Adenosine Deaminase Binding Protein, a New Diagnostic Marker for Kidney Disease

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This enzyme immunoassay detects adenosine deaminase binding protein (ABP), a glycoprotein that is shed from the brush border of the proximal tubule in kidney damage. Two monoclonal antibodies, URO-4 and URO-4a, each react with different epitopes on ABP and are used as the "sandwich" pair of antibodies. A linear standard curve can be generated by using partly purified ABP isolated from the urine of patients with kidney disease. Release of ABP into the urine appears to reflect the severity of the insult to the nephron. Therefore, measurement of ABP in urine may help distinguish between tubular disease and glomerular disease and indicate renal allograft rejection in renal-transplant patients.

Additional Keyphrases: enzyme immunoassay · monoclonal antibodies · renal transplant · urine · glycoproteins · tubular vs glomerular disease

The diagnosis of kidney disease relies heavily upon observation of changes in the concentrations of urea nitrogen and creatinine in blood, abnormalities on urinalysis, and measurement of urine output. Although useful, these measurements indicate only existing renal insults, do not give advance warning of pending renal damage, and may not adequately define the activity or reversibility of the disease process. Furthermore, because these measurements are indirect, they frequently do not indicate the anatomical site of the renal damage. Renal biopsy is often required to provide this information.

Attempts to improve diagnosis of kidney disorders have focused on the measurement of low-Mr plasma proteins and renal enzymes as possible indicators of kidney disease. Microsomal aminopeptidase (preferential for L-alanine; EC 3.4.11.2), N-acetyl-β-glucosaminidase (EC 3.2.1.30), and β2-microglobulin (I–5) have all been the analytes most frequently measured for this. However, doubts about the specificity and stability of these proteins (6, 7), combined with the presence of enzyme inhibitors and interfering material in urine, often complicate these measurements.

Radioimmunoassays with use of antisera to kidney membrane antigens have demonstrated the release of kidney antigens into urine in renal disease (8, 9). With the development of monoclonal antibodies to various kidney tissue antigens (10, 11), "sandwich"-type enzyme immunoassays have been developed (12, 13) that should be more specific than earlier immunoassays.

The use of monoclonal antibodies has led to the identification of unique renal markers that are specific to different anatomical sites in the nephron, and are negligibly present (as observed via cross reactivity) in other tissues (14). Given the commercial availability of URO-4 (S27) and URO-4a (S23) murine monoclonal antibodies, which recognize different epitopes of a 120 000-Da proximal tubule protein, designated gp120 (10), we developed a "sandwich"-type enzyme immunoassay to quantify this protein in urine. During the development of this immunoassay, gp120 was identified as adenosine deaminase binding protein (ABP) and URO-4 was shown to be capable of immunoprecipitating both the soluble and membrane forms of ABP (15). ABP has also been reported to be present in lung, liver, placenta, and plasma, and low concentrations have been detected in the urine of normal individuals (16–19).

We have discovered that, in renal disease, more ABP is released into the urine. Thus, its measurement could be the basis for a new approach to diagnoses of renal disorders. Here we present our initial evaluation of the release of this antigen in renal disease and demonstrate its usefulness as a diagnostic marker for tubular damage.

Materials and Methods

Materials

URO-4 and URO-4a were obtained as ascites fluid (Cambridge Research Laboratory, Cambridge, MA 02139). "Immulon 2" microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA 22314) were used as the solid supports. Horseradish peroxidase (HRP, EC 1.11.1.7, Type VI; Sigma Chemical Co., St. Louis, MO) was stored at −20 °C before use. o-Phenylenediamine (Eastman Organic Chemicals, Rochester, NY) was used as the chromogenic reagent. Benzamidine HCl (Sigma) was used to inhibit serine proteinase. Sephadex G200, Sephacryl S300, Protein A-Sephrose, and concanavalin A-Sepharose (Pharmacia, Piscataway, NJ) were used to purify monoclonal antibodies and urinary glycoproteins. We used an MR600 microtiter plate reader (Dynatech) to quantify color development. All other chemicals were of reagent grade or better.

Patients

The patients classified in this study as having glomerular disease had had renal biopsies demonstrating such lesions,
with no other renal pathology evident in the biopsy specimen, in the same week that their urines were assayed for ABP. Patients classified as having acute tubular necrosis had undergone an acute episode of hypotension, followed by oliguric renal failure \( \leq 72 \) h before ABP was measured.

