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Protease resistance and binding of Ig light chains in myeloma-associated tubulopathies

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Protease resistance and binding of Ig light chains in myeloma-associated tubulopathies. Kidney tubule dysfunction and lesions are frequent complications of myeloma, related to unknown properties of the monoclonal light chain. We have analyzed protease sensitivity and binding properties of urinary light chains from four patients with Fanconi's syndrome, 12 with cast nephropathy, and four control patients without myeloma-associated tubulopathy. All light chains were normal-sized, monomeric and/or dimeric, and none was N-glycosylated. Kinetic studies of light chain digestion by pepsin and the lysosomal enzyme cathepsin B showed the generation of a protease-resistant 12 kDa fragment, corresponding to the V domain of the κ chain in the four Fanconi's syndrome patients; in two out of four the V domain was also completely resistant to trypsin. Western and dot blots revealed similar patterns of reactivity of light chains from patients with the Fanconi's syndrome towards other light chains. Properties of cast-nephropathy light chains were more heterogeneous but clearly differed from those of Fanconi's syndrome: (i) 9 out of 12 were of the λ-type; (ii) only four yielded a transient 12 kDa fragment after cathepsin B digestion, but all showed some resistance to proteolysis of the entire molecule or a fragment thereof to at least one protease, at variance with control light chains; (iii) they displayed various patterns of reactivity with other light chains; (iv) 7 out of 12 reacted specifically with Tamm-Horsfall protein (THP) by ELISA, in contrast with those of Fanconi's syndrome. In one patient who presented with cast nephropathy and the Fanconi's syndrome, the light chain exhibited both partial resistance of the Vκ domain to cathepsin B and the highest reactivity with THP. These results suggest that light chain toxicity in Fanconi's syndrome is related to the resistance of the V domain to degradation in lysosomes of proximal tubule epithelial cells. In contrast, cast nephropathy is an heterogeneous entity whose pathogenesis may involve multiple factors such as protease resistance, in addition to light chain reactivity with THP.

Kidney tubule alterations related to monoclonal immunoglobulin (Ig) light chains are frequent complications of myeloma (1 case out of 3) [1]. They often lead to end-stage renal failure and may thus be considered as severe prognosis factors. The most common form is myeloma cast nephropathy, in which characteristic lesions consist of tubular atrophy associated with the presence of dense fractured casts surrounded by macrophagic cells predominantly located in the lumina of distal tubules and collecting ducts [2]. Formation of these casts, which mainly contain the light chain together with Tamm-Horsfall protein (THP) and few other protein components, is likely to be a major pathogenetic factor of renal impairment through tubular obstruction. Renal failure is also caused by associated lesions of tubule epithelial cells which are usually considered to be the result of light chain toxicity on the renal tubule [3].

Fanconi's syndrome is defined by specific alterations of proximal tubule functions, and may also complicate myeloma in rare instances [4–6]. Myeloma-associated Fanconi's syndrome is generally featured by crystal inclusions in proliferating plasma cells, macrophages and proximal-tubule epithelial cells. Although the light-chain content of the intracellular crystals has long been suggested by immunoperoxidase studies [7], we recently demonstrated in one case that their predominant component was a 107 amino acid fragment corresponding precisely to the variable domain of the light chain resistant to proteolysis and prone to crystallization [8]. Intracellular protein crystals may also be found in the tubules of patients with "classical" cast nephropathy [9], which suggests that Fanconi's syndrome and cast nephropathy could share common pathogenetic factors.

Intrinsic properties of light chains are likely to play a key pathogenetic role in myeloma tubulopathies as evidenced by lack of correlation between the amount of urinary light chain and renal lesions, improvement of renal function after antitumoral chemotherapy [10–12] and recurrence on grafted kidney [13]. Moreover, injection of pathogenic purified human light chains to mice [14, 15], as well as their infusion into rat renal tubules [16], induce the generation of myeloma casts or of crystals comparable to those found in the biopsy specimens. The lambda isotype may be more frequently responsible for cast nephropathy than the kappa [11], whereas the kappa isotype is usually involved in Fanconi's syndrome [5, 6], but these restrictions are far from being absolute [7]. Other structure-related properties, such as high isoelectric point of the light chains, have been thought to influence their propensity for interacting with THP and forming casts [17]; however, contradictory results question this hypothesis [11, 18, 19].

