Electrophoretic study of the physico-chemical characteristics of Bence-Jones proteinuria and its association with kidney damage

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Abstract

Aim—To identify a physico-chemical criterion, or set of criteria, explaining and possibly predicting the nephrotoxic behaviour of Bence-Jones proteins (BJP).

Methods—The electrophoretic mobility and isoelectric point (pI) of 92 BJP isolates were determined using various electrophoresis procedures on polyacrylamide gel. The proportions of monomers and dimers were determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) in 58 cases. PAGE data for 10 BJP isolates were used to construct Ferguson plots and titration curves.

Results—The distribution of electrophoretic mobility and pI values was bimodal and showed a positive correlation when the pI was above 6. The values of these two parameters in 22 patients with renal impairment were not significantly different from those in the patients without renal impairment, and the statistical analysis showed no predictive value for the onset of renal impairment. However, patients excreting the λ light chain isotype had a 2.8-fold higher risk of developing renal impairment compared with the other patients. Studies of the charge variation of the protein with pH indicated three types of behaviour, suggesting that the charge of BJP is highly variable at physiological pH.

Conclusion—It is important to study not only the positivity or negativity of the BJP charge at a given pH, but also its intensity. The study of the BJP titration curves in patients with renal impairment suggests that a low charge at physiological urinary pH could predict renal impairment.

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and in humans¹⁸⁻²² support this association. Indeed, light chain polymerisation seems to have a role in the development of renal lesions,¹⁸⁻²³ giving an importance to pI,¹¹⁻¹⁹ which has been contested.²⁴⁻²⁵ The degree of polymerisation is generally determined by denaturing electrophoresis which may affect the behaviour of BJP.

In patients with BJP and renal impairment we have noticed a peculiar migration of urinary BJP in polyacrylamide gel in native conditions, suggesting an unusual degree of polymerisation. This led us to study the degree of polymerisation in native conditions, in patients with Bence-Jones proteinuria, with and without renal impairment, and to compare the results with those obtained by methods involving denaturing media. Isoelectrofocusing was combined with these electrophoretic techniques, which were carried out in highly standardised conditions. The aim was to identify a physico-chemical criterion or set of criteria that could explain and possibly predict the nephrotoxicity of BJP.

Methods

We studied the urine samples of 92 patients (54 men and 38 women; mean age 64.9 and 63.8 years, respectively) with a monoclonal gammopathy detected and typed using electrophoresis and immunoelectrophoresis. The patients were recruited in comparable numbers by the Haematology, Internal Medicine, and Nephrology units of our institution (30, 22, and 21%, respectively). A smaller proportion of patients were recruited by the Rheumatology (10%) and other units (17%).

The underlying illnesses included lymphoproliferative syndromes with monoclonal IgG in 34% of cases (31/92), IgA in 24% (22/92), IgM in 9% (8/92), and IgD in 4% (4/92). Light chain abnormalities represented only 28% (25/92) of the cases. In this latter group the κ and λ isotype distribution was, respectively, 44% (11/25) and 56% (14/25), proportions equivalent to those in the overall population: 46% (42/92) and 54% (50/92).

At the time of the study 22 of the 92 patients had renal impairment, with creatinemia above 150 $\mu\text{mol/l}$. Clinical and laboratory findings in these patients at diagnosis are shown in table 1. The serum monoclonal constituents were distributed as follows: 55% (12/22) were free light chains (eight λ and four κ isotypes); 45% (10/22) were mono-

The presence of free monoclonal light chains—Bence-Jones protein (BJP)—in the urine of patients with myeloma is associated with the onset of kidney damage, a characteristic complication of this disease.¹⁻⁴

For reasons as yet undetermined, the severity of renal disease varies among patients.⁵⁻⁹ This depends both on the intrinsic properties of the protein (degree of polymerisation, isoelectric point (pI), and charge) and on the composition of serum and urine.¹⁰ Studies undertaken in recent years both in animals¹¹⁻¹⁷

