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Pathogenic potential of human monoclonal immunoglobulin light chains: Relationship of *in vitro* aggregation to *in vivo* organ deposition

(amyloidosis/Bence Jones proteins/multiple myeloma)

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ABSTRACT **The deposition of certain Bence Jones proteins as tubular casts, basement membrane precipitates, or amyloid fibrils results in the human light-chain-associated renal and systemic diseases—myeloma (cast) nephropathy, light-chain deposition disease, and immunocyte-derived (primary or AL) amyloidosis. To determine if light-chain nephrotoxicity or amyloidogenicity is related to the propensity of these components to form high molecular weight aggregates under physiological conditions, we used a size-exclusion chromatographic system to study 40 different Bence Jones proteins. Each sample was tested over a wide range of protein concentration in three different buffers varying in pH, osmolality, and the presence or absence of low concentrations of urea. Thirty-three of the 35 proteins found clinically and/or experimentally to form *in vivo* pathologic light-chain deposits were shown to undergo high-order self-association and form high molecular weight aggregates. In contrast, of five nonpathologic proteins, one showed polymerization under the chromatographic conditions used. The correlation between the *in vitro* results achieved by size-exclusion chromatography and that found *in vivo* provides (*i*) a rapid diagnostic method to identify potential nephrotoxic or amyloidogenic Bence Jones proteins and (*ii*) an experimental means to gain new insight into the physicochemical basis of light-chain aggregation and the treatment of those invariably fatal disorders associated with pathologic light-chain deposition.**

The human light-chain-related renal and systemic diseases—myeloma (cast) nephropathy, light-chain deposition disease, and immunocyte-derived (primary or AL) amyloidosis—result from the pathologic deposition of monoclonal light chains (i.e., Bence Jones proteins) in the form of casts, basement membrane precipitates, or fibrils, respectively (1). These light-chain deposits ultimately result in the impairment of renal and other organ function and account for much of the morbidity and mortality found in patients with these disorders. The fact that pathologic light-chain deposits are not an invariant accompaniment of clinical or experimental (1) Bence Jones proteinuria and are not necessarily directly related to the amount of monoclonal light chain synthesized or excreted implies that certain light chains are inherently nephrotoxic or amyloidogenic.

Several *in vitro* and *in vivo* models have been devised that provide an experimental means to assess the pathologic potential of Bence Jones proteins (2–5). For example, in one model we demonstrated that the injection into mice of certain Bence Jones proteins resulted in the deposition in the mouse kidney of the human proteins in the form of tubular casts, basement membrane precipitates, crystals, or amyloid fibrils (6). Through studies involving >40 different Bence Jones proteins, we found that the renal lesions induced experimentally were comparable to those of patients from whom the proteins were derived and that the experimental mouse model was capable of differentiating "nephrotoxic" from "nonnephrotoxic" Bence Jones proteins. These studies provided further evidence that the Bence Jones protein itself is primarily responsible for producing the distinctive types of deposition that occur in the light-chain-associated diseases.

The specific clinical or structural features that distinguish "pathologic" from "nonpathologic" light chains are presently unknown. We have previously postulated that Bence Jones proteins form casts, precipitates, or fibrils as a result of light-chain variable-domain (V_L) interactions that progress to insoluble aggregates (7). To determine the self-association properties of human light chains and to provide experimental data in support of this hypothesis, we applied the technique of size-exclusion chromatography to analyze a large number of structurally homologous Bence Jones proteins. Our studies demonstrated that the elution profile of these components was determined by their compositional nature—i.e., by the presence of covalent or noncovalent dimers, free monomers, or light-chain-related fragments as well as by the formation of higher-order aggregates resulting from solution-dependent affinities or other types of interactions. The concentration dependence of the elution profiles (i.e., relative decrease of high molecular weight components after dilution of the sample) confirmed the noncovalent nature of the aggregates and demonstrated that, in principle, this *in vitro* technique could be used to analyze quantitatively the affinity and kinetic properties of monoclonal light chains (8, 9).