In the present study, we purified monoclonal light chains from 12 patients with cast nephropathy and from four with the Fanconi's syndrome. We showed that biochemical properties of light chains, that is, binding to THP and resistance to proteases, were not uniform in cast nephropathy patients who made up a heterogeneous group both clinically and histologically. In contrast,

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Table 1. Characteristics of Fanconi's syndrome at initial presentation or biopsy time

Patient	Blood				Urine		Bone lesions
	Calcium *mM*	Phosphate *mM*	Uric acid *μM*	HCO_3^-	Glucose *mmol/24 hr*	Amino aciduria	
FS1	2.14	0.96	135	23.6	+++	↗	Osteomalacia, fractures
FS2	2.4	0.47	176	25	15	↗	None
FS3	2.3	1.24	101	22	10	↗	Osteoporosis
FS4	1.66	1.02	259	15	++	↗	Osteoporosis

[a] Normal range is 215 to 375 μM

Table 2. Clinical and biological data at the time of renal biopsy

Patient	Gender/ Age	M component				Marrow plasma cells %	Tumor mass	Serum creatinine μM	Course of renal failure
		Serum	Urine						
			LC	Polymerisation form	Amount *g/24 hr*				
FS1	F/65	—	κ	D/M	1.9 (60%)	2	low	168	Progressive
FS2	M/42	κ	κ	D/M	1.0 (50%)	9	low	274	Progressive
FS3	F/86	κ	κ	D/M	0.8 (80%)	6	low	200	Progressive
FS4	M/71	Gκ	κ	D/M	1.7 (50%)	7.5	low	428	Progressive
CN1	F/72	Aλ	λ	D	2.0 (90%)	86	high	2840	Irreversible
CN2	M/60	λ	λ	D/M	2.7 (78%)	42	high	805	Partially reversible
CN3	M/62	λ	λ	D/M	1.0 (60%)	18	high	1400	Irreversible
CN4	M/66	Dλ	λ	M	4.0 (80%)	20	low	130	Stable
CN5	M/69	Aλ	λ	D/M	7.5 (86%)	61	high	164	Irreversible
CN6	M/33	Gλ	λ	M	5.8 (95%)	64	high	1575	Reversible
CN7	M/74	Gλ	λ	D/M	2.4 (84%)	36	high	768	Irreversible
CN8	M/71	Gλ	λ	D	0.4 (60%)	7	high	945	Partially reversible
CN9	F/57	κ	κ	D or M	9.1 (80%)	65	high	141	Reversible
CN10	F/67	Gλ	λ	D/P	4.1 (90%)	15	high	220	Irreversible
CN11	M/34	κ	κ	M	3.1 (89%)	>90	high	698	Irreversible
CN12	M/47	Gκ	κ	M	3.0 (87%)	>50	high	812	Irreversible

FS, Fanconi's syndrome; CN, cast nephropathy; LC, light chain (Figures in brackets relate to % of LC M component in proteinuria); D, dimeric; M, monomeric (D/M means that the two polymerization forms could not be separated by chromatography); P, polymeric.

patients with the Fanconi's syndrome displayed remarkable clinical, histological and biochemical similarities, with their light chains being distinguished by prolonged resistance of the variable domain (Vκ) to proteolysis by pepsin and cathepsin B.

Methods

Patients

Characteristics of patients are depicted in Tables 1 to 3. Patients FS1 to FS4 presented with the main aspects of the Fanconi's syndrome (Table 1) including generalized aminoaciduria, glycosuria, and low or normal serum phosphate and uric acid levels despite mild to severe but progressive renal insufficiency. Typical osteomalacic bone lesions were seen in patient FS1. Bone X-rays were more difficult to interpret in the older patients (FS3 and FS4). The four patients with the Fanconi's syndrome had a κ-chain-secreting smoldering myeloma (Table 2).

In contrast, all patients except one with cast nephropathy were affected with a high tumor mass myeloma producing λ chains in 9 of them (Table 2). They also differed by higher amount of proteinuria and more severe and acute renal failure. The search for an associated Fanconi's syndrome was made difficult by markedly deteriorated renal function in most patients. Only patient CN9 showed the main signs of Fanconi's syndrome including glycosuria (3 g/day), generalized aminoaciduria, hypokaliemia (2.9 mEq/liter) and increased uric acid (27 ml/min) and phosphate clearance (27 ml/min) despite decreased creatinine clearance (37 ml/min). Significant amounts of glycosuria were also detected in patient CN1 who had corticosteroid-induced hyperglycemia, and in patient CN4 with a type-2 diabetes.

Renal biopsies of the four Fanconi's syndrome patients showed severe epithelial lesions with crystal formations observed in three cases in the cytoplasm of proximal tubule epithelial cells (Table 3). Casts were absent or hyaline, without features of myeloma casts. FS2 and FS3 were remarkable by the abundance of crystals in proximal tubule cells and their association with crystal inclusions in interstitium infiltrating cells.

