Complete Primary Sequences of Two λ Immunoglobulin Light Chains in Myelomas with Nonamyloid (Randall-Type) Light Chain Deposition Disease

Catherine Decourt,* Guy Touchard,[†]
Jean-Louis Preud'homme,[‡] Ruben Vidal,[§]
Hélène Beauvils,[¶] Marie-Claude Diemert,^{||} and
Michel Cogné* **

From the Laboratoire d'Immunologie,* Centre National de la Recherche Scientifique (CNRS) EP118, Faculté de Médecine, and Institut Universitaire de France,** Limoges, France; Département de Néphrologie,[†] Centre Hospitalier Universitaire La Milètrie, Poitiers, France; Laboratoire d'Immunologie,[‡] CNRS ESA 6031, CHU La Milètrie, Poitiers, France; Service d'Anatomie Pathologique,[§] INSERM U423 Hôpital Pitié-Salpêtrière, Paris; Service d'Immunochimie,[¶] Hôpital Pitié-Salpêtrière, Paris, France; and Department of Pathology,^{||} New York University Medical Center, New York, New York

We herein report on the first two primary sequences (BOU and RAC) of monoclonal light chains of the λ type responsible for nonamyloid λ light chain deposition disease. Both patients were affected with severe forms of myeloma complicated with renal failure. The pathological presentation typically featured Congo red-negative deposits along tubular basement membranes but differed somewhat from the typical "Randall-type" κ light chain deposition disease: they lacked the prominent glomerulosclerosis pattern often featuring nonamyloid κ deposits and were associated with cylinders or myeloma casts. Both protein sequences were deduced from those of the corresponding complementary DNAs in the bone marrow plasma cells. For each chain, products of three independent amplifications by polymerase chain reaction were sequenced and found to be identical. BOU and RAC λ mRNAs had a normal overall structure consisting of V λ 2 segments rearranged to J λ 2C λ 2 but displayed a number of unusual features within their primary sequences. These substitutions are likely responsible for changes in light chain conformation that promote their aggregation and deposition along renal tubule basement membranes. (*Am J Pathol* 1998, 153:313–318)

A number of diseases may result from monoclonal immunoglobulin (Ig) tissue deposition complicating malignant

or benign monoclonal B cell proliferations. These diseases are characterized by multivisceral Ig-related material deposits, most often corresponding to monoclonal light chains (LCs) or LC fragments and usually predominating in the kidney. The two most frequent pathological presentations are AL-amyloidosis and nonamyloid (Randall-type) LC deposition disease (LCDD): AL-amyloidosis deposits usually predominate in the glomerular mesangium and in arteriolar walls and mostly involve λ LCs highly organized in β -pleated sheet fibrils. By contrast, LCDD deposits (mostly of the κ type) are amorphous, predominate along the outer part of basement membranes in the distal tubule and the loop of Henlé, and often associate with marked nodular glomerulosclerosis.^{1–14} To date, many LCs implicated in AL-amyloidosis have been described, and a large amount of sequence data has allowed the formulation of hypotheses concerning the special role of some amino acid substitutions in the process of fibril formation.^{15–20} By contrast, only a few LCDD chains have been studied, all of the κ type,^{4,5,7–9,11–13} and the process by which LCs aggregate is still largely unknown.

We herein report on the first two λ LCs (BOU and RAC) implicated in nonamyloid LCDD. BOU and RAC belonged to the V λ 2 subgroup and included J λ 2/C λ 2 segments. In both cases, the renal lesions differed somewhat from those seen in most cases of κ nonamyloid deposits, because they did not reveal prominent glomerulosclerosis. In addition, for both patients, deposits along the tubular membrane basements were accompanied by tubular lesions resembling cast myeloma.

Supported by grants from the Association pour la Recherche sur le Cancer (Grant 9121), Fondation contre la Leucémie, Ligue Nationale contre le Cancer, and Conseil Régional du Limousin and by National Institutes of Health Grant AR02594 to Dr. Blas Frangione. CD is a recipient of a fellowship from the Association pour la Recherche sur le Cancer.

