Reactivity of Urinary Albumin (Microalbumin) Assays with Fragmented or Modified Albumin

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BACKGROUND: Controversy exists regarding occurrence and measurement of structural variants of albumin in urine. In this study, we examined cross-reactivity of in vitro modified albumins in assays for urine albumin (microalbumin).

METHODS: We analyzed albumin modified by reagents, trypsin, or physical treatments or differing in primary sequence (animal albumins) with an immunoturbidimetric assay (Beckman LX20) using goat antiserum and a competitive immunoassay (Siemens Immulite) using a monoclonal antibody. We assessed occurrence of albumin fragments in urine by use of Western blotting of 24 specimens.

RESULTS: Chemical modification, modest sequence substitution (gorilla albumin), or cleavage of albumin by cyanogen bromide (CNBr) had little effect on reactivity in the LX20 assay. Albumin extensively cleaved with trypsin retained partial reactivity. The Immulite assay generally was affected more severely by albumin modifications and sequence changes. Western blots of fresh urine specimens or specimens stored at –80 °C showed little albumin fragmentation, but some specimens stored for 3 years at –20 °C had extensively fragmented albumin that was detected by the LX20 but not the Immulite assay.

CONCLUSIONS: Nearly equivalent reactivity of intact albumin and CNBr fragments in the immunoturbidimetric assay indicates reactivity of antibodies with multiple epitopes throughout albumin. Therefore, it is difficult to abolish reactivity of albumin in this type of urine albumin assay. Differential sensitivity of 2 assays to albumin modification identifies a potential source of assay nonequivalence in measuring urinary albumin, particularly for specimens stored at –20 °C.

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Increased urinary excretion of albumin is recognized as an early indicator of glomerular injury in diabetes, before injury is indicated by assays for total urinary protein (1). Recently, albumin excretion, even within the normal range of <30 mg/day, has been identified as an indicator for risk of mortality and cardiovascular disease (2–4). These findings heighten interest in the accurate measurement of urinary albumin within the normal range and as in the microalbuminuric range (30–300 mg/day or 20–200 mg/g creatinine). At the same time, however, there is increased interest in accurately measuring low concentrations of urinary albumin.

Two issues have recently generated questions and controversy about the accuracy of urinary albumin measurements. First, there has been growing recognition of the occurrence of modified or fragmented forms of albumin in urine. Proportions of modified albumins such as glycated albumin and a C-terminal truncation product are higher in urine than serum, and isoelectric focusing shows that urinary albumin from people with diabetes appears to be more heterogeneous and acidic (5–7). Analysis of urinary proteins by Western blotting of 1-dimensional gels or by 2-dimensional gel electrophoresis shows the presence of large albumin fragments (8–11). Also, multiple small fragments of albumin have been detected in urine (12–14). Although urine may contain modified albumin or a variety of albumin fragments, assays for urine albumin are calibrated using serum albumin, and data regarding the influence of albumin fragments on assays for urine albumin are lacking.

A second source of uncertainty in urine albumin measurement is the lack of agreement of immunoassays for urine albumin with a size-exclusion chromatographic assay, which yields substantially higher values than immunoassays for many specimens from healthy individuals or diabetes patients (15–22). This bias has been hypothesized to result from modified forms of urinary albumin that are detected by the size-exclusion assay but not by immunoassays. Our laboratory, how-
ever, determined that size-exclusion chromatography does not resolve albumin from a number of other urinary proteins and is an assay for a mixture of albumin-sized molecules (ranging from about 40 000 to 80 000 Da) rather than an assay for albumin (21). This finding offers an alternative explanation for the difference between immunoassay and size-exclusion chromatography but does not completely rule out some contribution from modified forms of albumin with decreased immunoreactivity.

In this study, we sought to gain insights into the cross-reactivity of albumin fragments in immunoassays for urine albumin and to examine whether it is possible to modify albumin so as to markedly reduce its immunoreactivity.

