td>274.0 ± 8.4</td>
<td>3.0</td>
</tr>
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Table 2. Analytical Recovery

<table>
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<tr>
<th>Added</th>
<th>Recovered</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.5</td>
<td>24</td>
<td>94</td>
</tr>
<tr>
<td>51</td>
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<td>98</td>
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<td>76.5</td>
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<td>107</td>
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<td>102</td>
<td>105</td>
<td>103</td>
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<tr>
<td>128</td>
<td>137</td>
<td>107</td>
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</tbody>
</table>

Increasing amounts of human renin (international standard) were added to human plasma containing 22 ng of active renin per liter.

Curve parallel to the standard curve (analytical recovery values varied between 97% and 104%).

**Specificity of renin IRMA.** As shown in Figure 3, the concentration of renin measured by IRMA (460 ng/L) was not increased by trypsin treatment in the supernates from plasma incubated with MAb 4C1 affinity gel but was increased in plasma incubated with MAb 8A6 affinity gel (unrelated MAb, control experiment). Higher concentrations of trypsin in plasma decreased the concentration of renin measured by IRMA or by determination of plasma renin activity (data not shown), probably because of degradation of renin molecules.

We did not quantify the prorenin bound to the MAb 4C1 column because the elution procedure led to partial renin activation (data not shown).

**Normal values.** For apparently normal subjects studied in the upright position, 96% of the values were between 7 and 40 ng/L. For supine subjects, 92% of the values were between 7 and 19 ng/L (Table 3). As noted elsewhere, many factors (posture, age, sodium diet, menstrual cycle, etc.) can influence the plasma concentration of active renin (23).

**Discussion.** We have developed a new immunological sandwich assay method to measure directly and specifically the protein mass concentration of active renin in human plasma.

The pair of MAb chose for the renin IRMA, 3E8 (immobilized onto Magnoel) and 4G1 (iodinated), can distinguish active renin from inactive renin. In general, active renin represents <20% of total plasma renin; the other 80% represents inactive renin, the precursor of active renin. Several results demonstrate the specificity of the renin IRMA: MAb 4G1 recognizes active renin selectively (14); moreover, the active renin concentration in plasma, depleted of inactive renin by the use of an MAb directed against the human renin prosegment (MAb 4C1), does not change after exposure to trypsin (within the normal conditions of trypsin concentration).

Inactive renin was recognized and trapped by MAb 4C1 coupled to the affinity gel and could not be activated later and measured by IRMA renin. In the control experiment (MAb 8A6 gel), inactive renin was not retained by the column and could be activated and measured in the second step. In conclusion, inactive renin was not recognized by the renin IRMA, which measures active renin only.

The two MAb (3E8 and 4G1) recognize primate renin; renin from monkey serum (baboos) can be calibrated against the international standard and thus can be used as a source to establish a secondary standard. Using these reagents, we developed a highly sensitive solid-phase IRMA. We chose a two-step procedure instead of a one-step method to increase the slope of the dose-response curve and to allow measurement under equilibrium conditions. The active plasma renin concentration can be routinely measured over a large range of...
values with this assay. The inactive renin concentration could also be determined with our MAb 3E6 and 4G1 IRMA. After inactive renin was activated according to the method of Sealey (22), total renin was measured. The inactive renin concentration is the difference between the values of total and active renin (17).

A highly significant correlation between the IRMA for active renin and measurements of a conventional assay of plasma renin activity was obtained for plasma samples obtained from normotensive subjects (4, 17, 23). However, unlike normal subjects, patients with severe heart or liver failure have markedly lower concentrations of substrate for plasma renin, which leads to a considerable underestimation of the active renin concentration when it is measured as plasma renin activity (3, 4).

The IRMA for active renin concentration is independent of the plasma renin substrate, and measures the true concentration of active renin. Moreover, the renin IRMA can be used in studies where the renin–angiotensin system is blocked by a renin inhibitor; in such cases, the plasma concentrations of active and total renin are increased when measured by the IRMA (renin release), but plasma renin activity is suppressed (24).

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References