Biopsies from patients with cast nephropathy all contained typical fractured myeloma casts showing polychromatism upon staining with Masson's trichrome, and the casts were surrounded by multinucleated giant cells. Crystals were only occasionally detected within casts and in the cytoplasm of proximal and distal tubule cells (CN6 and CN9, Table 3). The biopsy from CN6 showed multiple fractured, bulky crystalline casts in the tubular lumina. Smaller crystals were present in phagolysosomes of distal tubule cells. Multiple crystalline casts with a variety of appearances were also found in CN9. This case was unusual in that

Table 3. Histological findings in patients with myeloma-associated tubulopathies

Patient	Glomeruli %		Tubules				Interstitium	
	Global scler	Periglom scler	Crystals	Epith lesions	Tubule atrophy	Casts[a]	Fibrosis	Crystals[b]
FS1	70	19	—	+++	+++	Hyaline	+++	–
FS2	60	10	+++ (Ep.)	+++	+	Hyaline	+	+
FS3	NA	NA	+++ (Ep.)	+++	++	None	++	+
FS4	50	9	++ (Ep.)	+++	+++	Hyaline	+++	–
CN1	0	30	0	++	++	++	++	–
CN2	20	10	0	++	+	++	++	–
CN3	0	20	0	++	++	++	++	–
CN4	10	0	0	+	+	+	+	–
CN5	40	10	0	++	++	++	+	–
CN6	0	0	+ (Casts)	++	0	++	0	–
CN7	45	27	0	++	+	++	++	–
CN8	7	14	0	+++	+	++	++	–
CN9	5	5	+ (Casts+Ep.)	++	+	++	+	–
CN10	20	20	0	+++	0	++	0	–
CN11	30	0	0	++	0	++	0	–
CN12	0	0	0	++	0	++	+	–

Abbreviations are: Global scler, global sclerosis; Periglom scler, periglomerular sclerosis; (Ep.), crystals in proximal tubule epithelial cells; Epith lesions, epithelial lesions; NA, not available.

[a] Typical myeloma casts were seen only in patients with cast nephropathy

[b] Presence of crystal inclusions in interstitium infiltrating cells

Table 4. Clinical and histological characteristics of control patients

Patients	Gender age	M component		Marrow plasma cells %	Tumor mass	Serum creatinine μM	Renal biopsy	Type of plasma cell dyscrasia	Comments
		Serum	Urine						
C1	M/76	Gκ	κ	23	High	187	Nonspecific CIN	Myeloma	
C2	F/64	Gκ	κ	?	High	N[a]	ND	Myeloma	
C3	M/81	κ	κ	15	Low	Dialysis[b]	AIN with eosinophils	Myeloma	Type 2 diabetes Diamicron-induced AIN[b]
C4	F/79	λ	λ	7	—	260	AL (λ) amyloid	"Primary" AL amyloidosis	Nephrotic syndrome

Abbreviations are: ND, not done; CIN, chronic interstitial nephritis; AIN, acute interstitial nephritis.

[a] Normal renal function over 6 years

[b] Recovery of renal function after stopping Diamicron™

proximal tubular cells were stuffed with similar crystals located in distended phagolysosomes.

No patient had glomerular lesions related to amyloid or deposition of light chains, but most had nonspecific globally sclerotic glomeruli, probably as a consequence of tubulointerstitial and vascular lesions.

Controls

Four control patients were also included in this study (Table 4). The first three had a multiple myeloma. C1 presented with nonspecific chronic interstitial nephritis. C2 did not develop renal failure over a 6-year follow-up. A diagnosis of Diamicron™-induced acute interstitial nephritis was made in C3 who rapidly recovered a normal renal function after eviction of the drug. The fourth control patient (C4) had "primary" AL amyloidosis without evidence of myeloma.

Light chain purification

Urine samples were concentrated by dialysis under reduced pressure and proteins were purified by a combination of DEAE-Trisacryl (Sepracor, Villeneuve la Garenne, France) chromatography in 10 mM Tris (pH 7.6 to 8 according to the light chain) with a 0 to 0.3 M NaCl linear gradient, and gel filtration on Sephadex G100, G150 or G200 Superfine (Pharmacia, Uppsala, Sweden). Fractions were concentrated as above and purity was controlled by thin layer agarose electrophoresis (Beckmann, Brea, CA, USA), immunoelectrophoresis and 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

N-glycosylation

N-glycosylation was searched for by treatment with endoglycosidase-F (Boehringer, Manheim, Germany) after denaturation of the light chain in 0.1% SDS at 100°C during two minutes and dilution in 0.5% nonidet P40, 0.1 M Tris pH 8.3; after a two hours incubation at 37°C with the enzyme (2 units per mg light chain), samples were analyzed by 12% SDS-PAGE in comparison with the native substrate.

Protease treatment

Pepsin (Boehringer) digestion of light chains (3 mg/ml) was performed at 37°C in 50 mM glycine pH 3.5, with an enzyme substrate ratio of 1:250. Light chains were similarly digested with

trypsin (Gibco, Paisley, UK) in 0.1 M NaCl, 50 mM TRIS, pH 8.2 with an enzyme substrate ratio of 1:50. Digestion by cathepsin B (Sigma, St. Louis, MO, USA) was performed at 37°C on 1 mg/ml light chain solutions in 80 mM sodium acetate, 8 mM L-cysteine, pH 5.0 (0.33 units for 10 μg light chain). The reaction was stopped by 10 mM iodoacetamide. After various incubation times from 1 to 24 hours, samples were diluted in SDS-PAGE sample buffer and frozen. When protease resistance was still detected at 24 hours of enzymatic treatment, digestions were also extended to 48 hours. Fifteen percent SDS-PAGE were stained by Coomassie R250 brilliant blue or electrotransferred onto nitrocellulose. Blots were saturated by 5% skimmed milk and revealed by biotinylated anti-kappa constant region (Cκ) monoclonal antibody (clone HP6053, a gift of Dr. G. Carlone, CDC, Atlanta, GA, USA), followed by alkaline phosphatase-conjugated streptavidin (Amersham International, Amersham, UK).

