Characterization of Renal Amyloid Derived From the Variable Region of the Lambda Light Chain Subgroup II

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Amyloid fibrils were extracted from the kidney of a patient (CHE) shown to have tetramers and dimers of a monoclonal λ light chain in his serum, and whose bone marrow cells in short-term culture synthesized these forms and a smaller λ fragment of approximately 10,000 to 12,000 daltons. Biochemical and serologic analysis of a fraction of a size (obtained from amyloid fibrils extracted from the kidney) similar to that synthesized by the bone marrow cells revealed a light chain fragment corresponding to the amino terminal end of the variable region of the λ light chain subgroup II. The presence of similarly sized short fragments of λ light chain in both the synthesized and deposited protein suggests that aberrant synthesis and/or proteolytic degradation may play a pathogenetic role in the process of amyloidogenesis. (Am J Pathol 1986, 124:82–87)

STUDIES of amyloid related to immunoglobulin light chains (AL) reveal that certain types of light chains are more "amyloidogenic" than others. Therefore, the possibility exists that these proteins may possess distinctive variable regions due to differences in their primary structure. Alternatively, the structure of these proteins may render them amyloidogenic due to their unusual susceptibility to proteolysis.[1] Collected data thus far reveal that λ light chain is more amyloidogenic than κ, and the variable region of the λ light chain subgroup VI ($V\lambda_{VI}$) appears to be preferentially associated with the amyloid process.[1]

An opportunity to study the protein synthesized by plasma cells and that deposited as amyloid fibrils occurs in individuals with AL amyloid. A comparison of the synthetic products and the tissue deposits may provide insight into the process of amyloidogenesis. This paper reports the chemical and serologic analysis of amyloid (CHE) extracted from the kidney obtained at necropsy from a patient whose bone marrow cells, at the time of the initial diagnosis, synthesized excess intact λ light chains, which were assembled into dimers and tetramers, and an additional λ fragment of 10,000–12,000 daltons.[2] The amyloid could not be classified by immunohistologic methods in frozen sections of kidney because the deposits were not reactive with antisera monospecific for κ or λ light chains or AA protein.[3]

Case Report

In 1974, a 52-year-old black male (CHE) presented with nephrotic syndrome, orthostatic hypotension, purpuric skin lesions, and bone marrow plasmacytosis. His serum contained tetramers and dimers of λ light chains. Biopsies of rectal mucosa, kidney, skin, and gastric mucosa all showed amyloid with conventional histologic techniques and Congo red staining. The patient was treated with alkylating agents intermittently for 2 years with the disappearance from the serum of the monoclonal λ chains, and stabilization of his clinical status. In late 1981 he became markedly azotemic, and chronic peritoneal dialysis was instituted. In March 1984 he noted the appearance of an enlarging mass in the right parietal area of the skull. Biopsy revealed metastatic renal cell carcinoma. Despite local radiotherapy, the patient followed a progressive downhill course and died 6 months later. A limited autopsy showed a primary renal carcinoma occupying 50% of the right

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kidney and multiple metastatic nodules in the liver. Histologic examination showed diffuse amyloid infiltration of both kidneys.

Materials and Methods

Morphologic Study

Blocks of kidney obtained at autopsy were routinely processed for light, immunofluorescence, and electron microscopy as previously described.[3,4] The extracted amyloid was monitored by examination of material on slides stained with Congo red viewed under polarized light and by electron-microscopic identification of fibrils on carbon-coated grids negatively stained with uranyl acetate.

Isolation and Purification of Tissue Amyloid

Unfixed kidney stored at −70 C, weighing 34 g, was available for chemical study. Amyloid was extracted with the use of a modification of the method of Pras.[5] The tissue was homogenized in three changes of 0.15 M saline, and the supernatants were discarded. The insoluble residue contained Congo-red–positive material that displayed green birefringence under polarized light and abundant fibrils typical of amyloid by electron microscopy. Aliquots of extracted material were dissolved in 6 ml of 6 M guanidine in Tris HCl buffer, pH 10.2, and made 0.17 M in dithiothreitol for reduction at room temperature with slow stirring. To this mixture 2 ml of 2 M guanidine in 4 M acetic acid was added; and the solution was centrifuged at 100,000*g*, filtered, and fractionated on a 60 × 1-inch column of Sephadex G-100 equilibrated with 5 M guanidine in 1 M acetic acid (Figure 1A). Another aliquot was treated with 15% formic acid in 5 M guanidine HCl prior to gel filtration. The column fractions were extensively dialyzed against distilled H_2O in dialysis tubing with a molecular weight cutoff approximately 3500 daltons and lyophilized. The purity and molecular weights of the fractions were determined by 17% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).[6]