Accepted for publication April 15, 1998.

Address reprint requests to Michel Cogné, Laboratoire d'Immunologie CNRS EP118, Faculté de Médecine, 2 rue du Dr Marcland, 87025 Limoges, France.

Figure 1. A: Immunofluorescence study of a kidney biopsy from patient BOU showing linear and diffuse staining of the tubular basement membranes and heavy staining of a myeloma cast (original magnification, $\times 800$). B: Light microscopy of periodic acid-Schiff-stained kidney from patient RAC showing thickening of the tubular basement membrane with a hyaline eosinophilic material (top center) and epithelial necrosis or vacuolation of other tubules (original magnification, $\times 450$). C: Immunofluorescence study of kidney from patient RAC with an anti-human λ LC conjugate, showing a strong fluorescence along tubular basement membranes (original magnification, $\times 450$).

Patients and Methods

Patients

Patient *BOU*, a 59-year-old French man, was referred in September 1993 for renal failure with anemia (hemoglobin, 8 g/100 ml). The creatininemia was 750 $\mu\text{mol/L}$, and proteinuria was abundant (8 g/24 hours) with a predominant λ Bence-Jones protein that was also detectable in serum in small amounts. Hypo- γ -globulinemia was marked (4 g/L). A moderate microscopic hematuria was found. Bone marrow smears showed 50% plasma cells. Light microscopic examination of a kidney biopsy showed myeloma cast nephropathy. Eighteen glomeruli were normal without nodular sclerosis. The interstitium was focally infiltrated by lymphocytes and plasma cells. Congo red staining was negative. Immunofluorescence study (Figure 1A) showed linear deposits along the tubular basement membranes, which only stained for λ chain. Electron microscopic study of a renal medulla fragment was essentially normal and did not show granular osmophilic deposits along tubular basement membranes.

From October 1993 to December 1993, the patient was given nine courses of plasmapheresis and two courses of vincristine (0.4 mg) and Adriamycin (9 mg/m²) combined the first day with prednisolone (1 mg/kg), which was continued for 4 further days. Despite this treatment, hemodialysis was required, and the patient died in December 1993 with 5% leukemic plasma cells.

Patient *RAC*, a 48-year-old Guyanese woman, was referred in September 1996 for persistent renal failure 2 months after a *Plasmodium falciparum* infection. The creatinine plasma level was 547 $\mu\text{mol/L}$, and there was an abundant proteinuria (7 to 9 g/24 hours) essentially consisting of monoclonal free λ chains that were also detectable in serum by immunofixation. There was no monoclonal IgD or IgE by immunofixation, and serum polyclonal Ig levels were moderately affected (9.7 g/L of IgG, 0.3 g/L of IgA, and 0.2 g/L of IgM). Bone marrow smears showed 13% dystrophic plasma cells and allowed the diagnosis of myeloma. Light microscopic examination of a kidney biopsy mainly showed severe and diffuse tubular lesions with a moderate inflammatory cell infiltrate (Figure 1B). Some tubules were lined by a flattened epithelium; other tubules were obstructed and dilated by proteic casts; some tubular basement membranes were enlarged with

Congo red-negative material; nodular glomerulosclerosis was not observed. By immunofluorescence (Figure 1C), tubular basement membrane deposits only stained with anti- λ LC antibodies and not with other anti-Ig chains or anti-complement antisera.

The patient was included in a protocol composed of two autologous bone marrow transplantations. One month after the first transplantation (January 1997), creatininemia dropped to 159 $\mu\text{mol/L}$ and proteinuria to 0.39 g/24 hours. The second transplantation was performed in April 1997; 2 months later the serum creatinine level was 120 $\mu\text{mol/L}$ and the proteinuria 0.26 g/L, without free LC detectable in the serum. However, 10 months later, a severe relapse occurred with a heavy plasma cell infiltrate in bone marrow and with high levels of free λ LC in urine.