Biotinylation

Samples (light chain and anti-Cκ antibody) were diluted to 1 mg/ml in 0.1 M sodium carbonate and incubated two hours at room temperature with 10 μg/ml sulfosuccinimidyl-6-(biotin-amido) hexanoate (Pierce, Rockford, IL, USA). The reaction was stopped by adding 10% 1 M NH_4Cl. Biotinylated products were purified on Sephadex G25 columns, and tested by a Western blot revealed with streptavidin-peroxidase; their concentration was evaluated by their absorbance at 280 nm ($E^{1\%}_{1\ cm} = 14.0$).

Light chain cross-reactivity

One microliter of each purified light chain (20 mg/ml) was deposited on nitrocellulose sheets, then saturated as above, incubated in biotinylated light chain solutions at 10 μg/ml in 2% bovine serum albumin (BSA)-PBS and revealed with streptavidin-peroxidase (Amersham).

Significant reactions of biotinylated light chains on dot blots were controlled by a Western blot technique: 20 μg of the corresponding light chain were migrated by thin layer agarose electrophoresis and transferred onto a nitrocellulose sheet by a single 10 minute pressure of 15 g/cm^2. Blots were saturated and revealed with biotinylated light chain as above.

Analysis of binding to Tamm-Horsfall protein (THP) by ELISA

Wells were coated overnight at 4°C with THP (2 μg/100 μl in PBS) or PBS alone as a control, then saturated with 2% BSA in PBS for one hour at room temperature. Biotinylated albumin (A), transferrin (T) and purified light chain (2.5 μg/100 μl) from each patient were incubated simultaneously in the same microplate for two hours at room temperature, followed by streptavidin-peroxidase (1:1000, 2 hr at room temperature). Enzymatic activity was revealed by 1 mg/ml O-phenylenediamine in 0.2 M citric acid, 0.2 M tri-sodium acetate 0.03% H_2O_2. The reaction was stopped by adding 1 N H_2SO_4 and read at 450 nm with a Titertek Multiskan spectrophotometer.

Simultaneously, specific binding of each light chain was assessed by preincubating an excess of non-biotinylated light chain (250 μg/100 μl for 2 hr at room temperature) before adding the biotinylated one.

Results were expressed as percentage of binding of CN1, the light chain with maximal binding to THP (mean ± SEM of 6 experiments). Specificity of binding was established by statistical comparison of values obtained in the absence and in the presence of an excess of non-biotinylated light chain, using the Student's *t* test.

Table 5. Light chain resistance to protease treatment

Protein	Trypsin		Pepsin		Cathepsin B	
	Entire light chain	12 kDa fragment	Entire light chain	12 kDa fragment	Entire light chain	12 kDa fragment
C1	−	−	−	−	−	−
C2	−	−	−	−	−	−
C3	−	−	−	−	−	−
C4	−	−	−	−	−	−
FS1	−	−	−	++	−	++
FS2	−	++	−	++	−	++
FS3	−	−	−	++	−	++
FS4	−	++	−	++	−	++
CN1	+	−	+	−	−	−
CN2	+	+	+	−	−	−
CN3	+	+	−	+	−	−
CN4	−	−	++	−	−	−
CN5	+	−	−	−	−	−
CN6	+	−	++	−	−	−
CN7	+	−	−	−	−	−
CN8	+	−	−	−	−	+
CN9	−	−	−	−	−	+
CN10	−	−	+	−	−	+
CN11	−	−	−	++	−	+
CN12	−	−	+	−	−	−

Symbols are: −, lack of resistance; +, partial resistance (defined as persistence of the entire LC or its 12 kDa fragment up to 24 hr of digestion); ++, complete resistance (over 48 hours of digestion).

Results

Light-chain purification and analyzes

Most purified light chains were mixtures of dimeric and/or monomeric forms, as shown by SDS-PAGE under non-reducing conditions (Table 2). In one case (CN9), we could completely separate monomeric and dimeric fractions. All light chains were normally sized and none was N-glycosylated, as suggested by absence of migration shift on SDS-PAGE after treatment with endoglycosidase F (not shown).