We have now extended our studies using size-exclusion chromatography to determine the capability of this *in vitro* technique to discriminate between pathologic (nephrotoxic and amyloidogenic) and nonpathologic light chains. Specifically, we tested the propensity of 40 different κ- and λ-type Bence Jones proteins, obtained from patients from whom renal functional and histological data were available [and which were also studied in the *in vivo* mouse model (6)], to form high molecular weight aggregates under specified physiological conditions of pH, salt, and urea concentration that would mimic those found within the nephron. Our studies showed that 33 of 35 clinically and/or experimentally proven nephrotoxic proteins formed noncovalent high molecular weight multimers *in vitro*. In contrast, only one of five nonnephrotoxic proteins aggregated under the experimental conditions used. The correlation between the behavior of the

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Abbreviations: V_L, light-chain variable domain; V_e, elution volume; V_t, total column volume.

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proteins *in vitro* and *in vivo* indicates the potential value of analytical size-exclusion chromatography to differentiate pathologic from nonpathologic human monoclonal light chains and to gain new insight into the pathogenesis of the human light-chain-associated renal and systemic diseases.

MATERIALS AND METHODS

Protein Preparation and Characterization. Bence Jones proteins were isolated and purified from the urine of patients with multiple myeloma or AL amyloidosis as described (10). The molecular composition of the proteins was determined on SDS/8–25% polyacrylamide gels in both the presence and absence of 2-mercaptoethanol by using the Phast system (Pharmacia LKB). The κ or λ isotype and the V_L subgroup of these monoclonal light chains were determined serologically by using polyclonal anti-light-chain antisera (10).

Assessment of Light-Chain-Related Pathology. The nature of clinical or experimentally induced light-chain deposits was determined in hematoxylin/eosin- and Congo red-stained biopsy or autopsy specimens by light and polarizing microscopy, respectively, and immunohistochemically by the immunoperoxidase method (6).

Size-Exclusion Chromatography. Chromatography experiments were performed at room temperature as follows. Superose 12 (Pharmacia LKB) was packed into 0.3-cm × 20- or 25-cm columns (Alltech Associates). Three different buffer solutions were used: buffer 1 was 50 mM sodium phosphate/0.10 M NaCl, pH 7.2 (PBS); buffer 2 was 50 mM sodium phosphate/0.4 M NaCl/0.4 M urea, pH 6.5 (urea buffer); and buffer 3 was 30 mM sodium acetate/0.245 M NaCl, pH 4.5 (acetate buffer). The buffers were delivered to the column at 0.06 ml/min with an LKB 2150 pump. Protein samples ranging in concentration from 0.02 to 8.0 mg/ml were injected in a volume of 5 μl, and the eluent was monitored simultaneously at 214 and 280 nm by an HP 1040 multiscan detector (Hewlett–Packard) during runs of 30 or 35 min. The data were collected and stored as described (9, 11). Chromatograms were normalized by summation of the absorbances at 1000 data points collected during the run and by scaling the data so that the integrated area under the elution profile was equal to 1.

In the absence of a quantitative method to estimate the multiple association constants that characterize high-order aggregation of light chains, and because of the heterogeneity of the samples obtained from the 40 patients, a subjective scoring of aggregation tendency was adopted for this study. No apparent concentration-dependent aggregation was scored as 0; a discernable forward shift of the dimer peak, as "+"; aggregation that led to elution of protein in a continuum from the excluded volume to the dimer position, as "+++"; and intermediate elution behavior, as "++". Because enhanced aggregation under any one of these conditions might enhance *in vivo* pathological tendency, the maximal observed aggregation state is reported.

RESULTS

SDS/Polyacrylamide Gel Electrophoresis. The molecular form of each Bence Jones protein studied was determined by SDS/polyacrylamide gel electrophoresis and gel filtration (data not shown). Each sample was free of high molecular weight contaminants that would account for aggregates observed by size-exclusion chromatography. Typically, λ light chains were found predominantly as covalent dimers, and κ chains as mixtures of predominately monomers, some covalent dimers, and, occasionally, fragments corresponding in molecular weight to a single domain. Low molecular weight components in Len and Cag samples were identified serologically as V_L-related fragments (10).

