Restricted Electrophoretic Heterogeneity of Immunoglobulin Light Chains in Urine: a Cause for Confusion with Bence Jones Protein

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The detection of Bence Jones protein, an important part of the investigation of suspected myeloma, is most commonly done by agarose or cellulose nitrate electrophoresis followed by immunofixation. Bence Jones protein is recognized as single or multiple bands of one type of light chain. Unfortunately, improvements in sensitivity of these techniques (use of high-affinity antisera and higher resolution electrophoresis) frequently allow detection of multiple light chain bands in the urine of patients who do not have a B-cell dyscrasia. The bands are usually kappa, although they may be accompanied by lambda bands. This pattern may lead to the misdiagnosis of Bence Jones protein and oligoclonal light chain production in patients. Here we show that this pattern is produced by polyclonal light chains; it is present in the urine of all patients with a tubular proteinuria of any etiology and may be induced in healthy individuals by blocking their renal tubular protein reabsorption. Polyclonal light chains separate into monomers and dimers on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and into four major bands with many minor bands by isoelectric focusing. This difference in charge and possibly size results in the banding pattern seen on good-quality electrophoresis and immunofixation.

Additional Keyphrases: electrophoresis, agarose - electrophoresis, cellulose - electrophoresis, polyacrylamide gel - immunofixation - proteinuria - myeloma - polyclonal light chains

Immunoglobulin synthesis by normal B lymphocytes results in the production by the cell of a small excess of free immunoglobulin light chains (1). These are filtered at the renal glomerulus and largely reabsorbed in the proximal tubule, with a small amount being excreted in the urine. Normal subjects excrete as much as 10 mg of these polyclonal light chains each day (2, 3). Patients with either increased filtration of light chains due to increased production from an immune response or failure of reabsorption due to proximal tubular damage may excrete five- to sixfold more light chains than do normal individuals (4, 5).

Single expanded clones of B cells, whether benign or malignant, may produce enough monoclonal light chains to be detected by their restricted electrophoretic mobility, and are known as Bence Jones protein (BJP).7 A restricted number of expanded clones may give rise to multiple (oligoclonal) bands. Small amounts of BJP may be present in the urine of patients with serum monoclonal components (MC) not associated with disease (monoclonal gammopathy of undetermined significance, MGUS) (6). Larger amounts, however, are more frequently associated with myeloma (7), which has led to the use of BJP as an important indicator of B-cell malignancy and as a means of differentiating MGUS from malignant monoclonal gammopathies (8). BJP is demonstrable in most patients with myeloma; in 15% to 20% of such patients, it is the only product secreted by the malignant clone, thus making its detection critically important for diagnosis (9).

Light chain fragments, probably produced by proteolytic degradation of BJP by macrophages in the tissues, are the major components of renal and systemic deposits that characterize amyloidosis AL. BJP is also responsible for light chain deposition disease (10); however, there is no quantitative relationship between the amount of BJP excreted in the urine and the presence or absence of tubular casts or other types of light chain deposition. Recently, Bellotti et al. (11) suggested the possibility that low-M, synthetic fragments of light chains may also be important in this condition. Often the clinical diagnosis of these diseases is first made by the detection of BJP, which has led in the last few years to the increasing need for, and use of, highly sensitive methods of detection. This is now obtained in most laboratories by urine concentration and immunofixation electrophoresis (IFE) with high-affinity antibodies and sensitive protein stains.

Using such techniques, investigators have not uncommonly observed multiple bands on immunofixation of urine with light chain antisera, in particular kappa bands, in patients without a urine or serum monoclonal gammopathy. This phenomenon provides problems in interpretation of results and in identifying BJP and oligoclonal light chain bands in good-quality electrophoresis. This problem has been very clearly recognized by

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Nonstandard abbreviations: BJP, Bence Jones protein; MC, monoclonal component; MGUS, monoclonal gammopathy of undetermined significance; IFE, immunofixation electrophoresis; and SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
Harrison (12), who highlights the need to distinguish the multiple bands from BJP and to understand their origins. No systematic study of these bands has yet been undertaken, the problem has not been detailed in the literature, and no suggestions have been made for their differentiation from BJP.