Table 1 Laboratory findings at diagnosis of renal failure

Case No	Sex/Age	Serum monoclonal components	Creatine ($\mu\text{mol/l}$)	Calcium (mmol/l)	Phosphorus (mmol/l)	Uric acid ($\mu\text{mol/l}$)	Precipitating events		
							Drugs	ECDH	IVP
1	M/75	IgA λ	800	2.27	1.8	336	—	—	—
2	F/46	λ	800	2.5	2.3	600	—	—	—
3	M/68	IgA λ + IgG λ + λ	648	2.01	1.44	569	Chemotherapy	—	—
4	F/67	λ	156	2.88	1.20	524	Chemotherapy	—	—
5	M/61	IgD λ	286	2.35	1.5	510	NSAIDs	—	—
6	F/66	κ	280	2.41	1.3	435	—	—	++
7	F/71	IgG κ	210	2.51	1.37	434	—	—	—
8	F/58	IgA λ + λ	457	3.24	2.6	511	—	—	—
9	M/49	κ	674	3.45	2.2	172	—	—	—
10	F/69	κ	720	3.0	2.3	528	NSAIDs	—	—
11	M/70	IgG κ	686	2.38	1.17	360	—	—	++
12	F/62	IgG λ + λ	600	2.25	0.88	218	—	—	—
13	M/70	IgA λ	300	2.3	1.16	333	—	—	—
14	M/72	IgD λ + λ	890	2.83	2.38	610	—	—	—
15	M/75	IgG λ + λ	517	NA	NA	NA	NA	NA	NA
16	F/58	λ	197	2.37	1.16	298	—	—	—
17	F/60	λ	870	2.17	2.43	330	—	++	—
18	F/63	λ	960	2.11	2.72	528	—	—	++
19	M/70	κ	251	2.3	1.04	441	NSAIDs	—	—
20	M/85	λ	258	NA	NA	NA	NA	NA	NA
21	M/70	λ	357	NA	NA	NA	NA	NA	NA
22	M/44	λ	600	2.19	1.6	490	—	—	—

ECDH = extra-cellular dehydration; IVP = intravenous pyelogram; NSAIDs = non-steroidal anti-inflammatory drugs; NA = not available.

clonal immunoglobulins, of which 18% (4/22) were IgA with λ light chains, 18% (4/22) were IgG with κ or λ light chains, and 9% (2/22) were IgD with λ light chains. None of the patients had detectable circulating monoclonal IgM. Seventy three per cent (16/22) of the urinary light chains were of the λ isotype in the patients with renal impairment, compared with 49% (34/70) in those with normal renal function.

In the group of patients with renal impairment for whom clinical and biological data were available (main data were not available for three patients), nine of 19 (47%) presented with acute renal failure as the initial manifestation of dysglobulinaemia. Eight of 19 (42%) whose presenting symptoms were more typical of multiple myeloma had had renal impairment before. Hypercalcaemia was found in five of 19 (26%) patients. Hyperuricaemia was seen in eight of 19 (42%) patients, exceeding 600 $\mu\text{mol/l}$ in one, while another was dehydrated. Radiocontrast studies were implicated in three patients and nephrotoxic drugs in five (three with non-steroidal anti-inflammatory agents and two with chemotherapy). Five (26%) patients were free of these factors.

Urinary BJP, measured by electrophoresis, represented at least 30% of total proteinuria in the 92 patients (total mean proteinuria = 1.3 g/l).

Twenty four hour urine specimens were collected at the bedside and stored in the laboratory for 72 hours at 4°C, without preservatives.

Samples containing more than 3 g/l of protein were diluted in appropriate buffer (depending on the electrophoresis method), or in distilled water (for isoelectric focusing). All electrophoretic migrations were carried out with the Phast-System (Pharmacia, Uppsala, Sweden).

Native polyacrylamide gel electrophoresis (PAGE) separates components on the basis of their size, net charge, and conformation. It is one of the methods used for studying the composition and structure of native proteins, without denaturing agents or detergents, such as sodium dodecyl sulphate (SDS).

One microlitre of urine from each patient was deposited on 8–25 Phastgel gradient (8% and 25% acrylamide gel gradient). Migration was run at a pH close to 8, according to the manufacturer's instructions, with a slight modification in the migration time (the end of migration was programmed at 350 Vh). Molecular mass calibration was carried out using the Pharmacia high molecular weight kit. When the BJP separated into several bands, only the main species was taken into account.

For denaturing, 58 of the 92 urine specimens were treated with 2.5% SDS, without mercaptoethanol, and heated for five minutes at 100°C. One microlitre of sample was deposited on an 8–25 Phastgel gradient. Electrophoresis was carried out according to the manufacturer's instructions, with slight modifications according to the method of Jackson *et al*²⁶ involving the migration time (end programmed at 90 Vh). The gel was calibrated with the Pharmacia low molecular weight kit.