RNA Preparation and Reverse Transcription-Polymerase Chain Reaction (PCR) Experiments

Total RNA was prepared by lysis of bone marrow cells in 4 mol/L guanidine isothiocyanate followed by centrifugation at $170,000 \times g$ for 18 hours on a 5.7 mol/L cesium chloride pad. Total RNA was analyzed on a 1% agarose, 1.7 mol/L formaldehyde gel in comparison with RNA from the human lymphoma cell line IARC 518 producing normal-sized κ mRNA and from the plasmacytoma cell line RPMI 8226 producing a normal-sized λ mRNA.²¹ They were transferred to nylon sheets and hybridized with either a C κ probe, a 2.5-kb *EcoRI* genomic fragment containing the human C κ exon, or a C λ probe, a 3.5-kb *EcoRI*-*HindIII* fragment containing the human C λ 2 exon. Total RNA was used as a template for synthesizing single-stranded cDNA using reverse transcriptase and an oligodeoxythymidylic acid primer (Boehringer Mannheim, Mannheim, Germany). PCR primers were a 5' primer corresponding to a V λ 1-V λ 2-V λ 3 consensus leader region (5'-ATGGCCKGSWYYSYTCTCCTC-3') and a 3' primer complementary to the consensus upstream part of the C λ exons (5'-CTCCCGGGTAGAAGTCACT-3'). Amplification of the cDNAs by PCR was performed with Taq polymerase (Pharmacia, Uppsala, Sweden) through 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds, and elongation at 72°C for 30 seconds.²² After amplification, PCR products were

fractionated on 1.2% agarose gels and sequenced by the dideoxy termination method,²³ using Taq polymerase and an automated laser fluorescent DNA sequencer (Perkin-Elmer, Branchburg, NJ).

Protein Analysis

Serum and urinary proteins were analyzed by conventional agarose gel zonal electrophoresis in nondenaturing conditions and by immunofixation.

For patient *RAC*, urinary proteins were purified using diethylaminoethyl-Trisacryl chromatography in 10 mmol/L Tris, pH 8.0, on a 0 to 0.3 mol/L sodium chloride gradient, followed by a second chromatography on Sephadex G100 in 0.1 mol/L Tris-0.5 mol/L NaCl, pH 8.0. An electrophoresis in nondenaturing conditions then showed that the protein was mainly present in dimeric form.

Purified urinary *RAC* protein (3 mg) was dissolved in 1 ml of 0.2 mol/L NH_4CO_3 and digested for 1 hour at 37°C with trypsin (modified, sequencing grade; EC 3.4.21.4) (Boehringer Mannheim) at an enzyme/protein ratio of 1:100 (w/w).

Proteolysis was terminated by freezing and freeze-drying. The resulting peptides were separated by reverse-phase high-performance liquid chromatography using a Vydac C18 (The Separations Group, Hesperia, CA) column (218TP52; 0.21 $\times$ 25 cm) and a 60-minute 0 to 60% linear gradient of acetonitrile-water (pH 2.1) at a flow rate of 0.2 ml/min. Automated Edman degradation sequence analysis of purified peptides was carried out on a 477A protein-peptide sequencer, and the resulting phenylthiohydantoin amino acid derivatives were identified using the online 120A PTH analyzer (Applied Biosystems, Foster City, CA).

Results

Using RNA extracted from bone marrow cells in two cases of myeloma-associated LCDDs, we studied λ LC mRNAs by Northern blotting: blots hybridized with a $\text{C}\lambda$ probe gave a strong signal at the expected correct size in both cases, although hybridization with a $\text{C}\kappa$ probe yielded no signal in the lanes corresponding to myeloma samples (Figure 2). These hybridization data indicated that the B cell population in both bone marrow samples mainly corresponded to the monoclonal λ -producing cells.