Patients classified as having acute renal allograft rejection had been diagnosed on the basis of (a) an increase in the concentration of creatinine in the serum more than two weeks after the transplant, and (b) a renal biopsy within 24 h of the ABP study that revealed acute rejection. For the purposes of this study, we defined renal transplant patients without rejection as those whose concentrations of serum creatinine remained stable at \( \leq 14 \) mg/L for at least one week before and after the ABP measurement.

Methods

**Purification of monoclonal antibodies:** We purified URO-4 and URO-4a on Protein A-Sepharose according to the methods of Ey et al. (20). Briefly, the procedure was as follows. We diluted 1 mL of ascitic fluid with an equal volume of Tris buffer (100 mmol/L, pH 8.5) and applied this to a 4-mL column of Protein A-Sepharose. After washing the column with five column volumes of more-dilute Tris buffer (10 mmol/L, pH 8.5) to remove nonbound proteins, we eluted the specific antibody with citrate buffer (10 mmol/L, pH 4.5) and collected 1-mL fractions into test tubes containing 1 mL of Tris buffer (100 mmol/L, pH 8.5).

**Preparation of monoclonal antibody-enzyme conjugate:** URO-4a was conjugated to HRP according to the method of Wilson and Nakane (21) by mixing 2 mg of URO-4a with 1 mg of periodate-oxidized HRP for 2 h at 4 °C. After reducing the conjugate with NaBH₄, we precipitated the product by half-saturation with (NH₄)₂SO₄ and dialyzed it overnight against 2 L of phosphate-buffered isotic saline (per liter, 10 mmol of Na₂HPO₄/NaH₂PO₄ and 150 mmol of NaCl, pH 7.4). In some cases the URO-4a-HRP conjugate was separated from unreactive HRP by gel filtration chromatography on Sephacryl S300.

**Preparation of antibody-coated microtiter plates:** Purified URO-4 was passively adsorbed onto the surfaces of Immulon 2 microtiter plates by leaving 125-μL aliquots of URO-4 solution (10 μg/mL, in Na₂CO₃/NaHCO₃ buffer, 50 mmol/L, pH 9.6) in each well to adsorb overnight at 4 °C. We then washed the plates with phosphate-buffered saline solution containing Tween 20 polyoxyethylene (20) sorbitan monolaurate (0.5 mL/L). Any remaining binding sites were blocked by use of a 10 g/L solution of bovine serum albumin in phosphate-buffered saline for 1 h at 25 °C. Before use, we washed the plates again three times with the Tween-containing phosphate-buffered saline.

**Urine samples:** Untimed urine specimens, collected from normal individuals and from patients with kidney disease who were being treated at the Massachusetts General Hospital, had preservatives added to give the following final concentrations: Tris 20 mmol/L, benzamidine 1 mmol/L, and EDTA 10 mmol/L. The pH of the specimens then ranged from 6.0 to 7.5. Cell debris and insoluble material were removed by centrifugation (1500 \( \times \) g, 10 min), then the specimens were stored at 4 °C until assayed.

**Preparation of antigen standards:** We prepared standards from a pool of patients' urine specimens with absorbances at 490 nm exceeding 2.0 in the assay described below. Urinary proteins were precipitated by 75% saturation with (NH₄)₂SO₄ for 1 h at 25 °C. After centrifugation, the pellet was redissolved and dialyzed overnight against acetate buffer (10 mmol/L, pH 6.0). At 4 °C. To the buffer we then added NaCl (final concentration 0.5 mol/L), CaCl₂ (1 mmol/L), MgCl₂ (1 mmol/L), and MnCl₂ (1 mmol/L) and applied this to a 1.5 \( \times \) 5 cm column of concanavalin A-Sepharose equilibrated in the same buffer. Nonbound proteins were removed by washing the column with five column volumes of the above buffer. Specifically bound glycoproteins were eluted with this buffer to which 0.2 mol of α-methyl-d-mannoside had been added per liter. We collected 5-mL fractions. ABP was eluted with the major protein peak. After dialyzing the ABP-containing portion of the eluate against phosphate-buffered saline, we stored this urinary antigen preparation at 4 °C in the presence of EDTA and benzamidine (10 and 1 mmol/L final concentration, respectively). To prepare standards, we diluted the antigen preparation with a 1 g/L solution of bovine serum albumin.