Ferguson plots were constructed for 10 BJP isolates, to study their charge and size. Relative mobility was determined using PAGE, with three known concentrations of acrylamide (T = 7.5, 12, and 20%) in native conditions.²⁷

For isoelectric focusing, 1 μl of untreated urine sample was deposited on the midline of Phastgel IEF 3–9 plates. Migration was run according to the manufacturer's instructions. The gel was calibrated using the Pharmacia isoelectric focusing protein calibration kit.

Figure 1 (a) Native polyacrylamide gradient gel electrophoresis pattern and (b) isoelectric focusing of a urinary BJP isolate (case 6). Lane 1, 24 hour urine; lane 2, fresh urine; lane 3, fresh urine collected on butanol; lane 4, fresh urine collected on a mixture of bovine serum albumin (not pure) and butanol; lane 5, fresh urine collected on 2-mercaptoethanol. The far right lane is the standard: (a) molecular masses in kilodaltons and (b) isoelectric point in pH units indicated on left.

For two-dimensional focusing, Phastgel IEF 3-9 was used, according to the manufacturer's instructions, by depositing 3 μ l of urine on the midline. The titration curves were established with 10 BJP isolates, six of which were from patients with renal impairment. Human albumin (Sigma, St Louis Missouri, USA) was used as a control (pI = 4.8).

With the aim of detecting BJP specifically, we applied a semi-dry electroblotting method (an extension of the Phast-System to Phast-Transfer) to the different types of gel cited above. Protein was transferred to nitrocellulose membranes (BA 85, 0.45 μ m Schleicher & Schull) over 10 to 15 minutes with a potential difference of 20 V to 25 mA, at 15°C, using a TRIS-glycine transfer buffer (TRIS 25 mM, glycine 192 mM, methanol 20% v/v), impregnating three sheets of filter paper (Phast Transfer filter paper, 50 $\times$ 50 mm) placed on each side of the nitrocellulose gel assembly. After transfer, the membrane was immersed in a saturating solution of skimmed powdered milk (Regilait, Lyon, France) at 50 g/l in 0.15 M NaCl for one hour at 45°C. After drying, the membrane was incubated for 40 minutes in goat monospecific antibodies for human free κ or λ light chains (Atlantic Antibodies, Stillwater, Minnesota, USA) and diluted 1 in 200 in the saturating solution containing Tween 20 (0.20 ml/l). After four five minute washes in 0.15 M NaCl-Tween

Figure 2 (a) Native polyacrylamide gradient gel electrophoresis pattern and (b) isoelectric focusing of four urinary BJP isolates, before and after storage for 72 hours at 4°C. Lanes 1-3-5-7, before storage; lanes 2-4-6-8, after storage.

20 (0.20 ml/l), the membrane was incubated with alkaline phosphatase labelled anti-goat IgG antibodies (EC 3.1.3.1; Jackson Immuno Research Labs. Inc., West Grove, Philadelphia, USA) and diluted 1 in 2000 in 0.15M NaCl. After four further washes (NaCl-Tween 20), enzyme activity was revealed with, as substrate, equal parts of naphthol-As-Mx (Sigma; 0.4 g/l) in TRIS-HCl 0.2M buffer and an aqueous solution of Fast Red (Sigma) at 6 g/l. Revelation was stopped after 10 minutes by rinsing with distilled water.

Mean values were compared by using Student's *t* test. The predictive value of the various factors (isotype of the light chain, "migrational" molecular mass, and pI) for the onset of renal impairment was assessed using unifactorial and then multifactorial logistic regression models. All calculations were performed on SAS software (SAS Institute Inc., Cary, North Carolina, USA).

Results

EFFECT OF URINE COLLECTION AND STORAGE CONDITIONS ON ELECTROPHORETIC PROFILE

The effects of urine collection and storage conditions on the electrophoretic profile are shown in figs 1 and 2. The results showed that relative to fresh urine, there was no change in native PAGE gradient electrophoresis and isoelectric focusing pattern after 24 hours of storage, with or without stabilisers (fig 1). After 72 hours of storage at 4°C, four different BJP isolates showed no change in the same electrophoretic profiles (fig 2).

Figure 3 Frequency distribution of the migrational molecular masses of 92 B β P isolates measured using 8–25% PAGE in native conditions.