Here we show the presence of kappa and lambda bands (on agarose and cellulose nitrate electrophoresis) in the urine of geriatric patients, patients with renal disease, patients with monoclonal gammopathy, and normal subjects in whom proximal tubular protein re-absorption has been blocked with arginine (13). Using isoelectric focusing and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), we demonstrate that the banding is due to restricted heterogeneity of charge and size in normal polyclonal urinary light chains and discuss the implications of these findings for the laboratory diagnosis of BJP.

Materials and Methods

Random (untimed), early morning urine specimens were collected into sodium azide (~0.01 mol/L). For agarose IFE these were concentrated 200-fold with Minicon concentrators supplied by Amicon (Div. of W. R. Grace, Danvers, MA). Electrophoresis of concentrated urine was performed on Litex HSA agarose (Park Scientific Ltd., Northampton, U.K.), 10 g/L in 0.06 mol/l barbital buffer, pH 8.6, as described by Johansson (14).

Agarose IFE was performed according to the technique described by Ritchie and Smith (15), with use of antisera from Atlantic Antibodies, Stillwater, MN; Dako, Copenhagen, Denmark; Behringwerke, Marburg, F.R.G.; Protein Reference Unit, Central Antiserum Purchasing Unit, Royal Hallamshire Hospital, Sheffield, U.K.; Unipath Ltd., Bedford, U.K.; and Oxford Reagents, Boehringer Corp. (London), Lewis, East Sussex, U.K. These antisera were diluted twofold with a 1 mmol/L solution of polyethylene glycol (Mw 6000) in 0.06 mol/l barbital buffer, pH 8.6.

For cellulose nitrate IFE (Super Sephaphore 111; Gelman, Ann Arbor, MI) the unconcentrated urine specimens were treated according to the procedure of Aguzzi and Rezzani (16), with antibodies from Dako. The proteins were detected with gold stain (Dasit, Breggio, Italy), as described by Aguzzi et al. (17). The immunofixed strips were evaluated by transillumination. The method could detect <1 mg/L of light chain in a single band.

$\alpha_1$-Microglobulin was measured either by radial immunodiffusion with Dako antibodies as described by Mancini et al. (18) or, at concentrations >10 mg/L, by latex agglutination (Rapitex alpha-1-microglobulin; Behringwerke). $\beta_2$-Microglobulin was measured by radial immunodiffusion with antisera supplied by Dako.

SDS-PAGE and silver staining of unconcentrated urines were carried out with the automated Phast System™ (Pharmacia AB, Uppsala, Sweden). The urine samples were not reduced. The separated proteins were identified by immunoblotting (19).

Agarose isoelectric focusing of unconcentrated urine specimens was performed over a range of pH 3–10.5 with Pharmalytes and agarose from Pharmacia. The proteins were identified by immunoblotting on polyvinylidene fluoride matrices (Millipore Corp., Bedford, MA) with biotinylated antisera and streptavidin–gold conjugate (Amersham International, Bucks., U.K.) with silver enhancement (Janssen, Olen, Belgium), according to the method described by Neepolo et al. (20).

Subjects

Arginine hydrochloride was given intravenously to volunteers as a 150 g/L aqueous solution over 30 min. The dose was 0.5 g/kg body weight, to a maximum of 30 g. The urine was collected immediately before the infusion and about every 30 min afterwards, for 3 h.

We studied three groups of patients:

1. 104 geriatric patients selected without conscious bias from the acute geriatric ward of the General Infirmary at Leeds and Ospedale di Stradella.
2. 1811 patients, known to have a monoclonal component in their serum, from the general hospital population of Ospedale di Stradella and San Carlo Borromeo, Milan.
3. Samples of urine, with proteinuria of known etiology, from the Unit for Cancer Research, University of Leeds.