**Assay Protocol**

Place duplicate 100-μL portions of patients' urines, assay controls, and known assay standards in previously prepared URO-4-coated microtiter plates, and incubate for 1 h at 25 °C. Wash the plates three times with the Tween-containing phosphate-buffered saline to remove nonbound antigen and other materials. Add 100 μL of URO-4a-HRP conjugate (0.4 mg/L) to each well and incubate for 1 h at 25 °C. Aspirate the unbound conjugate and wash three more times with Tween-containing phosphate-buffered saline. Then add 100 μL of the chromogen solution, which contains, per liter, 2 g of o-phenylenediamine and 500 μL of 30% H₂O₂ in citrate (19 mmol/L)–Na₂HPO₄ (62 mmol/L) buffer, pH 5.0. After 1 h at 25 °C, halt the enzymic action by adding 100 μL of 4.5 mol/L sulfuric acid. Quantify the enzymic activity by measuring, at 490 nm, the absorbance of the color that has developed. The standard curve is based on arbitrary units of reactivity, 1 arb, unit being defined in terms of concentration as the amount of ABP in 100 μL of sample that increased the absorbance at 490 nm by one absorbance unit (1.000 A) in the above assay. Determine ABP in patients' urines by comparison with the standard curve.

**Analytical Studies**

**Stability of ABP in urine:** To test the effect of pH on the stability of ABP, we added increasing volumes of HCl or NaOH (1.0 mol/L) to different aliquots of five patients' urines, generating a pH range of 4 to 9. We incubated the urine specimens at 37 °C for 3 h, then added the corresponding acid or base to neutralize the specimens before the assay. We corrected ABP concentrations for the change in volume from the addition of acid or base.

In another experiment, designed to assess the stability of ABP to freezing, urines from 72 patients were frozen overnight at \(-70\) °C, assayed, and the results compared with those for samples kept at 4 °C.

**Sample-collection time:** To assess the effect of sampling time on ABP content, we collected 24-h urine specimens from three renal-transplant patients and assayed each voiding separately. We also determined the concentration of creatinine in these samples by a modification of the Jaffé method (22).

**Molecular mass determination of ABP in urine:** We applied 1 mL of urine from each of three patients with high concentrations of ABP to a 1.5 \( \times \) 75 cm column of Sephadex G200 equilibrated in phosphate-buffered saline. We collected 1.5-mL fractions on washing the column at a flow rate of 5.0 mL/h with phosphate-buffered saline. We measured the absorbance at 280 nm to detect protein and measured ABP by the assay described above. We also ultracentrifuged urine from five renal-transplant patients (100,000 \( \times \) g, 75 min) to determine whether a membrane form of ABP was present.
Results

Optimization Studies

Optimum quantity of passively adsorbed antibody: We added URO-4 to microtiter plate wells in concentrations ranging from 0.5 to 50 mg/L. From the results, we chose 10 mg/L as the optimal concentration, one that would saturate the wells with the least URO-4 and still bind the standard sufficiently.

Optimum quantity of the enzyme conjugate: Tests of URO-4a–HRP added, in concentrations ranging from 0.1 to 10 mg/L, to microtiter-plate wells containing bound antigen showed that 0.4 mg/L was the optimal concentration of conjugate in this assay; higher concentrations increased the nonspecific binding and the blank value; lesser amounts gave insufficient color development.

Optimum standard curve: Because ABP is present in low concentrations in urine, we express its concentration in arbitrary units until a homogeneous preparation can be obtained for standardization. In our hands the standard curve (Figure 1) is linear from zero up to 2 arb. units. Above this point, the antigen binding sites begin to be saturated and nonlinearity results.

Reproducibility: We ran pooled urine specimens from renal-transplant patients 10 times to assess within-run variation, and another pool of urine specimens from renal-transplant patients 36 times to assess between-run variation. The resulting CVs were 12% or less (Table 1).

Stability of ABP in urine: As shown in Figure 2, results for measured ABP concentrations in the 3-h incubated urines were relatively consistent in the pH range 5.0 to 9.0, decreasing substantially at pH 4.0 and to a lesser extent at pH 4.5.