MOLECULAR MASSES IN NATIVE CONDITIONS

The frequency distribution of the molecular masses of B β P, measured in native conditions and identified by immunoblotting, is shown in fig 3. The latter shows a bimodal distribution, 93% of B β P having a "migrational" molecular mass of 50 to 450 kilodaltons, and 7% with values between 600 and 900 kilodaltons. This distribution was identical for κ and λ type B β P, and the "migrational" molecular masses were most often between 100 and 200 kilodaltons.

In the group of patients with renal impairment the mean "migrational" molecular mass was 206 kilodaltons, and was not significantly different from that in the group of patients with normal renal function ($\bar{x}$ = 215 kilodaltons). The distribution of the patients with renal impairment according to the "migrational" molecular mass, classified in increasing class values, showed that the proportion of patients with renal impairment increased from one class to another, although the difference between each class was not significant (fig 4).

MOLECULAR MASS IN DENATURING CONDITIONS

The study of the electrophoretic pattern obtained in a given gel in the presence of SDS showed the simultaneous presence of monomers (M) and dimers (D) in variable proportions in the 58 B β P isolates analysed. Sixty two per cent (36/58) of the B β P isolates were richer in dimers (M:D of <1) and showed a predominance of the λ isotype (κ : λ

Figure 4 Percentage distribution of the patients with renal impairment (■) and those with normal renal function (□), according to the mobility of the B β P isolate, expressed as "migrational" molecular mass by means of gradient PAGE, in native conditions. The most frequent molecular masses are divided into four classes from 0 to 400 kilodaltons.

Figure 5 Frequency distribution of the isoelectric points (pI) of 92 B β P isolates measured by isoelectric focusing on polyacrylamide gel. The pH gradient was from 3 to 9.

= 0.71); the opposite situation was found in the remaining 38% of cases (κ : λ = 1.2).

The 17 patients with renal impairment in this group showed an equivalent enrichment in dimers (59%—that is, 10/17 M:D of <1) or monomers (41% 7/17 M:D of >1), with a clear predominance of the λ isotype (κ : λ = 0.43 and 0.40, respectively) in each category.

In 25 of the 58 patients immunoblotting showed the presence of small fragments reacting with the free light chain antiserum, with a molecular mass estimated at 14 kilodaltons.

ISOELECTRIC POINTS

The frequency distribution of the pI showed a bimodal pattern: 55% (51/92) of the B β P isolates had a pI of <6 ($\bar{x}$ = 5.2) and 45% (41/92) had a pI of >6 ($\bar{x}$ = 7.5) (fig 5).

In 68 of the 92 patients in whom we characterised the isotype of a circulating monoclonal immunoglobulin heavy chain, monoclonal IgG (31/92 cases) was accompanied in 29% (9/31) of cases by a B β P with a pI above 6. This proportion was 45% (10/22) for IgA (22/92) and 63% (5/8) for IgM (8/92). In the patients whose circulating monoclonal protein was formed only of free light chains (25/92) 48% (12/25) of the urinary B β P isolates had a pI of >6.

Figure 6 Correlation between electrophoretic ("migrational" molecular mass) and the isoelectric point of 92 B β P isolates. The correlation is positive (r = 0.78) for the B β P isolates (n = 41) with a high isoelectric point (pI > 6).

Figure 7 Percentage distribution of 92 B β P isolates from patients with renal impairment (RI) and those with normal renal function (NIR), according to the isoelectric point (B β P of pI < 6, n = 51; B β P of pI > 6, n = 41), and the isotype of the light chain. RI κ : (■); RI λ : (□); NIR κ : (▨); NIR λ : (▩).

There was a correlation between the "migrational" molecular mass obtained in native conditions and the pI, when the latter was above 6 ($r = 0.78$; $n = 41$) (fig 6). This correlation was observed regardless of the monomer/dimer composition.

In the group of patients with renal impairment the mean pI was 6.3 compared with 6.1 in the patients with normal renal function. The difference was not significant, but there was a different distribution of B β P. Among the B β P isolates with a pI of <6, 18% (9/51) were from patients with renal impairment, while among those with a pI of >6, 32% (13/41) were from patients with renal impairment. In each of these categories the κ : λ ratio was less than 1 (respectively, 0.3 and 0.4), indicating a clear predominance of the λ isotype in the patients with renal impairment (fig 7).

