Monoclonal Antibody-Based Immunoenzymometric Assays of Retinol-Binding Protein

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Retinol-binding protein (RBP) is a low-molecular-mass protein (21 kDa), easily filtered in renal glomeruli and very efficiently reabsorbed by the proximal convoluted tubules (PCTs). In PCT dysfunction, high concentrations of RBP are found in urine. Several methods have been used to determine RBP in serum or urine. We describe the production, selection, labeling, and utilization of anti-RBP monoclonal antibodies in two- or one-step immunoenzymometric assays for the determination of RBP. The one-step assay has good precision, with within-run and between-run CVs < 6.6% and 5.9%, respectively. Comparison with radial immunodiffusion (x) showed good agreement: y = 0.068 mg/L + 0.899x (n = 24). Comparison between the one-step (y) and two-step (x) versions of the assay also showed a very good correlation: y = 212 µg/L + 0.910x. The one-step assay has been adopted for routine work; it detects transthyretin-bound as well as free RBP and may have clinical usefulness in evaluating the functional status of PCTs.

Low-molecular-mass proteins (<40 kDa) are easily filtered through glomerular capillaries. Proximal convoluted tubules (PCTs) in normal conditions reabsorb very efficiently the filtered load of proteins (1). Several of these proteins have been determined in clinical laboratories: light chains of immunoglobulins, lysozyme, β2-microglobulin, and retinol-binding protein (RBP). The excretion of such proteins is usually augmented when there is some dysfunction of PCTs. RBP is produced in hepatocytes and transports retinol to peripheral tissues (2–4). In circulation, ~90% of RBP is bound to transthyretin (TTR, prealbumin). After delivering retinol to different cells, RBP is freed from TTR, in its free form, with a molecular mass of 21 kDa, it is easily filtered in the glomeruli. PCTs almost completely reabsorb RBP.

The determination of RBP in urine offers advantages over that of other proteins because its production is relatively constant, there is no known clinical situation in which overproduction could lead to abnormal urinary concentrations, and it is very stable in the whole range of urinary pH (7). RBP has been determined in urine or serum by radial immunodiffusion (3), fluorometry (8), ELISA (9), RIA (10, 11), immunoturbidimetry (12), latex particle counting (13), and latex-enhanced immunoturbidimetry (13, 14). All these methods have utilized polyclonal antibodies; immunoassays based on monoclonal antibodies (mAbs) presumably could offer advantages over the previously reported methods for RBP determination. Here we describe the production and characterization of a group of such antibodies as well as the development of sensitive immunoenzymometric assays (IEMAs) for quantifying RBP. We also address some pertinent technical aspects, such as the protection of highly reactive amino groups in antibody molecules before biotin labeling and the utility of mAbs directed against hidden or only partially exposed epitopes in designing one-step assays with little or no hook effect.

Materials and Methods

Buffers

We used the following buffers:

- Phosphate-buffered saline (PBS), pH 7.4, containing, per liter, 20 mmol of phosphate (2.49 g of disodium phosphate, 0.34 g of monosodium phosphate), 9 g of NaCl, and 0.1 g of thimerosal.
- Coating buffer, pH 9.6: per liter, 60 mmol of carbonate/bicarbonate (1.8 g of sodium carbonate, 3.6 g of sodium hydrogen carbonate).
- Washing buffer, pH 7.4: PBS containing, per liter, 0.5 mL of Tween 20 (Sigma Chemical Co., St. Louis, MO).
- Assay buffer, pH 7.4: PBS containing, per liter, 0.5 mL of Tween 20 and 29.2 g of NaCl.
- Color reagent: 400 mg of o-phenylenediamine dihydrochloride (Sigma) and 100 µL of 300 mL/L H2O2 in 1 L of 0.1 mol/L citrate/phosphate buffer (per liter, 7.4 g of monohydrated citric acid and 9.9 g of disodium phosphate), pH 5.0.