Materials and Methods

Proteins, including human albumin, and reagents were obtained from Sigma-Aldrich, except as noted; cyanogen bromide (CNBr) was from Acros Chemicals and guanidine hydrochloride from Calbiochem. Other chemicals were reagent grade. Gels (4% to 12% gradient Bis-Tris gels), reagents, and SimpleBlue stain for SDS-PAGE were from Invitrogen. Western blotting on nitrocellulose used rabbit antiserum to human albumin and alkaline phosphatase–conjugated sheep antirabbit IgG. After blocking with SuperBlock T20 from Pierce Chemical, proteins, including 2 with diabetes, obtained from Dr. Jeffrey Kopp were diluted with PBS before analysis.

10 g/L cyanogen bromide (CNBr) was from Acros Chemicals and guanidine hydrochloride from Calbiochem. Other chemicals were reagent grade. Gels (4% to 12% gradient Bis-Tris gels), reagents, and SimpleBlue stain for SDS-PAGE were from Invitrogen. Western blotting on nitrocellulose used rabbit antiserum to human albumin and alkaline phosphatase–conjugated sheep antirabbit IgG. After blocking with SuperBlock T20 from Pierce Chemical, blots were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate.

Total protein (pyrogallol red) and immunotur- bidimetric urine albumin assay were performed on the Beckman–Coulter LX20 analyzer. A competitive immunoassay for urine albumin analysis was performed on the Siemens Immulite analyzer.

CNBr cleavage of albumin was conducted in 6 mol/L guanidine hydrochloride, 10% acetic acid, and 10 g/L cyanogen bromide (23) for 24 h at room temperature before dialysis vs PBS. Other incubations were conducted with 10 g/L albumin. Citraconylation used 0.1 mol/L citraconic anhydride in 1 mol/L N-methylmorpholine, pH 9.0. Incubations at 37 °C with 1% hydrogen peroxide or 10 mmol/L sodium ascorbate were in PBS, pH 7.4. Other incubations were at pH 2 (0.1 mol/L glycine HCl buffer) or pH 8 with 0.5 g/L trypsin (100 mmol/L Tris, 10 mmol/L CaCl2). Incubations for 5 days at 37 °C were in PBS containing 0.01% sodium azide or in acetate-buffered saline, pH 5.7. Additives were 10% glucose, 10 mmol/L sodium nitrite, or 10 mmol/L sodium ascorbate. Modification reactions were diluted with PBS before analysis.

For preparative size-exclusion chromatography of CNBr-digested albumin, we used a Pharmacia fast protein liquid chromatography system with a 1.6 by 60 cm Sephacryl S-200 column eluted at room temperature with PBS. This purified fractions enriched in fragments A and C, identified as residues 1–123 (A) and 299–584 (C) by electrophoresis and mass spectrometry. We performed reversed-phase chromatography of a size-exclusion chromatography fraction enriched in fragment B (residues 124–298) on a Waters 3.9 by 150 mm Symmetry 300 C4 column eluted at 1 mL/min with a linear gradient of acetonitrile and 0.1% trifluoroacetic acid, yielding purified fragment B. MALDI-TOF mass spectrometry of albumin fragments was performed using sinapinic acid as matrix (24).

Urine from 24-h collections submitted to the clinical laboratory were used anonymously as approved by an institutional review board. Specimens for Western blots consisted of 10 freshly collected specimens with ≥300 mg/L protein by dipstick and 5 specimens stored at −20 °C for 3 years, with albumin concentrations from 100 to 1700 mg/L (by LX20 assay), and 9 random urine specimens with albumin concentrations from 2 to 8 g/L from patients with focal segmental glomerulosclerosis, including 2 with diabetes, obtained from Dr. Jeffrey Kopp (protocol 94KD0127 approved by an institutional review board).

We examined the degree of identity of serum albumin sequences for different species in the UniProt database with the selected ion monitoring alignment tool using sequences in the UniProt database (www.expasy.org).