To assess the stability of ABP to freezing, we froze 72 renal-transplant urine samples at −70 °C and assayed them the next day. ABP concentrations in the samples stored frozen were the same as in samples of the same urine stored at 4 °C (Student’s t-test, p >0.05). To assess the long-term stability of ABP at 4 °C, we re-assayed 25 stored urine specimens from renal-transplant patients after four months; ABP concentrations decreased from 9 to 25% (Student’s t-test; p ≥0.01).

Effect of sampling time: ABP concentrations in the urine specimens collected during 24 h are shown in Figure 3A. For the values averaged for the 24 h, the CV for samples from an

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean (arb. units)</th>
<th>SD</th>
<th>CV, %</th>
</tr>
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<tbody>
<tr>
<td>Within-run (n = 10)</td>
<td>1.275</td>
<td>0.087</td>
<td>7.5</td>
</tr>
<tr>
<td>High control</td>
<td>0.438</td>
<td>0.014</td>
<td>3.2</td>
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<tr>
<td>Low control</td>
<td>0.328</td>
<td>0.018</td>
<td>5.6</td>
</tr>
<tr>
<td>Run-to-run (n = 36)</td>
<td>0.982</td>
<td>0.091</td>
<td>10.3</td>
</tr>
<tr>
<td>High control</td>
<td>0.992</td>
<td>0.091</td>
<td>9.1</td>
</tr>
<tr>
<td>Low control</td>
<td>0.332</td>
<td>0.038</td>
<td>11.1</td>
</tr>
</tbody>
</table>

Table 1. Precision Studies of the Enzyme Immunoassay for ABP

Fig. 2. Effect of urine pH on the stability of ABP

Urine from five different patients was assayed by the assay described above, spiked with radiolabeled ABP (specific activity 3.5 mg/μCi). Aliquots of each sample were incubated at 37 °C for 3 h, neutralized, and assayed for ABP

Fig. 3. Concentrations of ABP vs time of voiding for three transplant patients, without (A) and with (B) correction for urine flow (ABP arb. units/urinary creatinine, g/L)

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individual ranged from 8 to 20%, exceeding the fluctuation seen in the precision studies summarized in Table 1. We attributed this to changes in urine flow and tried to correct for sample volume by expressing ABP values as a function of urinary creatinine concentration. This did not, however, significantly reduce the variation observed between samples (Figure 3B).

Molecular-Mass Estimate for ABP

We collected urine samples from three renal-transplant patients with above-normal concentrations of ABP, and passed them through a column of Sephadex G200. ABP emerged ahead of rabbit IgG; we estimated its molecular mass to be about 200,000 Da. No high-Mₚ material appeared in the void volume of the column.

In a separate experiment, urine samples from five renal-transplant patients with above-normal concentrations of ABP were centrifuged for 75 min at 100,000 × g, conditions under which any membrane fragments would be in the pellet. ABP was quantitatively recovered in the supernate, suggesting that essentially all the ABP in the urine of these patients was in the soluble form.

Clinical Studies

In 30 urine samples from laboratory personnel who had no history of renal disease and were receiving no medication, the ABP concentrations averaged 0.08 arb. units (Figure 4). Table 2 summarizes our results for four groups of patients with clinically well-defined renal disease. For urine samples from the 10 patients with biopsy-proven glomerular disease, ABP concentrations were in each case well comparable with those found in the normal population, the mean value being 0.12 arb. units. Samples from the 20 patients with normal renal function after renal transplant had ABP concentrations only slightly higher than those of the normal population: range 0.10–0.32 arb. units, mean 0.26 arb. units. In contrast, values were significantly increased in the urine of patients with acute tubular necrosis and acute renal allograft rejection (mean 1.5 and 1.1 arb. units, respectively); both of these disorders are clear-cut disease processes that affect the proximal tubule of the kidney.

Discussion

This assay is easy to perform, undiluted urine is used, and we have seen no interference. Because purified ABP is not available, our standard curve is in arbitrary units; it is linear from 0 to 2 arb. units. The assay precision is similar to that of other "sandwich" immunosassays of this type.