Preparatory Procedures

Purification of RBP. RBP was purified from the urine of a patient with tubular proteinuria, as described by Bernard et al. (15) with modifications. Briefly, 3 L of urine was concentrated by ultrafiltration through PM 10 membranes (Amicon Div., W.R. Grace & Co., Danvers, MA) to 3 mL and chromatographed through a 2.0 × 80 cm column of Sephacryl S-200 (Pharmacia Fine Chemicals, Upsala, Sweden). Fractions containing RBP were pooled, reconcentrated through PM 10 membranes, and submitted to ion-exchange chromatography on a column of diethylaminoethyl–Sephacell (Pharmacia). RBP was eluted from this column as four different peaks. Two of them contained retinol, as judged by absorption at 330 nm. All peaks were pooled and this

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Nonstandard abbreviations: CSF, cerebrospinal fluid; DMA, dimethylaminoacid hydride; IEMA, immunoenzymometric assay; mAb, monoclonal antibody; NHS, N-hydroxysuccinimido; PBS, phosphate-buffered saline; PCT, proximal convoluted tubule; RBP, retinol-binding protein; and TTR, transthyretin.

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pool was used as the antigen in all of the following procedures.

Production of mAbs. Female Balb/c mice (6–8 weeks old) were immunized subcutaneously with 10 μg of purified RBP emulsified with Freund's complete adjuvant. Two booster injections with the same dose were given in 3-week intervals. Three days before fusion, the best responders were injected with 40 μg of RBP in saline intraperitoneally. The mice were killed, their spleens were collected aseptically, and the splenocytes were fused with SP2/0 myeloma cells, with use of polyethylene glycol 4000 (E. Merck, Darmstadt, Germany), as described by Goding (16). The supernates were assayed for antibody-producing hybrids by ELISA. Briefly, microtiter plates (Nunc A/S, Roskilde, Denmark) were coated overnight with purified RBP, 5.0 mg/L in coating buffer. The wells were washed three times with washing buffer; culture supernates were added and incubated for 2 h at 37 °C. After additional washings, sheep anti-mouse immunoglobulin–peroxidase conjugate was added and incubated for 1 h at 37 °C. Color was developed with color reagent for 30 min, the reaction being stopped by adding 25 μL of 0.5 mol H2SO4. The absorbance at 490 nm was measured with an automated plate reader (Model EL 311; Bio-Tek Instruments, Winooski, VT). Positive hybrids for antibody production were cloned by the method of limiting dilution. For production of ascitic fluid, we injected 106 cells into mice previously primed with pristane.

Purification of the mAbs. MAbs were purified from ascites with caprylic acid and ammonium sulfate precipitation (17). The immunoglobulins were subtyped by using a set of subclass-specific goat anti-mouse biotinylated antibodies (Amersham International plc, Amersham, UK).

Biotinylation of the mAbs. We labeled the mAbs with N-hydroxysuccinimido-biotin (NHS-biotin; Pierce, Rockford, IL), as described by Kendall et al. (18). The antibody (2 g/L) was dialyzed at 4 °C overnight against 0.1 mol/L NaHCO3 and mixed with 12 μL of 0.1 mol/L NHS-biotin in dimethylformamide. After incubation at room temperature for 4 h, with gentle agitation on a rotary mixer, the preparation was dialyzed against two changes of PBS at 4 °C for 48 h. The conjugate was then stored at −20 °C in glycerol, 500 mL/L. An alternative procedure for biotinylation utilized dimethylmaleic anhydride (DMA; Sigma) as a reversible blocking agent of amino groups, as described previously (19). Briefly, mAbs (2 g/L) were dialyzed against 0.05 mol/L borate buffer, pH 8.6 (per liter, 4.5 g of disodium tetraborate−10 H2O, 2.35 g of boric acid, and 8.2 g of NaCl), overnight at 4 °C, and 0.05 mL of 0.025 mol/L DMA in dimethylformamide was added; the solution was then incubated for 30 min, with constant stirring, on an ice-bath. After this pretreatment with DMA, the antibody was biotinylated as described above.