Solution Dependence of Light-Chain Aggregation. In the chromatograms shown in Figs. 1–4, light-chain dimers ($M_r \approx$ 45,000) were eluted at a position corresponding to a V_e/V_t ratio (elution volume/total column volume) of $\approx$0.6, whereas free monomers were eluted at $\approx$0.7. Proteins of $M_r > 200{,}000$ were excluded from the column and were eluted at the void volume position of 0.3.

Fig. 1 depicts the self-association properties of a κIV Bence Jones protein (Len) obtained from a patient who was excreting up to 50 g of this component daily and who had normal renal function despite the unusually high level of protein production. This light chain produced no evident pathology in the mouse model (6). The protein Len sample consisted of a mixture of covalent dimer, free monomer, and V_L fragment with M_r values of 45,000, 22,000, and 12,000, respectively. The light chain monomer and V_L fragment were capable of noncovalent association and were eluted from the column in a concentration-dependent manner corresponding to M_r values of 22,500–45,000 and 12,000–24,000, respectively. When tested at concentrations of 1–2 mg/ml in PBS or urea buffer, intact light-chain Len and the V_L fragment were eluted predominantly at a position close to that of the covalent dimer. Thus, under these conditions, the affinity between the V_L fragments was sufficient to maintain noncovalent association during passage through the chromatogra-

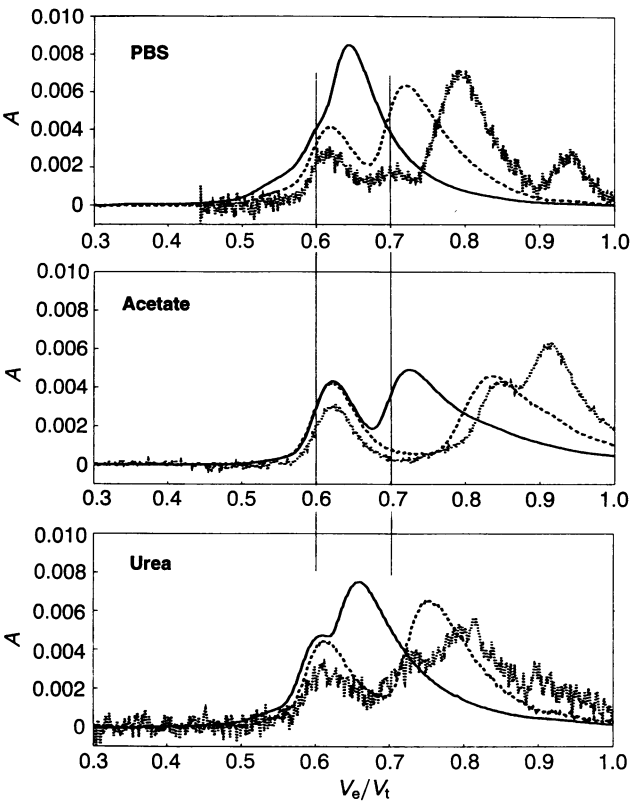

FIG. 1. Size-exclusion chromatograms of a nonnephrotoxic Bence Jones protein. Elution profiles of κIV protein Len in PBS buffer (*Top*) at 2.0 mg/ml (—), 0.2 mg/ml (---), and 0.02 mg/ml (· · ·); in acetate buffer (*Middle*) at 2.0 mg/ml (—), 1.0 mg/ml (---), and 0.2 mg/ml (· · ·); and in urea buffer (*Bottom*) at 1.0 mg/ml (—), 0.1 mg/ml (---), and 0.01 mg/ml (· · ·). Vertical lines at positions 0.6 and 0.7 indicate expected elution positions for the light-chain dimer and monomer, respectively. V_t is calculated from the physical dimensions of the column. The chromatograms in Figs. 1–4 have been normalized to facilitate comparisons of profiles generated over a range of protein concentration; for instance, in *Top*, the peaks at positions at 0.65, 0.62, and 0.62 were approximately 0.5, 0.05, and 0.0013 absorbance units (214 nm) respectively, in the descending concentration series.