In both cases, three independent reverse transcription-PCR amplifications were performed, and direct sequencing of PCR products yielded identical nucleotide sequences for all three independent amplification experiments. In addition, protein sequence analysis of urinary *RAC* tryptic peptides allowed the identification of peptide sequences (+9 $\rightarrow$ +21, +45 $\rightarrow$ +60, +66 $\rightarrow$ +88, and +156 $\rightarrow$ +166), which fell in complete agreement with the protein sequence deduced from *RAC* cDNA. From *BOU* and *RAC* cDNA nucleotide sequences, we could deduce complete amino acid sequences and assign both LCs to the $\text{V}\lambda 2$ subgroup. In both cases, the V region was normally rearranged to the $\text{J}\lambda 2\text{C}\lambda 2$ segment, which did not

Figure 2. Northern blot analysis of total bone marrow cell RNA from patients *BOU* and *RAC*. Total RNA from *BOU* (lane 1) and *RAC* (lane 2) were hybridized either with a $\text{C}\lambda$ probe or a $\text{C}\kappa$ probe, in comparison with RNA from the IARC 518 cell line, an IgA κ producer (lane 3), and RPMI 8226 cell line, a λ chain producer (lane 4).

present any sequence abnormality. On the contrary, comparison of $\text{V}\lambda$ sequences with previously reported λ chains pointed out several unusual features consisting of the appearance of residues never or rarely found at these positions (Figure 3).

In protein *BOU*, several substitutions unique among $\text{V}\lambda 2$ proteins were noticed: Ser+2 $\rightarrow$ Ala, Val+27F $\rightarrow$ Leu, Leu+46 $\rightarrow$ Ile, Ile+48 $\rightarrow$ Leu, Ser+65 $\rightarrow$ Phe, Ser/Cys+89 $\rightarrow$ Gly, Ser+90 $\rightarrow$ Leu, Ala+92 $\rightarrow$ Val, Ser/Asn+95 $\rightarrow$ Arg, and Thr95A $\rightarrow$ Leu. Some other substitutions have been rarely observed among $\text{V}\lambda 2$ proteins: Ala+43 $\rightarrow$ Val and Thr+70 $\rightarrow$ Ala. Two other rare substitutions were encoded at the VJ junction (Val+96 $\rightarrow$ Trp) and within $\text{J}\lambda 2$ (Lys103 $\rightarrow$ Arg). Another feature of the *BOU* sequence is a three-codon deletion within CDR1 involving codons +28 to +30.

In protein *RAC*, unique substitutions included Thr+5 $\rightarrow$ Val, Pro+7 $\rightarrow$ Leu, Thr/Ser+27 $\rightarrow$ Gly, Ser+29 $\rightarrow$ Thr, Val+33 $\rightarrow$ Leu, Ile+48 $\rightarrow$ Leu, Thr+70 $\rightarrow$ Ser, and Ala+84 $\rightarrow$ Gly.

Rare substitutions included Gly+28 $\rightarrow$ Thr, Asn+31 $\rightarrow$ Lys, and Lys+42 $\rightarrow$ Ile. Two other rare substitutions were common with *BOU*, Val+96 $\rightarrow$ Trp at the VJ junction and Lys103 $\rightarrow$ Arg within $\text{J}\lambda 2$.