Results

Albumin was subjected to a variety of treatments that potentially alter its conformation, chemically modify amino acid side chains, or cleave its polypeptide chain. Analysis by SDS-PAGE, with approximately equivalent inputs of protein or peptide in each lane, examined products of the modification reactions (Fig. 1, top panel without reduction and bottom panel with reduction). SDS-PAGE indicated that almost no intact albumin remained after extensive digestion with trypsin (lane 2) or CNBr (lane 3), which should cleave albumin at 6 methionine residues, yielding 3 major fragments from residues 1–123 (A), 124–298 (B), and 299–585 (C) (25). A and C fragments should contain 1 and 3 internal cleavages, respectively, but are held together by disulfide bonds. The 3 CNBr fragments obtained by chromatographic purification are shown (lanes 4, 5, and 6). Based on amino acid sequence, the C fragment should yield 4 fragments following reduction, but 6 fragments were observed (bottom panel, lane 4), possibly related to incomplete cleavage at some sites or at residue 365, at an acid-labile Asp-Pro bond. Citracon-
nylation of albumin resulted in altered electrophoretic mobility, showing that all albumin molecules were modified (lane 7). Incubation of albumin with sodium ascorbate for 1 day led to cleavage of some molecules, evident mainly on reduction (lane 8, bottom panel), indicating that most cleavages yielded nicked molecules held together by disulfides. Incubation with ascorbate for 5 days yielded extensive fragmentation of albumin, particularly at neutral pH (not shown). Cleavage of albumin by ascorbate possibly results from free radicals generated by trace amounts of metals as described (26).

We examined how various treatments of albumin affected its reactivity in 2 assays for urinary albumin (Table 1). Exposure to low pH, heat, and chemical modifications, such as citraconylation and exposure to hydrogen peroxide, had only a small effect on the reaction of albumin in the LX20 assay. The total digestion of albumin by CNBr also exhibited little change in assay response vs intact albumin. Even isolated CNBr fragments cross-reacted strongly in the LX20 assay. The only treatment that severely decreased measured albumin was extensive digestion with trypsin. The decrement in measured albumin became pronounced only with extensive digestion of albumin, with a high ratio of trypsin to albumin (1:20 by weight). Trypsin-treated albumin retained >20% cross-reactivity in both urine albumin assays, although electrophoresis indicated that virtually no intact albumin remained; thus trypsin-cleaved albumin cross-reacted at least partially in the assays. Less extensive tryptic digestion yielded

### Table 1. Comparison of albumin:total protein ratios for modified or fragmented albumins analyzed by 2 different assays for urinary albumin.

<table>
<thead>
<tr>
<th>Albumin preparation*</th>
<th>Albumin:total protein</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>LX20</td>
</tr>
<tr>
<td>Treatment for 24 h</td>
<td></td>
</tr>
<tr>
<td>Albumin (saline control)</td>
<td>1.03</td>
</tr>
<tr>
<td>CNBr digest, total</td>
<td>1.09</td>
</tr>
<tr>
<td>CNBr fragment A</td>
<td>0.85</td>
</tr>
<tr>
<td>CNBr fragment B</td>
<td>0.93</td>
</tr>
<tr>
<td>CNBr fragment C</td>
<td>1.58</td>
</tr>
<tr>
<td>Trypsin digest, total</td>
<td>0.21</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>1.04</td>
</tr>
<tr>
<td>Citraconic anhydride</td>
<td>0.94</td>
</tr>
<tr>
<td>Incubated at pH 2</td>
<td>1.05</td>
</tr>
<tr>
<td>Incubated at 25 °C</td>
<td>0.95</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>0.98</td>
</tr>
<tr>
<td>Treatment for 5 d</td>
<td></td>
</tr>
<tr>
<td>Albumin (control), pH 7.2</td>
<td>1.04</td>
</tr>
<tr>
<td>Albumin (control), pH 5.7</td>
<td>0.92</td>
</tr>
<tr>
<td>10% glucose, pH 7.2</td>
<td>1.01</td>
</tr>
<tr>
<td>10% glucose, pH 5.7</td>
<td>0.94</td>
</tr>
<tr>
<td>Nitrite, pH 7.2</td>
<td>1.03</td>
</tr>
<tr>
<td>Nitrite, pH 5.7</td>
<td>0.96</td>
</tr>
<tr>
<td>Ascorbate, pH 7.2</td>
<td>0.65</td>
</tr>
<tr>
<td>Ascorbate, pH 5.7</td>
<td>0.94</td>
</tr>
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*Modified albumins were all prepared from purified plasma albumin.
larger fragments and less effect on immunoreactivity, as described in a preliminary report (27).