Protease treatment

In all cases of Fanconi's syndrome, studies of protease digestion showed the generation of a 12 kDa fragment which was resistant to further digestion by pepsin (Table 5) and cathepsin B (Fig. 1); in two out of four cases, the fragment was also resistant to trypsin (Table 5). After 48 hours of incubation, the amount of fragment remained unchanged. Western blot analysis with anti-C_κ monoclonal antibody suggested that these fragments corresponded to the light chain variable regions (Fig. 1).

Light chains from all patients with cast nephropathy were normally digested by cathepsin B, except for four: CN8 (Fig. 1) to CN11 yielded small amounts of a 12 kDa fragment, but the latter was only partially resistant since it was no longer observed at 48 hours of digestion. In addition, the entire light chain from 10 patients with cast nephropathy showed significant resistance to trypsin and/or pepsin, but in only one case (CN11) was generated a 12 kDa fragment totally resistant to pepsin (Table 5).

In the four control patients, no light chain resistance was

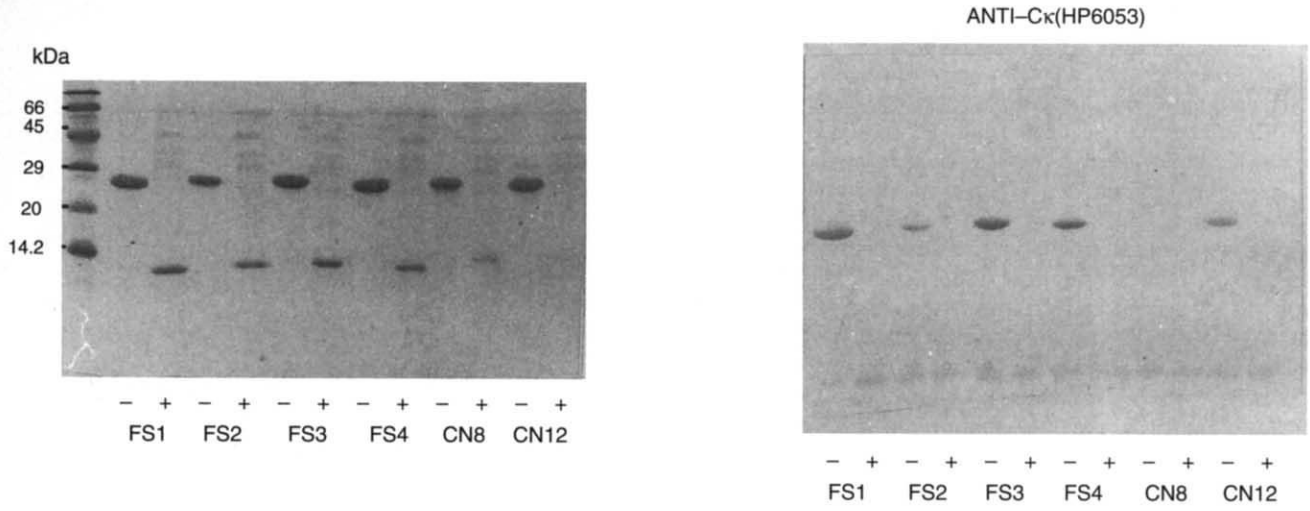


Fig. 1. *15% SDS-PAGE and Western blot analyzes of cathepsin B digestion products.* The 4 κ light chains purified from patients with Fanconi's syndrome (FS1, FS2, FS3, FS4) as well as 2 light chains purified from patients with cast nephropathy (CN8=λ, CN12=κ) were submitted to 15% SDS-PAGE (**Left panel**) before (−) and after a 24 hour digestion with cathepsin B (+). Molecular weight markers (Pharmacia) were migrated in the left lane. Western blot (**Right panel**) was revealed by biotinylated anti-kappa monoclonal antibody HP 6053 followed by alkaline-phosphatase conjugated streptavidin.

Table 6. Light chain—light chain reactivity

LC: Immobilized / Biotinylated	C1	C2	FS1	FS2	FS3	FS4	CN1	CN2	CN3	CN4	CN5	CN6	CN7	CN8	CN9	CN10	CN11	CN12
C1	−	−	−	−	+	−	−	+	+	−	−	−	−	−	−	−	−	−
C2	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
FS1	−	−	−	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−
FS2	−	−	−	+	+	−	−	+	+	−	−	−	+	−	−	−	−	−
FS3	+	−	−	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−
FS4	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−
CN1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
CN2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
CN3	−	−	+	+	+	−	−	−	−	−	−	−	−	−	+	−	−	−
CN4	−	−	−	+	−	+	−	−	−	−	−	−	−	−	+	−	+	−
CN5	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
CN6	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	−
CN7	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
CN8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
CN9	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
CN10	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
CN11	−	−	−	−	−	−	−	+	+	−	−	−	−	−	+	−	−	−
CN12	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−

observed. Only C3 showed transient appearance of the 12 kDa fragment which completely disappeared by 24 hours.