Discussion

A dozen monoclonal LCs involved in LCDDs, all of the κ type and with an overrepresentation of the $\text{V}\kappa 4$ subgroup, have been sequenced to date.^{4,5,7-12} We herein describe two λ chains forming amorphous deposits in patients' kidneys. The *BOU* and *RAC* LCs were responsible for nonamyloid LC deposits stacked to tubular membrane basements; both cases lacked the prominent glomerulosclerosis that often features Randall-type κ nonamyloid LCDD. Indeed, it has been previously noticed that morphological changes were often less severe and nodular sclerosis was less frequent in λ LCDD than in κ LCDD

+1										+17									
BOU	CAG	GCT	GCC	CTG	ACT	CAG	CCT	GCC	TCC	GTG	TCT	GGT	TCT	CCT	GGA	CAG			
RAC	---	T---	---	T---	GT---	---	T---	CG---	---	A---	---	---	---	---	---	---			
BOU	Gln	Ala	Ala	Leu	Thr	Gln	Pro	Ala	Ser	Val	Ser	Gly	Ser	Pro	Gly	Gln			
	(Ser)																		
RAC	-	Ser	-	-	Val	-	Leu	Arg	-	-	-	-	-	-	-	-			
					(Thr)		(Pro)												
+18										+27 27D 27E 27F +28 +30									
BOU	TCG	ATC	ACC	ATT	TCG	TGC	ACT	GGA	ACC	TCC	AAT	AAT	CTT	...	...	...			
RAC	---	A---	---	---	---	---	---	---	---	GG---	---	G---	G---	ACC	ACT	TAT			
BOU	Ser	Ile	Thr	Ile	Ser	Cys	Thr	Gly	Thr	Ser	Asn	Asn	Leu	...	...	...			
											(Ser Asp Val Gly Ser Tyr)								
RAC	-	Val	-	-	-	-	-	-	-	Gly	Ser	Asp	Val	Thr	Thr	Tyr			
										(Ser)				(Gly Gly)					
+31										+46									
BOU	CAT	TTC	GTC	TCC	TGG	TAC	CAA	CAA	CAC	CCA	GGC	AAA	GTC	CCC	AAA	ATC			
RAC	A-A	-A-	C-	---	---	---	---	---	---	---	---	---	T-	-C-	---	C-			
BOU	His	Phe	Val	Ser	Trp	Tyr	Gln	Gln	His	Pro	Gly	Lys	Val	Pro	Lys	Ile			
	(Asn Leu)												(Ala)		(Leu)				
RAC	Lys	Tyr	Leu	-	-	-	-	-	-	-	-	Ile	Ala	-	-	Leu			
	(Asn)	(Val)										(Lys)							
+47										+62									
BOU	CTT	CTT	TAT	GAG	GTC	TCT	AAG	CGG	CCC	TCA	GGG	GTT	TCT	AAT	CGC	TTC			
RAC	A-A	---	---	---	---	AA-	---	---	---	---	---	---	---	---	---	---			
BOU	Leu	Leu	Tyr	Glu	Val	Ser	Lys	Arg	Pro	Ser	Gly	Val	Ser	Asn	Arg	Phe			
	(Met Ile)				(Gly)														
RAC	Ile	-	-	Asp	-	Asn	-	-	-	-	-	-	Pro	-	-	-			
	(Met Ile)					(Ser)							(Asp)						
+63										+78									
BOU	TCC	GGC	TTC	AAG	TCT	GGC	AAC	GCG	GCC	TCC	CTG	ACA	ATC	TCT	GGA	CTC			
RAC	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---			
BOU	Ser	Gly	Phe	Lys	Ser	Gly	Asn	Ala	Ala	Ser	Leu	Thr	Ile	Ser	Gly	Leu			
		(Ser)					(Thr)												
RAC	-	-	Ser	-	-	-	-	-	-	-	-	-	-	-	-	-			
							(Thr)												
+79										+94									
BOU	CAG	TCT	GAG	GAC	GAG	GCT	GAC	TAT	TAC	TGC	GGC	TTA	TAT	GTC	GGT	GAT			
RAC	---	G---	---	---	---	---	---	---	---	---	---	---	---	---	---	---			
BOU	Gln	Ser	Glu	Asp	Glu	Ala	Asp	Tyr	Tyr	Cys	Gly	Leu	Tyr	Val	Gly	Asp			
	(Ala)									(Cys Ser)	(Ala)	(Ser)							
RAC	-	Ala	Asp	-	-	Gly	-	-	-	-	Cys	Ser	-	Thr	-	Thr			
		(Glu)			(Ala)									(Ala)	(Ser)				
+95 95A										+108									
BOU	AGA	CTT	TGG	GTG	TTT	GGC	GGA	GGG	ACC	AGG	TTG	ACC	GTC	CTA	GGT	CAG			
RAC	TCC	AC-	---	---	---	---	---	---	---	---	---	---	---	---	---	---			
BOU	Arg	Leu	Trp	Val	Phe	Gly	Gly	Thr	Arg	Leu	Thr	Val	Leu	Gly	Gln				
	(Ser Tyr Phe)																		
RAC	Ser	Thr	Trp	-	-	-	-	-	-	Arg	-	-	-	-	-	-			