There is the potential for fragmented antigens to inhibit immunoturbidimetric reactions. To test this possibility, we prepared small tryptic fragments of albumin by ultrafiltration through a 5000-Da cutoff membrane. These fragments had little measurable cross-reactivity in the LX20 assay. Results are not included in Table 1, because total protein content of small peptides could not be standardized using the pyrogallol red assay. Small albumin fragments were mixed with albumin to examine whether the fragments would inhibit the LX20 assay, and no evidence of inhibition was obtained (data not shown).

The Immulite assay generally showed greater sensitivity to modification or fragmentation of albumin than the LX20 assay, except in the case of albumin digested by trypsin, for which severe reduction in cross-reactivity occurred for both assays (Table 1). Several treatments, such as digestion with CNBr, citraconylation, or incubation with ascorbate, glucose, or nitrite, affected the Immulite assay more than the LX20 assay. The greatest difference between assays was evident for albumin cleaved by CNBr and prolonged incubation with ascorbate.

It was considered that modified albumins might change response curves owing to altered affinity for antibodies or loss of reactivity of subsets of reactive epitopes. However, analysis of a series of dilutions of the CNBr digest of albumin indicated that responses were linear throughout the assay ranges (Fig. 2).

Serum albumins from different animal species were examined as another model for structural modification of albumin (Table 2). The LX20 assay, which uses polyclonal goat antiserum, had strong cross-reactivity with baboon albumin, 30% to 40% reactivity with albumins from rat, mouse, pig, and rabbit, low cross-reactivity with albumin from cow, and no measurable reactivity with sheep albumin. The Immulite assay, which uses a mouse monoclonal antibody, strongly cross-reacted with albumin from baboon and rabbit, but did not cross-react with albumins from other species. Thus, the monoclonal antibody-based assay tended to respond to sequence changes in an all-or-none rather than a graded fashion. By serial dilution of albumins from baboon, rabbit, and pig, we found that LX20 responses were linear over a wide concentration range (data not shown).

As described in the Introduction, urine has been noted to contain albumin fragments (8–14). We analyzed 24 urine specimens by Western blotting to examine the occurrence of large albumin fragments. Comparison of analyses under reducing and nonreducing conditions examined the occurrence of internally cleaved forms of albumin held together by disulfide bonds. Results are shown for a representative sampling of 6 frozen (at –80 °C) and thawed specimens from patients with focal segmental glomerulosclerosis [Fig. 3, lanes 1–6, panels A (nonreduced) and B (reduced)]. Only trace amounts of fragments were observed, almost exclusively under reducing conditions. Also, small amounts of higher-molecular-weight forms were observed under nonreducing conditions. Disappearance of these components under reducing conditions suggested that they were disulfide-linked dimers. Fresh urine specimens randomly selected for proteinuria also showed low amounts of albumin fragments [lanes 10–12, panels E (nonreduced) and F (reduced)]. Analyses...
of 3 proteinuric specimens frozen for 3 years at −20 °C showed variable degradation of albumin [lanes 7–9, panels C (nonreduced) and D (reduced)]. One specimen consisted primarily of intact albumin and the other 2 specimens showed mostly albumin fragments.

The 3 specimens in lanes 7–9, with prolonged storage at −20 °C, all yielded high measured values of albumin by the LX20 assay—1700, 1690, and 202 mg/L—and responses were approximately linear with dilution (Fig. 4). Total protein measurements by pyrogallol red assay were 2000, 1600, and 550 mg/L, respectively. The Immulite assay found a high concentration of albumin—1510 mg/L—and linear responses for 1 sample but values below the limit of detection in the other 2 specimens at all assayed dilutions. Apparently, the Immulite assay had low cross-reactivity with fragmented albumin in the latter 2 specimens, whereas the albumin fragments were detected by the LX20 assay.