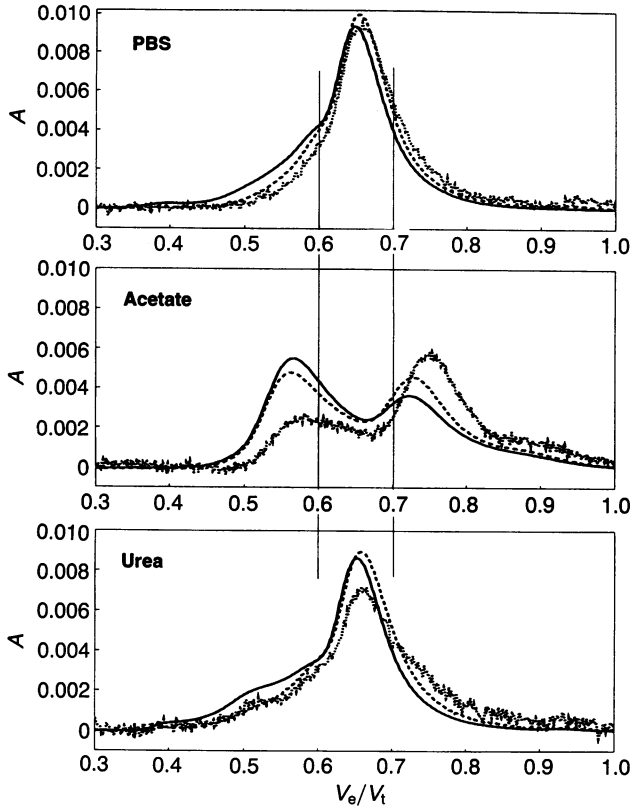

FIG. 2. Size-exclusion chromatograms of a cast-forming Bence Jones protein. Elution profiles of κII protein Cag in PBS buffer (*Top*) at 2.0 mg/ml (—), 0.2 mg/ml (---), and 0.02 mg/ml (· · ·); in acetate buffer (*Middle*) at 3.8 mg/ml (—), 1.0 mg/ml (---), and 0.10 mg/ml (· · ·); and in urea buffer (pH 6.5) (*Bottom*) at 2.0 mg/ml (—), 0.2 mg/ml (---), and 0.02 mg/ml (· · ·).

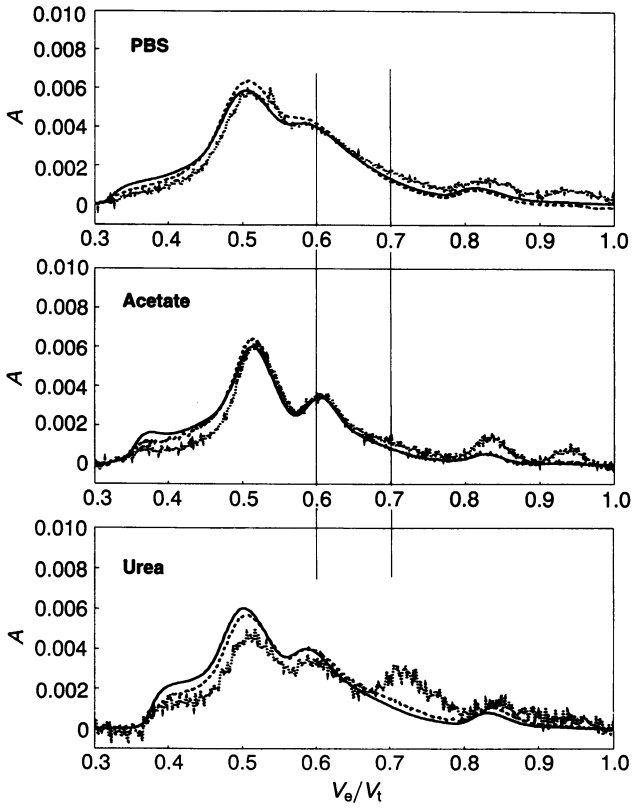

FIG. 3. Size-exclusion chromatograms of an amyloid-associated Bence Jones protein. Elution profiles of λI protein She in PBS buffer (*Top*) at 2.0 mg/ml (—), 0.4 mg/ml (---), and 0.08 mg/ml (· · ·); in acetate buffer (*Middle*) at 2.5 mg/ml (—), 0.2 mg/ml (---), and 0.02 mg/ml (· · ·); and in urea buffer (*Bottom*) at 2.0 mg/ml (—), 0.2 mg/ml (---), and 0.02 mg/ml (· · ·).

phy column. At lower protein concentrations, the three species were clearly resolved.