Assay Development

Selection of antibodies. For the selection of the mAbs, we performed two- and one-step assays (see below) because reactivity of the antibodies was shown to differ in these two versions of the assay. For these tests we used only one concentration of RBP, 100 μg/L.

Two-step assay. Wells of microtiter plates were coated with 100 μL of a solution containing mAb, 10 mg/L, in coating buffer. After an overnight incubation at 4 °C for the adsorption of the antibodies to the plastic surface of the wells, the wells were washed three times with washing buffer. Plasma standard (Behring Institut, Marburg, Germany), diluted in the assay buffer to give final concentrations of 1.6 to 100 μg/L, or samples (ordinary urine specimens diluted 10-fold) were added to the wells at a volume of 100 μL. After being incubated for 2 h at 37 °C, the wells were washed three times with washing buffer and the biotinylated antibody, usually diluted 1000-fold in the assay buffer, was added and incubated for 1 h at 37 °C. After three washings, streptavidin–peroxidase (Amersham), diluted 1000-fold, was added (100 μL per well) and incubated for 1 h (37 °C); after four more washings, color was developed by adding color reagent. Color development was allowed for 30 min at room temperature, and interrupted by adding 25 μL of 0.5 mol/L H2SO4 per well. Absorbances were read at 490 nm in a plate reader. Concentrations in the samples were calculated by comparison with the standard curve, prepared by utilizing nonlinear regression, usually as a third-degree polynomial.

One-step assay. In this variant, the biotinylated antibody was diluted 500-fold and added (50 μL per well) simultaneously with the standards or samples (also at 50 μL per well). After incubation for 2 h, the wells were washed three times with washing buffer and the streptavidin–peroxidase was added (100 μL per well). The remaining steps were the same as for the two-step version of the assay.

Other Methods

RID. For comparison, we also determined the concentrations of RBP in selected urine samples of patients with tubular proteinurias by radial immunodiffusion (Behringwerke AG, Marburg, Germany).

Gel-filtration analyses. Samples (1.7 mL) of serum, urine, or cerebrospinal fluid (CSF) were filtered through a 170-mL column of Sephacryl S-200. The column was equilibrated with PBS and 2-mL fractions were collected at a flow rate of 20 mL/h. RBP in each fraction was quantitatively assayed by the described IEMA; IgM, IgG, albumin, and β2-microglobulin were also determined as molecular size markers.

Samples

Random (untimed) urine samples were collected without any preservative from 95 apparently healthy adults, 56 women and 39 men, ages 19 to 56 years, and screened with Combur 9 strips (BM Test; MB Bioquimica Ltd., Rio de Janeiro, Brazil). The urine samples with no abnormal results were kept at −20 °C for subsequent analysis. Urine creatinine was determined by an alkaline picrate kinetic method.
Results

Hybridoma production. Seven mAbs to RBP were selected from two independent fusions, based on the reaction of the antibodies with purified RBP adsorbed onto the wells of microtiter plates. Six of them were of IgG1 subclass and one (mAb H2P1) of IgM class.

Efficiency of conjugation. When the mAbs were labeled with biotin, two of them (H2P1 and B12P5) lost their antigen-binding activity. However, efficient biotin labeling could be obtained when their most reactive amino groups were protected with the reversible blocking agent DMA, before the biotinylation (data not shown).

Selection of antibodies. We evaluated the binding sites of the mAbs on the RBP molecule with two-step and one-step sandwich assays. In these assays the sandwich can be completed only if capturing and labeled antibodies bind to different epitopes on the surface of the antigen molecule, with little or no steric hindrance. Although all the mAbs had initially been selected on the basis of their ability to bind to RBP adsorbed onto the solid-phase, two of them (mAb H2P1 and B12P5) failed to capture RBP in the two-step assay. Interestingly, however, use of these mAbs as capturing antibodies in the one-step assay yielded an efficient capturing activity (Figure 1). Apparently, these mAbs recognize an epitope that is exposed only when RBP is adsorbed onto plastic surfaces or after its reaction with one of the other antibodies.