Figure 3. Comparison of λ chains BOU and RAC. The nucleotide sequences are indicated together with the deduced amino acid sequences. Dashes indicate identities, points indicate deletions, unique residues are underlined with continuous lines, and rare residues are underlined with broken lines. Residues normally encoded by the most related germline V λ 2 segments, V1-7 and DPL12, are indicated in parentheses below the peptide sequences of BOU and RAC, respectively. Positions are numbered according to Kabat et al.²⁸

(13% versus 49% among LCDD cases reported in the literature).¹² Deposits evidenced only by immunofluorescence, without gross morphological alterations of the kidney structures, have also been reported in an experimental model of κ LCDD that represented an initial step of the disease.²⁴ The constellation of LCDD and myeloma casts that we report in patient BOU also appear to be unusual and was only present in 2 of 24 patients with LCDD in the study of Strøm et al.¹²

Strikingly, both chains are related to the same V λ 2 subgroup²⁵ and encoded by a V λ segment normally rearranged to J λ 2 and spliced to C λ 2.²⁶ However, given their numerous differences, BOU and RAC nucleotide sequences likely derived from two different members of the V λ 2 gene subgroup: BOU is mostly related to the V λ gene V1-7 (90.8% identity),²⁷ whereas RAC is mostly related to DPL12 (90.5% identity).²⁵ None of them encodes any potential N-glycosylation site, but they display a number of features unique among V λ 2 chains: RAC and BOU share a few amino acid substitutions, and both

display several replacements introducing hydrophobic residues.

The BOU sequence harbors several unusual features within the variable region, both in framework (FR) and in complementary determining regions (CDRs). A striking feature of BOU is a three-residue deletion within the CDR1 between the +28 and +30 positions (according to the numbering of Kabat et al.²⁸), which had never been previously found in any λ 2 chain. This three-codon deletion shortens the L1 binding loop and may change its conformation, which is normally helical in λ chains.²⁹ Apart from the CDR1, important changes were also noticed in the CDR3 region, with four unique and two rare amino acid substitutions. In particular, Ser+90→Leu, Ala+92→Val, Ser/Asn+95→Arg, and Thr+95a→Leu may disturb the CDR3 structure by strongly increasing the hydrophobicity of the L3 binding loop.²⁹ As for the FR regions, unique or rare substitutions altogether increasing hydrophobicity include: Ser+2→Ala in the FR1, Ala+43→Val in the FR2, and Ser+65→Phe and Thr+70→Ala in the FR3. The Leu+46→Ile and Ile+48→Leu unique substitutions probably have a limited effect on the protein structure. However, it is noticeable that the Ile+48→Leu substitution is common to BOU and RAC and affects an invariant Ile supposedly implicated in the maintenance of the L2 loop conformation.²⁹ The presence in both proteins of an Asn+60 residue, germinally encoded in BOU and resulting from a substitution in RAC, may also be destabilizing: most LCs carry a negatively charged residue at this position, in the vicinity of a critical Arg+61 to Asp+82 salt bridge usually stabilizing V domains.³⁰ Two other unusual features common to BOU and RAC are the presence of a Trp+96 residue at the end of the CDR3 encoded at the VJ junction and a Lys+103→Arg substitution in the FR4; strikingly, a solvent-exposed Trp features all κ LCDD proteins sequenced to date.