At acidic pH, the affinity of interaction between the monomeric forms of protein Len was diminished. At a concentration of 2 mg/ml, there was significant resolution of the peaks that corresponded in molecular weight to species that were covalently linked and noncovalently associated. Representative of the majority of "benign" light chains tested, protein Len showed no significant tendency to aggregate (beyond dimerization) under any of the conditions examined.

Under identical chromatographic conditions, a considerably different pattern was found for a κII Bence Jones protein (Cag) that formed tubular casts both clinically and experimentally. Protein Cag exhibited high-order aggregation during chromatography in PBS and the nondenaturing urea buffer, as evidenced by its elution as a continuum ranging from position 0.6 (dimer) to position 0.35 (excluded volume) (Fig. 2). The presence of protein in the void volume indicated the existence of aggregates of $M_r > 200{,}000$. Under these conditions, the elution profiles were relatively insensitive to protein concentration, suggesting high-affinity aggregation. Under acidic conditions, protein Cag had a predominately bimodal elution pattern with a principle elution peak at $V_e/V_t = 0.55$, corresponding to the position of a light-chain tetramer ($M_r \approx 90{,}000$). In contrast to aggregation in PBS and urea buffer, the ratio of tetramer to dimer was highly concentration dependent.

The most extensive aggregation was observed in the elution patterns of an amyloid-associated λI Bence Jones protein (She). Qualitatively, elution profiles of this light chain were the same under the three buffer conditions used (Fig. 3). Protein She exhibited aggregation with the majority of material eluted at positions corresponding to molecular weights much higher than that of a light-chain dimer. Under all three conditions, protein was present at the excluded volume of the column.

Another Bence Jones protein (κI protein Borf) exhibited a small degree of aggregation when examined chromatographically in PBS (Fig. 4). Such multimers were not observed at low pH or in the presence of urea. Patient Borf had multiple myeloma and, despite the excretion of 16 g of Bence Jones protein daily, had normal renal function. When tested in the

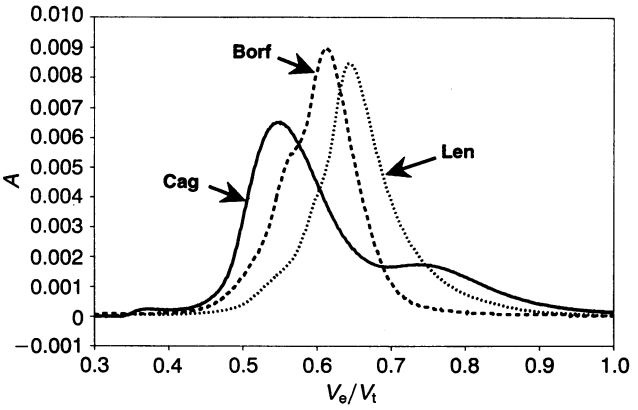

FIG. 4. Comparative elution profiles of the cast-forming κII Bence Jones protein Cag [in acetate buffer (pH 4.5) at 3.8 mg/ml (—)] to minimally nephrotoxic κI Bence Jones protein Borf [in PBS at 2.0 mg/ml (---)] and to nonnephrotoxic κIV Bence Jones protein Len [in PBS at 2.0 mg/ml (· · ·)].

mouse model, minimal basement membrane precipitates and tubular casts were found.

The results of our *in vitro* analyses of 40 different Bence Jones proteins are summarized in Table 1. The presence or absence of light-chain-related pathology (cast formation, membrane deposition, crystals, and amyloid formation) was established in 18 cases clinically by biopsy or autopsy. For the remaining 22, the nature of the light-chain deposition was demonstrated experimentally in the mouse model (6). Four proteins were nonnephrotoxic and exhibited no aggregation in the *in vitro* chromatographic system. In contrast, 33 of the 35 nephrotoxic proteins demonstrated oligomerization/aggregation under the experimental conditions used.