FERGUSON PLOTS

These plots, established with 10 B β P isolates (fig 8), showed that variations in B β P mobility as a function of the acrylamide concentration were linear. The slope of the lines was related to the size of the protein, and interestingly the lines had the same slope. This indicates that the B β P isolates studied had the same retardation coefficient and thus the same size. The different intersections extrapolated on the y axis also showed large differences in the net charges of the B β P.

TITRATION CURVES

The titration curves for 10 B β P isolates selected according to their pI (table 2) showed that the B β P behaved differently when placed in the same pH gradient. Three types of behaviour were defined (fig 9):

Figure 8 Ferguson plots: migration of 10 B β P isolates in polyacrylamide gels at three different concentrations ($T = 7.5, 12.5$, and 20%).

(1) B β P type a, with acid pI (in our study 4.7 and 4.9), which were the most mobile, even at pH values close to their pI. They were strongly charged and behaved similarly to albumin ($n = 2$).

(2) B β P type b, with $5.7 < \text{pI} < 6.5$, with poor mobility, even when the pH varied by one unit either side of the pI, probably indicating a weaker charge ($n = 7$).

(3) B β P type c, with a pI above 7, which only took a positive charge. Its mobility increased gradually as the pH fell, indicating a strong positive charge at acid pH ($n = 1$).

Of the 10 titration curves, six were for B β P from patients with renal impairment, and five of these six took a weak negative charge at pH 7. These 10 titration curves also enabled us to detect the emergence of isoforms at distinct acid or alkaline pH values in three cases (fig 10).

STATISTICAL ANALYSIS

The data for the patients with renal impairment were examined to determine their predictive value for the onset of renal impairment. Three factors were taken into account: the isotype of the light chains; their "migrational" molecular mass in an acrylamide gradient, and their pI. The results of the unifactorial analysis showed that only the isotype of the light chain was predictive of the onset of renal impairment ($p < 0.05$). Patients excreting a λ type B β P had a 2.8-fold higher risk of developing renal impairment compared

Table 2 Physico-chemical characteristics of 10 B β P isolates from patients with renal impairment ($n = 6$) and patients with normal renal function ($n = 4$) for which titration curves were constructed

B β P isolate	Serum monoclonal component	Molecular mass (kilodaltons)	Isoelectric point	B β P charge at pH 7	Renal Impairment
A	κ	180	5.8	—	0
B (case 19)	κ	200	6.7	—	+
C	λ	135	6.1	— —	0
D (case 15)	IgG λ + λ	100	5.9	—	+
E	IgA κ + κ	>700	8–9	++	0
F	IgA κ + κ	130	4.7	— — —	0
G (case 1)	IgA λ	210	6.5	—	+
H (case 8)	IgA λ + λ	140	4.9	—	+
I (case 3)	IgA λ + IgG λ + λ	85	5.6	— — —	+
J (case 14)	IgD λ + λ	250	6.5	—	+

Figure 9 Schematic representation of the three types of titration curves obtained with 10 B β P isolates. Type a: $n = 2$ ($pI < 5$); type b: $n = 7$ ($5.7 < pI < 6.5$); type c: $n = 1$ ($pI > 7$).

with those excreting κ type BJP. The multifactorial analysis confirmed that the isotype of the light chain was the only predictive factor.

Discussion

This study of 92 BJP isolates using polyacrylamide gradient electrophoresis in native conditions shows that most BJP isolates had an apparent molecular mass greater than 50 kilodaltons, while the expected molecular mass was, at most, that of a dimer of two light chains of 214 amino acids and generally estimated at 45 kilodaltons. It was tempting to interpret these migration patterns as those of polymers or aggregates, which have often been reported.^{20 28-31}

Electrophoresis in the presence of SDS (in denaturing conditions) cannot show up this characteristic, given the dissociating effect of SDS. We found, in these conditions, that molecular mass values reflected the presence of monomers (molecular mass = 22.5 kilodaltons) and dimers (molecular mass = 45 kilodaltons), as generally described for this type of electrophoresis.