Routine IEMA for determining RBP. From seven mAbs produced, we selected two (A8P3 and E9P6) on the basis of the useful range of determination and sensitivity they provided in the assay, as well as stability of the clone. Using this pair of mAbs, we have developed two-step and one-step assays, with A8P3 as the capture antibody and E9P6 as the biotinylated antibody. The results obtained by the two versions of the assay show excellent correlation (Figure 2). After this initial evaluation, the one-step assay was selected for routine work, being the faster and simpler assay. Besides, it shows a hook effect only at very high concentrations of RBP; i.e., >50.0 mg/L in the well. Given that unknown urine samples are routinely diluted at least 10-fold, this value would correspond to ≥500 mg/L. RBP values that high have not been found, so far, in our laboratory. Except for the analyses of the urine samples from the normal control subjects and for the comparison between one-step and two-step versions of the assay, all other results of RBP determination in the present work were obtained with the one-step assay. To avoid interference from heterophile antibodies in urine reactive with mouse IgG, a situation theoretically anticipated for patients with nephrotic syndrome and massive proteinuria, we routinely add normal mouse serum, 10 mL/L (final concentration), to the biotinylated mAb.

Analytical variables of the IEMA. Figure 3 shows representative standard curves for the one-step assay, with a working range of 1.6–100.0 μg/L. The minimal

![Fig. 1. Complementarity of anti-RBP mAbs](image)

![Fig. 2. Correlation of RBP measured in urine samples tested in both one-step and two-step assays](image)

![Fig. 3. Standard curves obtained with serial dilutions of standard plasma (Behring), normal human serum, purified RBP, and urine with a high concentration of RBP](image)
detectable concentration per well, calculated from the mean plus 2 SD of the zero point, was 0.6 μg/L. When serial twofold dilutions of serum, urine with high concentrations of RBP, and RBP that had been purified from urine were tested, the curves could be superimposed on the RBP standard curve (standard plasma from Behring). These results demonstrate that endogenous serum or urine constituents do not usually interfere with the assay. When diluted standard plasma (100 μg/L RBP) was added to normal urine samples (n = 5), the analytical recovery ranged from 98% to 109% (mean ± SD = 101.8% ± 4.66%). The within-run imprecision was evaluated by assaying three different urine samples 10 times each within a single run. Between-run imprecision was evaluated by testing, in 12 different days, three other samples (Table 1).

**Comparison with other methods.** IEMA results (y) were compared with those obtained by radial immunodiffusion (x) within a range of RBP concentrations of 25–132 mg/L. The correlation coefficient (r) was 0.949 and the linear-regression equation was y = 0.068 ±3.667 + 0.899 (±0.064)x (Sxy = 7.073, n = 24).

**Reference interval.** In urine samples of the 95 normal adults, we measured RBP concentrations ranging from 4 to 385 μg/L. Log-transformed results showed a mean of 58.5 μg/L and the reference interval (mean ± 2 SD) was 13–315 μg/L. The median of the results observed for the 56 women (65 μg/L) was not significantly different (Mann–Whitney test) from that for the 39 men (83 μg/L). When corrected for urinary creatinine, the P2.5, median, and P7.5 values were respectively 0.9, 6.5, and 23.9 mg of RBP per mole of creatine. These values closely agree with other published results (3, 11, 13).