Ethics committee approval was obtained for the study, with both patients and volunteers giving informed consent.

Results

Urine specimens from geriatric patients. Agarose IFE of 54 urine specimens concentrated 200-fold demonstrated multiple kappa bands in 39 (72%). The urine specimens, which initially appeared negative, were subsequently shown to contain these bands after either diluting the urine and so decreasing the background or by concentrating it further as much as 600-fold.

Immunofixation with antisera to lambda light chains showed two to five less-dense bands in 14 of the 54 (26%) urine specimens when concentrated 200-fold. Bands were detected in a further five urine specimens when we increased the concentration to 600-fold. No additional urine specimens with bands were detected by reducing the concentration. These bands matched the mobility of some of the kappa bands.

Cellulose nitrate IFE with gold staining, performed on a further 50 unconcentrated urine specimens, gave comparable findings. Figure 1 demonstrates four to eight kappa bands and two to five lambda bands of various concentrations in the urine specimens examined. In >90% of the urine specimens containing such bands, the $\alpha_1$-microglobulin concentration was >10 mg/L, consistent with a tubular proteinuria.

Urine specimens from patients with an MC in serum. We performed cellulose nitrate IFE on 1811 urine specimens collected from patients showing an MC on serum electrophoresis; >90% of these were patients with MGUS. Of the urine specimens examined, 25% had
multiple kappa bands, 7% had multiple lambda bands, and 27% had BJP. In those with BJP, 37% had multiple kappa bands in addition to the BJP band itself. Kappa BJP was present in 60.5% of cases and lambda BJP in 39.5%. In all cases of malignant MC, the BJP was of the same light chain type as the serum MC.

Proteinuria in renal disease. Multiple kappa bands were found in all specimens of urine (concentrated 200-fold) examined from patients with proteinuric renal disease associated with diabetes (seven cases), pre-eclampsia (31), nephrotic syndrome (three), Fanconi syndrome (four), glomerulonephritis (four), rejected renal transplant (two), and systemic lupus erythematosus (two). These examples of proteinuria were tubular, glomerular, and mixed tubuloglomerular.

Arginine-induced proteinuria. A transient tubular proteinuria was induced in healthy volunteers by infusing intravenous arginine. Agarose IFE of these urine specimens collected at 30-min intervals after the infusion demonstrates the appearance and disappearance of the multiple kappa bands. Samples were also separated by SDS-PAGE and silver-stained (Figure 2), which confirmed a normal pattern of excretion of proteins before the infusion (track 1) and demonstrated a reversible tubular proteinuria (tracks 2 to 6). Agarose IFE of these urine specimens demonstrates the transient appearance of multiple kappa bands with the tubular proteinuria (Figure 3).

Characterization of kappa and lambda bands. The finding of multiple kappa and lambda bands was confirmed with antisera from the six independent sources examined. Immunofixation of urine specimens with 21 specific antisera to other serum proteins demonstrated only one discrete band in the region occupied by the light chain bands; this reacted with anti-β₂-microglobulin. Ouchterlony double diffusion showed no cross-reactivity between this antiserum and those directed against light chains.

Molecular-mass separation of kappa chains. We analyzed by SDS-PAGE 27 unconcentrated urine specimens from eight geriatric patients, seven women with pre-eclampsia, three diabetics, four healthy subjects post-arginine infusion, and five patients with renal disease, and identified the kappa light chains by immunoblotting. Kappa bands were identified as monomer and dimer and also as associated with heavy chain as intact IgG. There was some nonspecific binding of the antibody to albumin (Figure 4).