Complete Reduction and Alkylation and Deblocking of the Amino Terminal End

Peak 4 (2.1 mg) (Figure 1A) was completely reduced in 6 M guanidine/0.6 M Tris HCl/0.001 M EDTA/20 mM dithiothreitol, pH 8.2, for 1 hour at 37 C and alkylated by the addition of iodo[^{14}C] acetic acid (0.7 mCi/mmol, New England Nuclear, 1 Ci = 3.7 × 10^{10} Bq) to a final concentration of 40 mM for 1 hour at 37 C. The material was dialyzed against deblocking buffer (0.1 M disodium phosphate, adjusted to pH 8.0 with 0.1 M monosodium phosphate and made 5 mM in dithiothreitol, 10 mM in disodium EDTA, 5% in glycerin, (vol/vol), and adjusted to pH 8.0 with 1 N NaOH) and then treated with pyroglutamate aminopeptidase (L-pyroglutamyl peptide hydrolase, EDC 3.4.11.8) (Boehringer Mannheim Biochemicals, Indianapolis, Ind), according to the method of Podel and Abraham.[7]

Amino Acid Analysis and Amino Acid Sequence

Amino acid analysis was performed on a Durrum Model D-500 amino acid analyzer (Durrum Instrument, Sunnyvale, Calif). Samples were hydrolyzed in 2 ml of 6 N hydrochloride under vacuum for 24 hours at 110 C. To prevent degradation of tyrosine, 40 µl of a 10% aqueous solution of phenol was added. Automated amino acid sequencing was performed on a Beckman 890C sequencer (Beckman Instruments, Fullerton, Calif). Phenylthiohydantoin amino acids were identified by high pressure liquid chromatography (HPLC) performed on a Waters HPLC Model ALC/GPC-201 (Waters Instruments, Milford, Mass), prepared with a C18 Bondapak column (Waters Instruments), and eluted with a methanol–water gradient.

Antigenic and Immunoblotting Analysis

Antigenic analysis of amyloid protein was performed by the method of Ouchterlony in 1% agar in 0.025 M barbital buffer, pH 8.6, using standard anti-λ and anti-κ light chain and anti-AA antisera.[8,9]

After SDS-PAGE the proteins (from Peaks 4,5, and 6, Figure 1A) were transferred to nitrocellulose paper (0.45-µ pore size, Schleicher & Schuell, Inc., Keene, NH) by overnight electrophoresis, then soaked in phosphate-buffered saline (PBS) containing 0.2% gelatin with gentle shaking for 2–3 hours at room temperature to block free binding sites, and incubated at 4 C overnight in standard rabbit anti-human λ or anti-κ light chain, anti-AA antisera, or normal rabbit serum, diluted 1:50 with PBS/0.2% gelatin containing 0.1% Triton X-100. After thorough washing in PBS/0.2% gelatin/0.1% Triton X-100 (six washes for 30 minutes each), the nitrocellulose strips were incubated overnight at 4 C in swine anti-rabbit IgG antibody conjugated with peroxidase, diluted 1:1000 (DAKO) in PBS/0.2% gelatin/0.1% Triton X-100. The nitrocellulose strips were transferred to the substrate solution, prepared by adding 50 mg of diaminobenzidine (Sigma Chemical Co., St. Louis, Mo) to 100 ml of 50 mM Tris HCl, pH 7.6, followed by 5–10 µl of 30% hydrogen peroxide. After the desired signal was obtained (usually within several seconds), the reac-