Light chain reactivity

Western blot and dot blot experiments revealed similar patterns of reactivity of light chains from the four Fanconi's syndrome patients towards other light chains, especially immobilized FS2, CN2 and CN3 (Table 6 and Fig. 2). Only FS2 showed self-reactivity. Light chains from patients without the Fanconi's syndrome displayed various patterns of reactivity. Immobilized CN9 had the broadest reactivity with other cast nephropathy light chains. Reactivity of the same immobilized and biotinylated light chain was not identical, suggesting that the form of presentation of the light chain could influence its reactivity. No significant binding was observed with normal human serum or with a human monoclonal IgG, in the same experimental conditions.

Binding to THP

Albumin, transferrin as well as control and Fanconi's syndrome light chains did not bind significantly to THP. Seven light chains from cast nephropathy patients bound to THP (Fig. 3). Binding was specific since it could be significantly reduced by a preincubation with an excess of the non-biotinylated homologous light chain.

Discussion

In this work, we have investigated some physicochemical properties of myeloma light chains which might be responsible for tubular lesions. We decided to analyze comparatively myeloma-associated Fanconi's syndrome and cast nephropathy because these two complications, although both affecting the renal tubule, occur in different settings. As illustrated by our own series as well

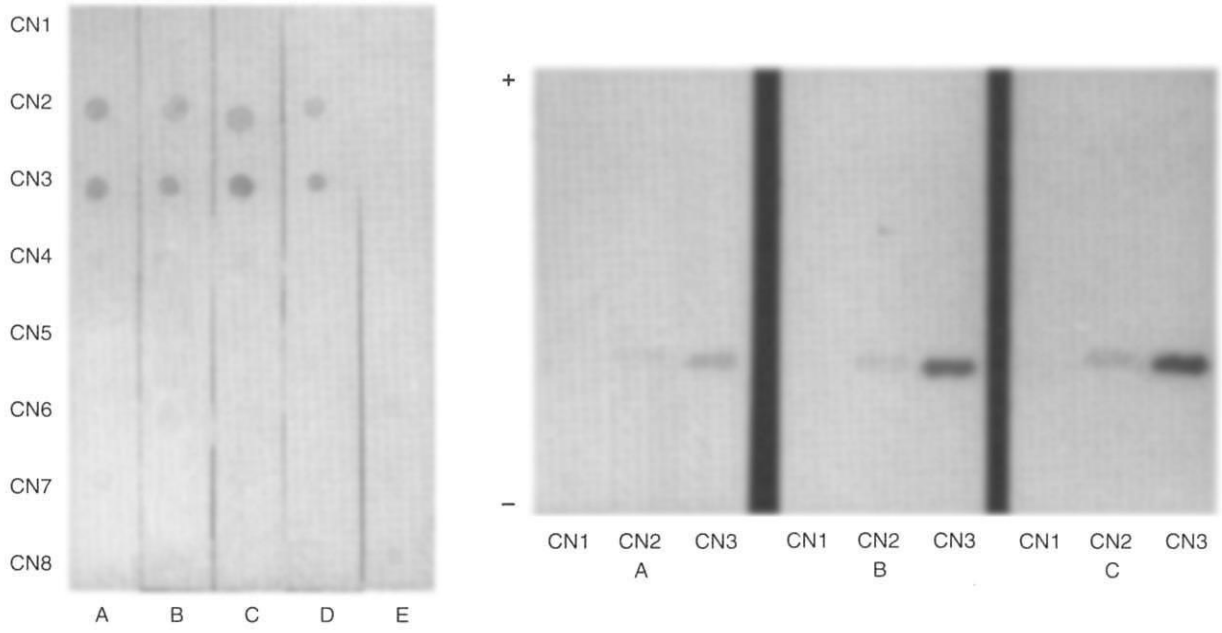


Fig. 2. *Light chain-light chain reactivity studies.* Binding of biotinylated light chain to light chain immobilized on nitrocellulose was analyzed by dot blot (**Left panel**. 20 μg of light chain deposited per spot) and Western blot experiments after thin layer agarose electrophoresis (**Right panel**. 20 μg of light chain deposited per lane). The object of the Western blot was to demonstrate that reactivity was specifically directed to the light chain (identified by its electrophoretic mobility). CN1 to CN8 (left) and CN1 to CN3 (right) are immobilized light chains from patients with cast nephropathy. They were reacted with 10 μg/ml of biotinylated light chain from patients with Fanconi's syndrome (A = FS1, B = FS2, C = FS3, D = FS4) or with cast nephropathy (E = CN3).

as others, FS usually appears in the course of smoldering myeloma with low tumor mass [6], whereas CN occurs early in patients with high tumor mass [11]. This contrasting time-course as well as the different pattern of clinical and pathological expression suggested that distinct properties of light chains might be implicated.