DISCUSSION

Our data show that under physiologically relevant conditions—i.e., environments comparable to those found within the kidney—and at nondenaturing temperatures, many Bence Jones proteins are capable of forming high molecular weight aggregates *in vitro*. It is probable that these protein interactions found *in vitro* also occur *in vivo* and may account for the strong correlation between high-order *in vitro* self-association and the propensity of monoclonal light chains to form pathologic deposits *in vivo* (Table 1).

The results described in this report provide evidence that many Bence Jones proteins (perhaps the majority) are capable of forming high molecular weight aggregates *in vitro* when tested under appropriate conditions. The heterogeneity of aggregation properties exhibited by this family of proteins is consistent with previous observations of Putnam and coworkers (12, 13), who quantitatively analyzed the temperature dependence of Bence Jones protein solubility as a function of pH, ionic strength, and solvent composition. In addition to systematic differences in the solubility of κ and λ proteins, significant variations in protein-to-protein pH, ionic strength, and temperature effects were found.

Table 1. Correlation of *in vivo* pathology with *in vitro* aggregation of Bence Jones proteins

Light-chain deposition	Proteins					
	κ			λ		
None	Borf	(I)	+	Kir	(III)	0
	Fin	(II)	0			
	Kin	(I)	0			
	Len	(IV)	0			
Casts (renal tubules)	Cag	(II)	+++	Wild	(III)	+++
	Edm	(I)	+++	Biv	(VIII)	++
	Dru	(I)	++	Cle	(III)	++
	Mcc	(III)	++	Lev	(II)	++
	Pri	(I)	++	Mora	(II)	++
	Hol	(I)	+	Pug	(III)	++
	Pat	(II)	+	Wilc	(I)	++
	Rhy	(III)	+	Wit	(III)	+
	Scu	(I)	+	Loc	(I)	0
	Wat	(I)	+			
Precipitates (basement membrane)	Burn	(IV)	++	Eve	(II)	+++
	Kel	(III)	++	Han	(III)	++
	Mon	(I)	++	Cox	(I)	0
Crystals (renal tubules)	Wms	(I)	+++	Sho	(III)	++
Amyloid fibrils	Cro	(I)	+	Doy	(III)	+++
				She	(I)	+++
				Tyl	(III)	+++
				Sut	(VI)	++
				Emm	(I)	+
				Mor	(VI)	+

Roman numerals in parentheses refer to the V_κ or V_λ subgroup. The scoring criteria are as follows: +++, extensive aggregation; ++, intermediate aggregation; +, discernable aggregation; 0, no aggregation. Scores reflect maximal aggregation tendency under one or more solution conditions tested (PBS, urea, or acetate).

The three buffers (PBS, acetate, and urea) used in our chromatographic studies were chosen to reflect environments that light chains would be exposed to within the nephron (14). Buffer 1 was isotonic with serum and represented conditions expected during transport of protein in the bloodstream and filtration in the glomerulus. Buffer 2 contained urea and salt to emulate the microenvironment of the distal tubule. The salt concentration was at the hyperosmotic end of the normal range as would occur during partial dehydration, a condition that significantly exacerbates renal pathology associated with Bence Jones proteins (15), and the urea concentration was considerably less than that typically required to solubilize proteins. Because acidification has been implicated as a contributing factor to the nephrotoxicity of Bence Jones proteins (16), buffer 3 provided the condition of low pH found in the renal proximal tubule (the site of light-chain catabolism as well as urine acidification).

Relationship Between *in Vitro* Light-Chain Aggregation and *in Vivo* Pathology. Our data suggest that light-chain deposition as casts, precipitates, or fibrils depends upon physicochemically determined association phenomena inherent to the proteins themselves. Tetramer and higher order polymeric forms of Bence Jones proteins have been found (17–19) in the serum of patients with multiple myeloma; however, the pathologic import of such components has not been established. We posit that the tissue deposition of light chains is governed by the concepts of mass action that underlie all molecular interactions. In addition, host-related factors, such as dehydration, affect cast formation in the kidney (20–22). Since this process results in an increased protein concentration—a condition we find to increase aggregate formation exponentially—this phenomenon could account for the renal tubular deposition of an apparently nontoxic Bence Jones protein (23). Alterations in osmolality, urea concentration, and pH modulate parameters that we have shown to affect interaction. Other generic host-related factors that may contribute to the development or stabilization of light-chain deposition include Tamm–Horsfall protein (24, 33), amyloid enhancing factor (25), amyloid P component (26), and glycosoaminoglycans (27).