Discussion

There are numerous ways in which urinary albumin could differ structurally from plasma albumin, so as to alter the protein’s immunoreactivity. These include changes of conformation, chemical modifications of amino acid side chains, cleavage of the polypeptide chain, covalent homo- or heterodimer formation via an unpaired cysteine residue, noncovalent aggregation, changes in ligand concentration, or proteolytic fragmentation. In the urine environment, albumin can be exposed to a wide range of ionic strength, pH, urea concentrations approaching 1 mol/L, high concentrations of glucose and ascorbic acid, aerobic oxidizing conditions, increased amounts of nitrite in cases of urinary tract infections, and a variety of proteases along the urinary epithelium (14). We examined the effects of exposures of albumin to a variety of denaturing conditions or reactants on its cross-reactivity in assays for urinary albumin.

Albumin consists of 3 globular domains stabilized by 17 internal disulfide bonds (25, 28). Albumin resists denaturation during heating for 10 h at 60 °C as performed for commercial albumin preparations (25). Exposure to low pH or to high concentrations of chaotropes results in reversible denaturation (25), and we observed no effects of these treatments on measured albumin. Therefore, exposure of albumin to the usual range of urinary pH, urea, and salt concentrations appears unlikely to induce an irreversible conformational change. When albumin does denature at extremely high temperature or pH, it aggregates irreversibly; this leads to precipitation of albumin rather than additional forms in solution (25). Some dimerization of albumin is observed with many preparations, including purified plasma albumin (21, 25), but as in previously reported studies, we found albumin dimers to be minor components in urine. Aggregation of albumin with other components such as the Tamm-Horsfall protein would result in elution in size-exclusion chromatography as a much larger complex, and that has not been observed (15, 21). Albumin binds many ligands, and ligand binding may induce a change in conformation of albumin (reviewed in 25). Such factors may be of interest in examining matrix effects of urine on albumin measurements, but analyses in this study used a simple saline matrix.

Urinary albumin is enriched for glycated forms (5, 6), but enrichment for other modifications of amino acid side chains has not been described. We
found only small effects of exposure to high concentrations of glucose on cross-reactivity of albumin in the assays for urine albumin. Another reagent, citraconic anhydride, which also modifies lysine residues, had little effect on the LX20 assay but decreased cross-reactivity in the Immulite assay by approximately 50%.

We anticipated that albumin modification or fragmentation would diminish reactivity in immunoassays for urine albumin owing to loss of functional epitopes and decreased ability to form cross-linked complexes in immunoturbidimetric assays. Surprisingly, the immunoturbidimetric LX20 assay reacted nearly equivalently with albumin and with most preparations of fragmented or chemically modified albumin. Even isolated CNBr fragments representing 3 separate segments of albumin had nearly similar reactivity. These findings indicate a broad distribution of reactive epitopes across the albumin molecule that still allow antibody-mediated cross-linking reactions to occur for fragments representing only about one-third of the intact molecule. Proteolysis and chemical modification of albumin probably destroy some epitopes, but, apparently, polyclonal antibodies still react with enough epitopes to sustain turbidimetric reactions. A previous study of trypsin digestion of albumin (29), using a lower ratio of trypsin to albumin, concluded, “Many techniques used to assay patient urine samples are unable to detect fragmented albumin...” In one respect, our results agree with that conclusion; low cross-reactivity was observed for small fragments isolated by ultrafiltration. However, our results differ in noting significant cross-reactivity of tryptic digests containing very little intact albumin. Therefore, we conclude that large fragments and internally nicked...
forms of albumin from trypsin digestion retained significant cross-reactivity in 2 assays for urine albumin. The experiments with CNBr cleavage and incubation with ascorbate provide even clearer demonstration of the reactivity of albumin fragments in an immunonephelometric assay and the differential reactivity of albumin assays based on different assay principles.