All four patients with Fanconi's syndrome excreted κ-type light chains, both in dimeric and monomeric forms, which were remarkable by their pattern of proteolytic resistance and their reactivity with other light chains. The four light chains yielded, after cathepsin B treatment, a 12 kDa fragment which was resistant to further proteolytic attack over a 48 hour period. This fragment was most likely made of the κ light chain variable region (Vκ) as it was not recognized by an anti-C_κ antibody. Resistance of the V domain to cathepsin B over 48 hours was specific for Fanconi's syndrome κ chains. It was not observed with control or cast-nephropathy light chains. A similar pattern of proteolysis was noted with pepsin. These data confirm and extend a previous single observation made on FS2 which, unlike four control κ light chains from myeloma patients without renal involvement, showed resistance of its Vκ domain to trypsin and pepsin [8]; however, cathepsin B sensitivity had not been evaluated in this initial study. Vκ was the essential component of crystals forming spontaneously from the patient's urine, and could also be crystallized alone using the hanging drop technique [8]. We now show that the same proteolysis resistant fragment is specifically generated from four different light chains by cathepsin B, an enzyme normally present in lysosomes. This suggests that complete proteolysis cannot occur *in vivo* after endocytosis of the light chain in proximal tubule epithelial cells, leading to accumulation of the V fragment within the cells in the lysosomal compartment.

A second characteristic of light chains from Fanconi's syndrome is their peculiar proneness to react with other light chains. Although self-reactivity of FS2 [8] was not verified with the other three light chains, we did find an unusual pattern of reactivity characterized by intense specific binding to immobilized FS2, CN2 and CN3 light chains. Since a low amount of polyclonal IgG is continuously filtered through the glomerulus and then absorbed by proximal tubule cells, reactivity of light chains with normal IgG may serve as a nucleation starter for crystal formation. Alternatively, this low affinity reactivity could merely reflect a restriction of the V segment repertoire in light chains causing the Fanconi's syndrome. Indeed, preliminary results show that they all belong to the $V_{\kappa I}$ variability subgroup (Rocca et al, manuscript in preparation). In contrast, these chains did not show any reactivity with THP, a protein absent from the proximal tubule.

It must be noted that cathepsin B resistance of the Vκ domain was observed irrespective of the abundance of crystals in the biopsy sample, suggesting that it is a common feature of Fanconi's syndrome and possibly a more reliable marker than crystals themselves. Accumulation of the V domain fragment generated by lysosomal enzymes and favored by light chain reactivity may alter apical membrane recycling and/or ATP production (hence Na^+,K^+-ATPase functioning), as suggested by the presence of mitochondrial lesions [5], leading to progressive impairment of sodium-dependent apical transports. This hypothesis is currently being investigated in our laboratory using proximal cell culture models.

Physicochemical properties of cast nephropathy light chains were more heterogeneous, but several common features contrasting with those of Fanconi's syndrome are worth noting. (i) The majority of light chains (9 of 12) were of the λ-type. (ii) Only four of them yielded a 12 kDa fragment after cathepsin B digestion,