**Gel-filtration analyses.** We analyzed by chromatographic gel filtration sera (Figure 4) from a normal control and two patients with chronic renal failure, as well as urine and CSF. RBP in the effluent was quantified by the new assay. We found that the mAbs used in the IEMA recognize free RBP as well as TTR-bound RBP. As previously described, in plasma from healthy subjects, RBP is found mainly bound to TTR, the free form corresponding to <10% of the protein. The concentration of this free component usually increases when the glomerular filtration rate decreases (6). In CSF, as previously demonstrated (data not shown here), RBP is found mainly in its free form, although TTR is well known to be more concentrated in CSF than in plasma. In urine of patients with proximal tubular dysfunction,

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Table 1. Imprecision Profile of the Monoclonal Antibody-Based IEMA of RBP

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<tr>
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<th>Within-run (n = 10)</th>
<th>Between-run (n = 12)</th>
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<tbody>
<tr>
<td>Mean, μg/L*</td>
<td>CV, %</td>
<td>Mean, μg/L</td>
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<tr>
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<td>2.6</td>
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<tr>
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<td>4.2</td>
<td>12.9</td>
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*Concentration in the well.

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**Discussion**

Here we describe the production of new anti-RBP mAbs and the development of a sensitive and reproducible mAb-based IEMA for quantifying RBP. The assay was performed in either two-step or one-step variations, with use of the seven mAbs we obtained. These mAbs could be separated into three groups, according to their behavior in recognizing epitopes on the RBP molecule. The first group comprises the mAbs (A8P3, F2P8, H9P2, and H8P5) that recognize epitopes naturally exposed on the RBP molecule—of them could capture the analyte in the two-step assay. The mAbs of the second group (H2P1 and B12P5) react with a class of different epitopes, epitopes that are exposed only after a previous interaction with another antibody (this could be verified only when we used both two- and one-step assays). Another mAb, E9P6, seems to recognize a partially hidden epitope; when this mAb was used as capturing and A8P3 as tracer antibody, the detection limit of 10 μg/L in the two-step assay could be improved 10-fold, to 1 μg/L, in the one-step assay. The observation that a mixture of mAbs may have higher affinity than an individual antibody has been reported for monoclonal antibodies against human chorionic gonadotropin (20). Still to be determined is whether the binding of one or several antibody molecules to the antigen alters its spatial configuration, resulting in the exposure of new determinants, or whether the different reactivities are merely a consequence of allosteric effects.

Although RBP is known to consist of a single polypeptide chain with 182 amino acid residues (21), there is little information concerning its immunological determinants. From the epitope analysis, four determinants could be tentatively defined through the use of this panel of seven mAbs. These findings are consistent with the data previously presented by Vahlquist and Peterson (22).
Total or partial loss of antigen-binding activity after antibody-labeling procedures has been described previously. Endo et al. (19) circumvented this problem, when conjugating methotrexate to mAbs, by reversibly reacting the amino groups on the antibody molecules with DMA, before the conjugation with methotrexate. Interestingly, our two mAbs of the second group lost their activity when labeled with biotin. They were, however, efficiently protected by DMA. In the neutral conditions of the subsequent dialysis, the DMA was hydrolyzed and removed. This conjugate was highly stable even without subsequent treatment with hydroxylamine (data not shown). We have previously had problems in obtaining adequate sensitivity as well as reproducibility when we used polyclonal antibodies in an IEMA for RBP determination (data not shown). Use of the selected mAbs, A8P3 used for capturing the antigen and E9P6 as the labeled antibody, has enabled these problems to be overcome. The mAb E9P6, being directed toward a partially hidden epitope, presents an additional advantage. When it is utilized either as capturing or as labeled antibody in the one-step modality of the assay, a hook effect is observed only at very high concentrations of urinary RBP, >500 mg/L in the original specimen. The highest concentration of urinary RBP we have found so far was 255 mg/L, in the urine of a kidney-transplanted patient with acute cellular rejection and acute tubular necrosis. The anti-RBP mAbs and the IEMAs described in this paper may have clinical application in the diagnosis of proximal tubular dysfunctions.

References