Charge separation of the kappa bands. Twenty-one unconcentrated urine specimens from 11 geriatric patients, seven women with pre-eclampsia, and three healthy subjects post-arginine infusion were separated by isoelectric focusing (pI 3–10.5). Identification of the kappa bands by immunoblotting defined at least four major bands with isoelectric points of 6.5, 8.0, 8.5, and 9.2 and many minor bands (Figure 5).
Discussion

The presence of light chain bands on agarose or cellulose nitrate IFE has, so far as we are aware, not been previously investigated. The recognition of such a finding is of great importance for avoiding confusion with BJP.

The diagnosis of BJP depends upon finding one or more (in the case of dimer or higher polymer formation) bands of a single type of light chain in urine (21). The restricted heterogeneity of polyclonal light chains we observed results in the finding of multiple bands in the urine whenever light chains are present. The number of light-chain bands seen depends on the amount of light chains in the urine and the sensitivity of IFE. We have shown in patients with renal disease and in normal subjects infused with arginine (which blocks renal tubular reabsorption of protein) that the phenomenon occurs if renal tubular reabsorption of protein is impaired. Unfortunately, because tubular damage or transient tubular impairment is common in elderly patients, precisely those who are at risk from myeloma, the detection of these light chains is likely to result in misdiagnosis of BJP.

How then may the polyclonal light chains be distinguished from BJP? The polyclonal pattern of three to eight bands is seen on IFE with anti-kappa antisera. The pattern has a distinctive, evenly spaced "ladder" appearance (12) (Figure 1). The bands are of maximum intensity at the cathodal side of the center of the pattern and diminish anodally and cathodally. In about 25% of cases, this pattern may be accompanied by two to five lambda bands, which are fainter than those of the kappa bands, even when a variety of antisera are used for fixation. The presence of BJP is suggested by the finding of a kappa or lambda band of increased density or a band that disrupts the regular pattern (Figure 6, kappa track 3 and 5; lambda track 5). It is important to appreciate that BJP is frequently associated with tubular proteinuria and that the BJP occurs in addition to normal banding. To differentiate the polyclonal banding we have described from BJP requires good-quality electrophoresis (22) and may indeed be difficult or impossible in some cases if the bands of BJP overlap one of the polyclonal bands. Thus, if the BJP band in Figure 6 (lambda, track 5) had more cathodal mobility, it would have been lost among the polyclonal bands. In particular, the use of inadequate separation methods may result in compression of the typical polyclonal pattern, making it indistinguishable from true BJP.

The main difference between the two methods we used is that one requires concentrated urine. Both methods gave comparable results, as shown by the study of urine specimens from geriatric patients—an important finding because of controversy regarding the possibility of arti-
facts caused by concentrating fluids. The banding pattern demonstrated here is not due to such an artifact. However, the possible loss of small-M openings that are implicated in amyloidogenicity (II) may be a serious limitation of methods involving concentration.

A recent report exemplifies the confusion that arises from this phenomenon in suggesting that patients with AIDS have oligoclonal light-chain bands in their urine (23). The pattern in these patients is similar to the multiple kappa bands we have observed. On the basis of observations of isoelectric focusing (24), Kahn (25) recently suggested that the multiple kappa bands observed in AIDS may in fact be due to polyclonal light chains and not an oligoclonal response. On the basis of our studies, we would concur with this.

We have shown that the banding seen on agarose and cellulose nitrate IFE in the urine from patients and healthy individuals with an induced proteinuria is polyclonal. It can be separated into monomers and dimers on SDS-PAGE and into four main groups on isoelectric focusing with pIs of 6.5, 8.0, 8.5, and 9.2. There are many small bands also. This restriction in charge of polyclonal urine light chains results in a banding pattern on IFE when good-quality electrophoresis is used.

The finding of multiple kappa, and less frequently lambda, bands on IFE of polyclonal light chains is a result of impaired tubular reabsorption and may result in an incorrect diagnosis, when used with common analytical techniques. It is important to be able to differentiate these bands from those for BJ, because geriatric patients, the group most frequently screened for myeloma, frequently have a tubular proteinuria.

References