All of the other unusual features of protein RAC differ from those of BOU. Although the CDR2 and CDR3 were almost completely canonical, the CDR1 presents three unique substitutions: Ser/Thr+27→Gly; Gly/Ser+29→Thr; and Val+33→Leu and two rare residues, Thr+28 and Lys+31. Hydrophobic amino acids appear in the FR1, Thr+5→Val and Pro+7→Leu, the latter affecting an invariant Pro residue and likely destabilizing the FR1 conformation. Finally, the RAC chain presents an additional Lys+42→Ile substitution in the FR2 and two substitutions within the FR3: Thr+70→Ser and Ala+84→Gly.

The herein reported BOU and RAC λ chain sequences are the first to be documented in Randall-type LCDD, in which monoclonal κ chains strongly predominate. The presence of V region peculiarities in LC BOU and RAC was expected from previous sequence analysis of LCDD κ chains^{4,5,7,8,11,12} and from direct evidence for the involvement of V region abnormalities obtained in a murine model of human κ LCDD.²⁴ It is striking that the herein reported λ chains are closely related. By analogy to the strong implication of λ 6 chains in AL-amyloidosis, it may be tempting to speculate that germinally encoded residues of some V λ 2 domains may promote LC aggregation and deposition. However, it is known that the V λ 2 sub-

group is overexpressed in myeloma and Waldenström macroglobulinemia (28% of monoclonal Ig λ , instead of 3% of Ig λ from normal sera),^{31,32} and it is clear that not all λ 2 chains are responsible for LCDD. In addition, a dozen AL-amyloidosis cases implicating V λ 2 subgroup LC have been reported.^{31,33–35} A likely hypothesis is thus that the λ 2 germinally encoded sequences may somehow favor LC aggregation (and in some instances lead to amyloidosis) but that a definitive role in the process of tissue deposition in patients *BOU* and *RAC* is played by specific amino acid replacements resulting from somatic mutations and facilitating hydrophobic interactions between LC monomers or dimers. Such replacements would further promote the destabilization and deposition of LC. It is indeed striking that *BOU* and *RAC* λ chains carry a number of unusual replacements, several of them introducing hydrophobic residues. Although crystallization of the proteins will be needed for a definitive assignment of substitutions to solvent-exposed portions of the molecules, several such hydrophobic residues located in the amino-terminal part of the molecule (Ala+2 in *BOU* and Val+5 and Leu+7 in *RAC*) or in CDR regions (Val+51, Leu+90, Val+92, and Leu+95A in *BOU*, and Leu+33 in *RAC*) are likely to altogether increase hydrophobicity at the surface of both LCs and may play a destabilizing role. Hydrophobic residues may participate in interchain hydrophobic interactions leading to aggregation and thus promote tissue deposition. Similarly, substitutions introducing hydrophobic residues in solvent-exposed portions of LCs have been previously pointed out for several κ LCs implicated in LCDDs^{4,8,11,12} as well as for LCs involved in amyloid fibril formation.^{15–17} These new sequences extend the short list of characterized LCs involved in LCDDs cases and will hopefully help to understand the process by which LCs of decreased solubility or stability can interact, aggregate in high-order polymers, and form deposits in tissues. It also appears likely that structural properties of a given LC are directly correlated to the extent of visceral deposition occurring in the patient and to the prognosis. In that sense, the herein reported λ LCDD cases with high-level secretion of the LC and minimal pathological alterations strikingly contrast with many reported κ LCDD cases in which low or even undetectable secretion of the LC is associated with extensive visceral deposition and renal morphological alterations.^{5,7–9,12–14}

Acknowledgment

We thank Dr F. Stevens for helpful comments on λ LC sequences.

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