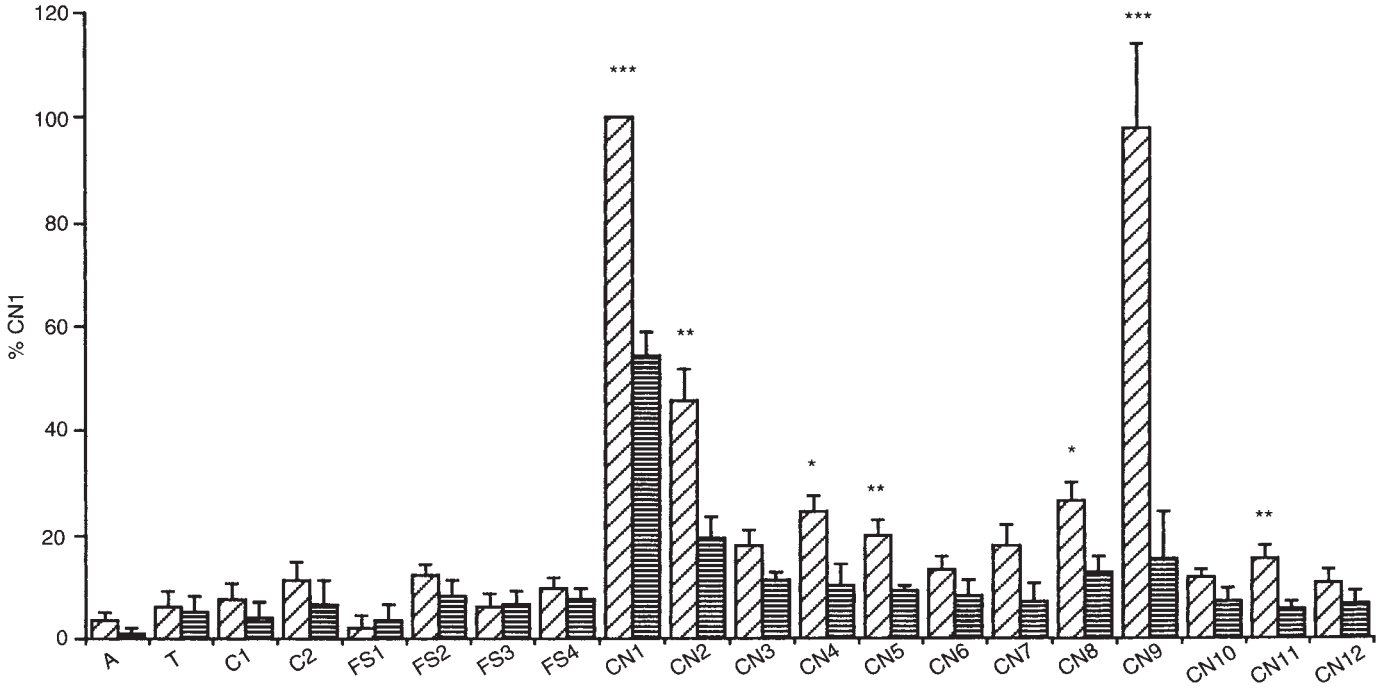


Fig. 3. *Reactivity of light chains with Tamm-Horsfall protein (THP).* Wells coated with THP were incubated with biotinylated albumin (A), transferrin (T), and light chains purified from 2 control (C1, C2), 4 Fanconi's syndrome (FS1 to FS4), and 12 cast nephropathy (CN1 to CN12) patients. These proteins were added simultaneously to the same microplate at the concentration of 2.5 μg/100 μl, in the absence (▨) or in the presence (▤) of an excess (×100) of the nonbiotinylated protein (see *Methods* for details). Results are the mean ± SEM of 6 experiments, and are expressed as the percent of binding of the light chain with maximum reactivity (CN1). Specificity of binding was assessed by statistical comparison of values obtained in the absence and in the presence of an excess of the nonbiotinylated protein. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

but contrary to light chains from patients with the Fanconi's syndrome, this fragment disappeared when digestion was prolonged over 24 hours. Only one of the four patients (CN9) exhibited crystals in casts and proximal tubule epithelial cells. (iii) Cast nephropathy light chains did not show self-reactivity, and none of them reproduced the pattern of reactivity of FS light chains with FS2, CN2 and CN3. (iv) Seven out of the 12 light chains showed significant reactivity with THP, in keeping with previous data by Sanders et al [16, 20, 21] supporting a role for coaggregation of human light chains with THP in cast formation. The same authors demonstrated that heterotypic aggregation of THP and light chain was promoted by a common peptide segment of THP, but the carbohydrate moiety of THP was also required, perhaps facilitating homotypic aggregation of the glycoprotein [20]. When separation of monomers and dimers could be achieved (CN9), reactivity with THP of the dimer was higher than that of the monomer, as was reactivity with other light chains (not shown); this may point to the role of a pseudo-antibody formed by association of light chain variable domains.

That 5 of 12 cast nephropathy light chains did not react with THP by ELISA may be explained by low affinity of certain light chains [20], but also suggests heterogeneity of pathogenetic mechanisms leading to cast formation. Myeloma casts occasionally do not stain for THP in human biopsies [22]. Moreover, in some rat experimental studies, casts induced by human myeloma light chains did not contain THP for the first 24 hours after injection [14]. These observations suggest that some light chains may undergo homotypic aggregation or precipitation in the absence of THP, as shown *in vitro* by Myatt et al [23]. On the other hand, since in all studied cases of cast nephropathy, the light chains displayed a complete or partial resistance of either the entire molecule or a fragment thereof to at least one protease, we hypothesize that resistance to light chain catabolism by urinary and macrophage-released proteases might be a pathogenic factor. In contrast, all tested control light chains in this and our previous study [8] were completely digested by proteases.

It is worth noting that in patient CN9, who presented with cast nephropathy and features of the Fanconi's syndrome including accumulation of crystals inside proximal tubule epithelial cells, the light chain exhibited both partial resistance of a 12 kDa fragment to cathepsin B and the highest reactivity with THP. Such boundary forms of myeloma-associated tubulopathies were already described morphologically by Pirani and associates [9], who found crystals in proximal tubule cells in 5 of 24 cast nephropathies. Our data may provide a biochemical basis to these observations. It is also of interest that, at variance with the four patients with "pure" Fanconi's syndrome, CN9 had an overt myeloma with 65% plasmocytes and a high tumor burden.

Cast nephropathy appears to be an heterogeneous entity as far as clinical, pathological [9, 24, 25] and molecular aspects are concerned. Its pathogenesis is complex, involving intrinsic properties of urinary light chains, concentration and carbohydrate content of THP [16], and extrinsic precipitants which modify the distal nephron milieu [26]. Elucidation of the different pathogenic factors in correlation with pathological forms would require studies on a larger group of patients. In contrast, myeloma-associated Fanconi's syndrome is a rather homogeneous entity characterized by prolonged resistance of the light-chain variable

region to proteolysis whose consequences need to be further investigated at the cellular level.

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