The mechanism that we consider most likely to influence the measurement of urinary albumin by immunoadsorbs is cleavage of its polypeptide chain, and this was previously suggested by Osicka and Comper (15) as a mechanism for generating internally nicked forms of albumin with decreased immunoreactivity. Several studies have noted the presence of albumin fragments in urine (8–14), although there has been controversy regarding the abundance and sources of small peptides in urine (30–32). Our results suggest that most freshly collected urine specimens or specimens frozen at −80 °C do not contain substantial amounts of large albumin fragments detectable by Western blotting, but extensive cleavage of albumin can occur in some urine specimens stored frozen for an extended period at −20 °C. This result bears further investigation, as we had only a few such samples for analysis. Albumin degradation could result from either action of proteases or free radicals. Several reports offer varying views regarding the stability of urine specimens stored at −20 °C (33–37). The recent report by Brinkman et al. (37) notes a progressive decline in results by immunonephelometric assay for specimens stored at −20 °C, but an even greater decline for results by size-exclusion chromatography. Those results suggest that the size-exclusion assay is more sensitive to structural changes of albumin during storage than is the immunonephelometric assay, countering a previous hypothesis that analysis by size exclusion is less sensitive to albumin degradation (15, 16).

Our experiments demonstrate that it is extremely difficult to modify albumin so as to abrogate immunoreactivity with polyclonal antibodies. The amino acid sequence of albumin diverges substantially through phylogeny (25), although there is conservation of the cysteine residues that form disulfides that serve as stabilizing cross-links for albumin’s globular structure. Human albumin has about 94% sequence identity with albumin from other primates such as the macaque. In the present study, albumin from gorilla was observed to have strong cross-reactivity in the 2 assays for urine albumin (human). Sequence identity with human albumin decreases to 72%–77% for albumins from rabbit, sheep, cow, rat, and mouse, and amino acid substitutions between species are relatively evenly distributed through the albumin molecule. Therefore, goats immunized with human albumin will be presented with an antigen that differs in about 150 of 585 amino acid residues relative to goat albumin. (The sequence of albumin from sheep, which phylogenetically is closely related to goat, was used to estimate identity between goat and human albumin, since goat albumin is not in the sequence database.) The >100 amino acid differences should lead goats to develop a diverse repertoire of antibodies directed at multiple sites in human albumin, and it explains how a urine albumin assay using goat antibodies vs human albumin can react with a variety of modified albumins, albumins from other animal species, or albumin fragments. It is not plausible, as recently reported, that there is a modified form of albumin failing to cross-react with 20 different polyclonal antisera (16). Those findings are strong evidence that the putative “immunounreactive albumin” actually is a different protein than albumin.

Use of a monoclonal antibody in an assay for urine albumin leads to a very different sensitivity to structural modification of albumin, as it depends on reactivity with a single specific epitope expressed by albumin. Consequently, much greater sensitivity to albumin modification was observed for the Immulite assay, which uses a monoclonal antibody, than for the LX20 assay using polyclonal goat antibodies. The differential reactivity of assays to modified albumins is likely to be primarily related to the type of antibodies employed, although there may also be differences between competitive and noncompetitive assay formats. Previously, differential reactivity of monkey albumin was noted in 2 urine albumin assays. Using the particle-enhanced turbidity inhibition assay (a competitive assay) for human albumin on the DuPont analyzer suggest a loss of reactivity with albumin fragments similar to the competitive assay on the Immulite (27). Those reports, together with findings in this report, suggest that assay design probably influences the reactivity of urine albumin assays with modified or fragmented albumin, and this is a potential source of non-equivalence between assays in the analysis of urinary albumin, although assays may react equivalently with serum albumin added to calibrators, control, or proficiency testing materials.

There are many commercially available assays for urine albumin. The present study focused primarily on model systems to examine the sensitivity of urine albumin assays to structural modification or fragmentation of albumin. Our results suggest a need for evaluation of the reactivity of different assays with modified or fragmented albumin and for further investigation into occurrence and formation of modified or fragmented albumin in urine specimens. Further
development of reference methods for the measurement of urinary albumin such as recent efforts to apply new electrophoretic (39) or mass spectrometric (40) methods might contribute to progress in this area.

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References