The Ferguson plots for 10 BJP isolates, in native conditions, suggest that the "migrational" molecular masses in these conditions are simply apparent masses. They are, it seems, the expression of a particular protein charge, which confers poor mobility at the electrophoretic pH, and an apparently raised molecular mass in the polyacrylamide gradients. This characteristic, already observed in gel filtration,³² was also reported in a study involving two-dimensional high resolution electrophoresis.³³

The pI values of the BJP isolates we studied confirm the variability of this parameter.^{24 25 34} Two groups of BJP were also identified by Norden *et al*,²⁴ with a pI distribution similar to that in our study. Among the BJP isolates with a pI of more than 6, there was a positive correlation between the pI and apparent masses. This observation, first made by Hill *et al*,¹⁸ who measured the mobility, relative to albumin, of 17 BJP isolates in agarose (pH 8.6), has also been observed by Norden *et al*²⁴ for eight BJP isolates in agarose at pH 6, using titration curves.

Figure 10 Titration curves for two B β P isolates (B β P A: $pI = 5.8$; B β P C: $pI = 6.1$ (table 2) showing the existence of isoforms in distinct pH zones (a: B β P A: two isoforms; b: B β P C: three isoforms).

We found three types of BJP, whose mobility was differently influenced by the pH, indicating three different charges at physiological pH. The presence of isoforms identified from the titration curves at different pH zones according to the BJP type explains some of the microheterogeneity observed in acrylamide gradient electrophoresis and in isoelectric focusing. A triple band in electrophoresis could be because of the charge difference of the three isoforms of a given molecule, which are separated at the precise pH at which the electrophoresis is carried out.

When these different parameters were examined according to renal function, we observed that the extreme characteristics (apparent masses and pI) of the BJP isolate from case E (table 2) were never found in the other patients with renal impairment. However, we found an association between these two parameters and renal impairment. Indeed the latter seemed to be more common when the apparent mass of the BJP was raised and, as a result, its mobility lower. This association has been described by Hill *et al*¹⁸ in cast nephropathy, with an excellent correlation between poor mobility and the severity of renal impairment.

We found that measurement of the pI distinguished two sets of patients with BJP, with most pI values falling between 4.5 and 6, or between 6.5 and 8. The cut off value of 6 did not, however, identify a population at a higher risk of renal impairment. Various authors have raised the possible involvement of high pI values in the onset of renal impairment,^{11 19} but studies of patients with renal impairment have not provided proof that this is the case,²⁴⁻²⁵ and this is also true of our data.

The variations in the mobility of BJP according to pH should be taken into account, as we found that mobility diminished when the pI was >6. The titration curves represent this mobility as a function of pH and thus of the charge. In the nephron this phenomenon might enable interactions between BJP and other proteins to be predicted according to the pH.

The curves suggest that the BJP charge was low at intratubular pH in five of the six cases of renal impairment in which they were constructed. Renal impairment in case 3 (tables 1 and 2), whose BJP had a high charge at pH 7, only started after about 10 years' follow up, suggesting that a high charge might delay the onset of renal impairment.

In a study of cast nephropathy Sanders *et al* showed the importance of urinary ion composition.¹⁷ They reported a protective effect of albumin which, by increasing chloride absorption, countered the formation of obstructive casts. They found that BJP with a high pI (7.7) behaved similarly—although capable of forming aggregates *in vitro* in the presence of Tamm-Horsfall protein, it did not give rise to this nephropathy in rats. More recently, the same authors showed that furosemide could aggravate the course of cast nephropathy, favouring cast formation by increasing the luminal sodium chloride concentration.³⁵ These findings suggest that an interaction between the ion composition of intratubular urine and the degree of charge on the BJP could contribute to the nephrotoxicity of the latter.

In our study five of the 19 patients for whom data on possible confounding factors were available had no factors other than BJP likely to contribute to renal failure. This, together with the fact that none of these five patients recovered normal renal function after the episode of acute impairment, points to the nephrotoxic potential of BJP.

We did not find that electrophoretic physico-chemical characteristics of BJP (apparent mass and pI) were predictive of the onset of renal impairment. However, immunochemical identification of the light chain isotype can provide information on the risk of renal failure, as previously reported by Alexanian *et al*.³⁶ The pI remains informative, as it can be used to predict the BJP charge according to urinary pH. None the less this criterion does not take into account the degree of charge, which may explain the interactions of BJP with other components of urine or tubule cells. Apparent masses, which can be measured in acrylamide gradients, would

seem to reflect this charge. Only titration curves can describe the charge behaviour of the molecule, its variability at different pH values, and the possible existence of isoforms.

Studies of a wider variety of BJP will no doubt permit these proteins to be classified according to the type of renal impairment and help explain *in vivo* toxicity mechanisms.

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