Host-related factors may contribute to the limited number of "false" negative and positive analyses. Alternatively, the false negatives could indicate that the protein was tested at nonoptimal conditions or concentrations or that the light-chain sample recovered from the urine of the patient was not representative of the material that was deposited physiologically. Categorizing the chromatographic behavior of protein Borf as a false positive may be incorrect. In contrast to protein Cag, which exhibited strong aggregation in nephron-like low pH medium, the solubility of protein Borf increased under these conditions. Thus, the comparative elution properties of this protein may be consistent with its physiological behavior. Although not nephrotoxic, the high-order self-association properties that were observed could indicate a tendency for other physiological deposition, such as amyloid formation, whose presence was not clinically evident. As noted above, when tested in the mouse model, protein Borf did exhibit minimal but observable cast formation and precipitation and could arguably be classified as pathological on that basis. Further study will be needed to address these issues and will require characterization of additional clinically infrequent nonpathological light chains.

Relationship of *in Vivo* Pathogenesis and Light-Chain Primary Structure and Conformation. The dimerization of antibody light chains is mediated by interface residues through which light chains and heavy chains assemble to form a

functional antibody (7). Higher-order aggregation of light-chain dimers represents an anomalous self-association that most likely involves other surfaces of the light chain. If both V_L complementarity-determining and framework residues are involved in higher-order assembly, then certain clinical observations can be rationalized: the mode and propensity of pathological deposition are (*i*) protein specific (i.e., idiosyncratic to each protein) and (*ii*) correlated with particular light-chain V_L type and subgroup.

The fact that individual light chains differ in their capacity to form high-order aggregates or polymers implies that the complementarity-determining residues, because of extensive sequence variability, are the segments responsible for this phenomenon. Therefore, the extent of polymerization and optimal conditions for polymerization will be highly protein specific. Alternatively, because light-chain V_L subgroups are identified on the basis of conserved framework-residue sequences (28), the participation of at least some of these segments in aggregation must also be considered. Thus, each relevant subgroup-characteristic residue or peptide segment represents a potential sequence-dependent contribution to aggregation (the magnitude of which will be shared with most subgroup members) that differs from the corresponding contributions by proteins of different V_L subgroups. Amino acid substitutions in the framework residues that are located on the outside surface (rather than the interior) of the V_L domain could modulate the ability of each subgroup to interact with other proteins or receptors. Although light chains of the $V_{\lambda VI}$ subgroup are invariably associated with AL amyloidosis (29), we have not as yet found a relationship (clinically or experimentally) between V_L subgroups and modes of light-chain nephrotoxicity.

The correlation of pathologic properties with light-chain type (κ or λ) may similarly be attributed to conserved structural features of κ and λ V_L domains. Although it has been observed that the light-chain constant domain (C_L) is not required for experimental light-chain deposition (23), it is well established that the distribution of κ and λ light chains differs among the light-chain-related pathologies. For instance, in contrast to the normal $\approx$2:1 ratio of κ to λ chains among human immunoglobulins, this ratio is reversed in AL amyloidosis. Conversely, κ chains predominate in light-chain deposition disease (30). In addition to V_κ-specific and V_λ-specific features, C_κ and C_λ contribute differentially to the pathological processes because of potential differences in certain intrinsic properties such as susceptibility to proteolysis (31).

SUMMARY

The propensity for monoclonal light chains to deposit as casts, precipitates, or fibrils reflects in part the extensive variability in protein primary structure. The results of our studies suggest that the capability of a light chain to aggregate *in vitro* reflects intrinsic light-chain-specific physicochemical properties that contribute to protein deposition phenomena observed *in vivo*. The ability to readily identify such proteins by size-exclusion chromatography under specified conditions has prognostic and therapeutic importance. This technique also provides a unique means to study factors that accelerate or prevent light-chain aggregation and thus provides an experimental approach to study the pathogenesis and treatment of the light-chain-associated renal and systemic diseases (32).

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