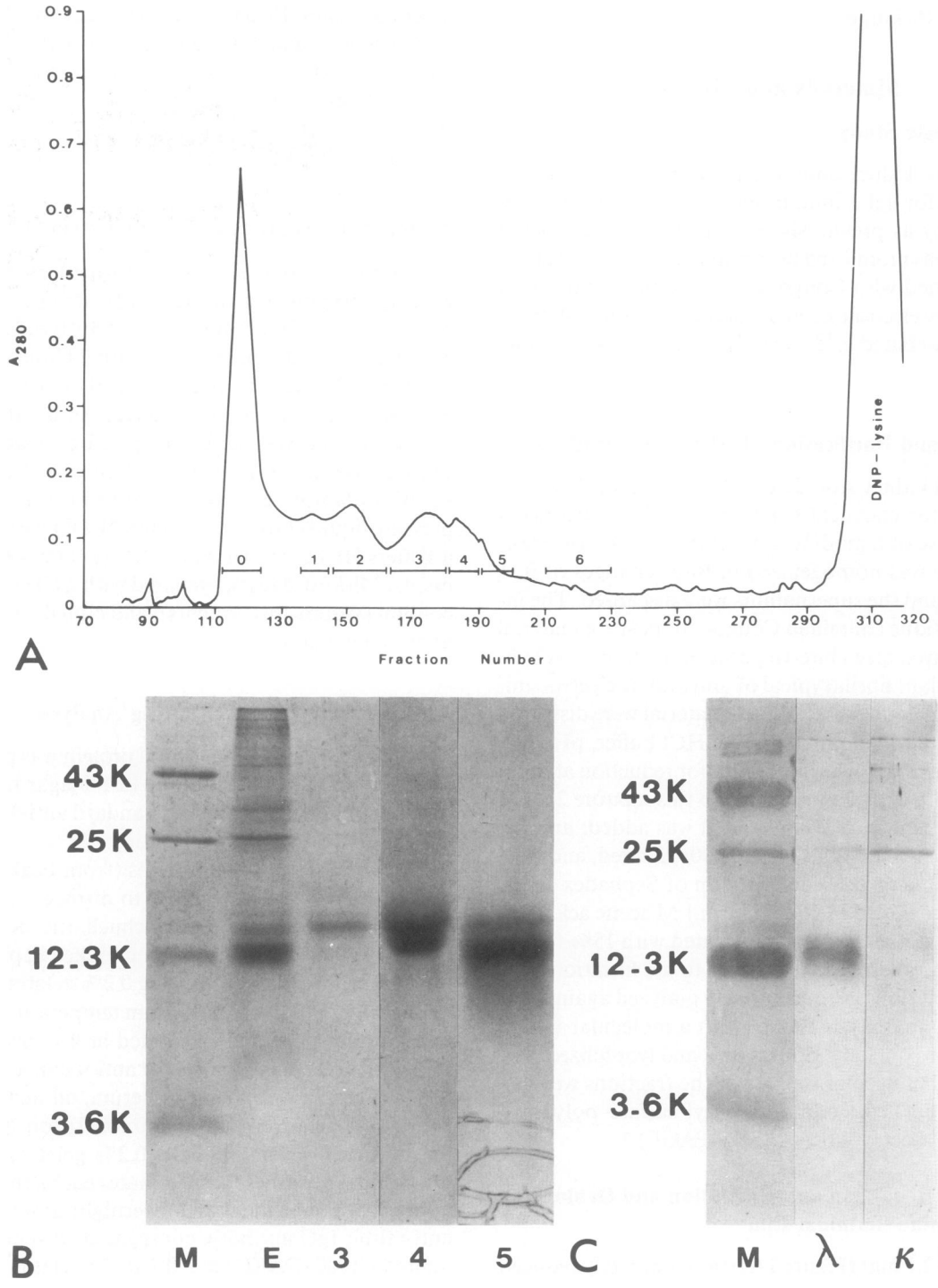

Figure 1A—Elution profile of amyloid protein extracted from the kidney of patient CHE and fractionated on a Sephadex G-100 column equilibrated with 5 M guanidine HCl in 1 M acetic acid. The tubes were pooled into six fractions as shown. The molecular weights of Fractions 2, 3, 5, and 6 were: 25,000 daltons; 13,900 daltons; 12,400 daltons, and 9000 daltons, respectively (not shown). **B**—SDS-PAGE, 17% slab gel under reduced conditions, of material extracted from kidney. Markers (*M*): ovalbumin, 43,000 daltons; chymotrypsinogen A, 25,000 daltons; cytochrome *c*, 12,300 daltons; insulin, 3600 daltons. *E*, extracted material before fractionation. Colums 3, 4, and 5 correspond to fractions shown in **A**. **C**—Immunoblot analysis of Fraction 4 shows reactivity with anti-λ antibody but not with anti-κ antibody. *M*, markers.

tion was stopped by washing the nitrocellulose strips with H_2O. Strips for controls were developed for at least 2 minutes.

Results

Tissue Studies

Diffuse deposits of amyloid, which displayed metachromasia in crystal violet stains and green birefringence under polarized light in Congo red stains, were present in glomeruli, tubular basement membranes, and vessels. By electron microscopy there were randomly oriented fibrils typical of amyloid. Immunofluorescence microscopy of frozen sections revealed no significant staining of amyloid deposits for λ or κ light chains, γ, α, and μ heavy chains, or AA protein; the anti-light chain antisera used for immunoblotting analysis also showed no significant staining of amyloid in tissue.