ABP has been reported to be an integral part of the cellular membrane (23); however, depending on the isolation protocol, it has also been found in a soluble form (19, 24), as we find it here. ABP evidently is shed from the proximal tubule in response to injury, and this release of ABP corresponds with increased amounts of the previously described proximal tubule markers, microsomal aminopeptidase and N-acetyl-β-glucosaminidase (data not shown). Unlike microsomal aminopeptidase, which has both a soluble and a membrane form (1, 25), ABP appears to be released only in a soluble form in renal-transplant patients, as evidenced above. The estimated molecular mass of ABP, approximately 200,000 Da, corresponds to the soluble dimeric form of the protein reported by other investigators (16, 26). The mechanism of dimerization of the 120,000-Da monomeric protein and the means by which ABP is released during cell damage are unknown.

In experiments designed to simulate conditions in the bladder, ABP was stable from pH 5.0 to 9.0. ABP loses approximately 75% of its reactivity within 3 h at pH 4.0, a pH that urine seldom reaches (27). The stability at pH 5.0 is extremely advantageous because markers such as β₂-microglobulin cannot be validly assessed if the pH of the sample is below 6.0 (7, 27). ABP survives at least one freeze–thaw cycle but 9 to 25% is lost after four months at 4°C.

The ability to obtain diagnostically useful information from an untimed urine specimen is desirable, given the difficulty and inconvenience in collecting accurate 24-h urine specimens or first-morning-void specimens. ABP concentrations varied during a 24-h collection period for the three renal-transplant patients studied (Figure 3A). The variation between sampling times exceeded the limits of precision of this assay for two of the three patients. Expressing ABP concentrations as a function of urinary creatinine concentration (Figure 3B) did not significantly reduce this variation, even though a similar approach had been useful in reducing variations in measurements of N-acetyl-β-glucosaminidase (28). We found that, in some cases, the use of a single untimed urine specimen instead of a 24-h specimen introduced an additional 16% uncertainty in the measurements of ABP. However, this variation is acceptable, given the large difference between the concentrations of ABP in urine of patients with and without active acute tubular disease (Table 2).

We have confirmed the previous finding of ABP in serum (19) and, with this assay, detected in six normal individuals concentrations of ABP ranging from 0.26 to 0.40 arb. units (mean 0.31, SD 0.04). ABP has also been reported in the glomerulus (29) but this was not confirmed in a later study from the same laboratory, using fixed tissues (17). In a recent study with frozen tissues, URO-4 bound weakly to the epithelial cells of Bowman's capsule (14). In glomerular disease, ABP may leak into the urine from the blood, and could also be shed from the glomerulus, resulting in increased concentrations of it in urine. However, our data show that patients with glomerular disease have ABP concentrations only slightly higher than the insignificant amounts found in normal individuals, whereas patients

![Image](https://example.com/image.png)

**Fig. 4.** Concentrations of ABP in urines from 30 normal adults.

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. of patients</th>
<th>ABP, arb. units</th>
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<tbody>
<tr>
<td>Glomerular disease</td>
<td>10</td>
<td>0.08–0.18</td>
</tr>
<tr>
<td>Acute tubular necrosis</td>
<td>25</td>
<td>0.80–2.0</td>
</tr>
<tr>
<td>Renal allograft recipients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with acute rejection</td>
<td>11</td>
<td>0.56–2.0</td>
</tr>
<tr>
<td>without rejection</td>
<td>16</td>
<td>0.10–0.32</td>
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</tbody>
</table>

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with tubular disease have a greatly increased concentration of ABP. These findings suggest that this assay is highly specific and clinically useful in delineating the anatomic site of disease activity within the kidney.

This assay may have a variety of uses in clinical medicine: to locate the site of disease activity in the kidney, to define the activity and reversibility of the disease process and, perhaps, to provide an early warning of renal injury before the concentration of creatinine significantly increases in serum and when such disease processes as transplant rejection or drug toxicity might be still reversible. Studies to test these hypotheses are currently in progress.

In conclusion, this "sandwich"-type assay measures the release of a protein, ABP, which shows promise as a diagnostic marker for proximal tubule damage. In most cases the release of ABP precedes the increase of serum creatinine (30), one of the current diagnostic markers for renal disease. ABP exists in a soluble form, is not affected by inhibitors in the urine or by pH, and is present in low concentrations in normal individuals. Other proposed markers do not have these advantages. Measurement of ABP in the urine differentiates between glomerular and tubular disease and may facilitate the early diagnosis of transplant rejection.

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References

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