Purification of Amyloid Proteins

The elution profile of the extracted material fractionated on a Sephadex G-100 column is shown in Figure 1A. Treatment of the extracted material with 15% formic acid in 5 M guanidine HCl prior to gel filtration gave similar results (not shown). The purity of the fractions was determined by SDS-PAGE (Figure 1B). Peak 0 was not studied further. Peak 1 contained polymers of light chains, and Peak 2 contained protein of 25,000 daltons, which on further analysis was heterogeneous (not shown). The molecular weight of proteins in Peaks 3, 4, and 5 ranged from 13,900 to 12,400 daltons. Peak 4 was composed of a mixture of peaks 3 and 5, whereas Peak 5 contained only a small admixture (less than 10%) of Fraction 3. The amino acid composition of Fraction 5 is shown in Table 1; this fraction could not be sequenced, presumably because it was blocked at the amino terminal end. The adjacent Fraction 4 (2.1 mg protein) was deblocked with L-pyroglutamyl peptide hydrolase; the result of the main protein sequenced for 24 steps is shown in Figure 2. The main sequence of Fraction 4 corresponds to the amino terminal end of the variable region of the λ light chain subgroup II (V λ_{II}) protein.[10] A second protein present in Peak 4 (Figure 1B, Column 4) was sequenced through 26 steps representing approximately 30% of the total amount of protein. This second sequence appears to correspond to a degradation product of the V λ_{II} from Position 62 to Position 87. Its tentative sequence is: [Phe-Gly-Val-Ser-Lys-Ser-Gly-Thr-(Ile)-Ala( )Leu( )Ile-(Thr)-Gly-Leu-Ala-Ala-Glu-Asp-Glu-Ala-Asn-Tyr-Tyr]. Attempts to type the subgroup of Peak 5 serologically were inconclusive. Immunoblotting analysis of Peaks 4 and 5 revealed positive reactions with anti-λ light chain antiserum (Figure 1C) but not with anti-κ (Figure 1C) or anti-AA antisera.

Table 1—Amino Acid Composition of Amyloid Protein Extracted From Kidney, Fraction 5 (Figure 1A) (Residues/mol)

Cys*	3.3
Asp	9.0
Thr	4.0
Glu	14.15
Pro	7.1
Gly	10.7
Ala	9.0
Val	2.25
Met	0.9
Ile	4.6
Leu	8.9
Tyr	4.4
Phe	3.4
His	1.6
Lys	3.0
Arg	6.2
Ser	10.7
Trp†	

* Determined as cysteic acid.
† Not done.

A small amount of protein (approximately 9000 daltons) in Peak 6, by amino acid analysis, was similar to Peak 5 (not shown); this protein also appeared to be blocked at the amino terminal end, because it could not be sequenced. Immunoblotting analysis of Peak 6 revealed no reaction with anti-λ or anti-κ light chains or with anti-AA antisera and thus appears to be a further degradation product of the λ light chain.

Discussion

In this study we present data on the chemical characterization of the amyloid protein (CHE) extracted postmortem from the kidney of a patient whose serum

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                    5                  10                  15                  20
CHE   PCA-Ser-Ala-Leu-Thr-Gln-Pro-Ala-Ser-Val-Ser-Gly-Ser-Pro-Gly-Gln(Ser)Ile-Thr-Ile-Ser-Cys-Ile-Gly
NEI   ————————————————————————————————————————————————————————————————————————————————— Thr —
BUR   ————————————————————————————————————————————————————————————————————————————————— Thr —
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Figure 2—The amino terminal sequence of amyloid protein CHE in comparison with protein NEI and BUR of the same light chain type (λ) and V region subgroup ($V_{\lambda II}$)[10]. —— indicates indentity; ( ), uncertain.

contained monoclonal λ light chain dimers and tetramers and whose bone marrow cells in culture synthesized λ light chains assembled into dimers and tetramers. Reduction and alkylation of the synthesized molecules precipitable with anti-light chain antisera showed λ light chain monomer and a smaller fragment of approximately 10,000–12,000 daltons.[2]

The relationship between synthetic fragments and deposited fibrils in a single patient has never been studied. In our study of the fibrils extracted from the kidney we characterized Fractions 4 and 5 (Figure 1A and B, Columns 4 and 5) of protein similar in size to that obtained in the biosynthetic studies. Biochemical and serologic analysis of this fraction proved that it was composed of two closely related proteins that were reactive with the anti-λ antiserum, but not with anti-κ (Figure 1C) or anti-AA protein antisera (not shown). The amino acid analysis revealed the presence of cysteic acid and an amino acid composition compatible with a fragment of λ light chain (Table 1). Amino acid sequencing performed on deblocked protein from Peak 4 showed two closely related sequences, the main one corresponding to the amino terminal end of V λ_{II} and the second corresponding to a degradation product of the V λ_{II}, which begins at the third framework region and presumably contains a fragment of the constant region. Apparently the same second protein was also present in Peak 5, but its admixture was too small (less than 10%) to be sequenced. However, the presence of this second protein containing a fragment of the constant region in Peaks 4 and 5 could be responsible for the positive reaction with anti-λ light chain antibody in the immunoblotting.

AL amyloid has been found to vary in size from 5000 to 23,000 daltons. Although sequence data exist for only the amino terminal region, it is assumed that the AL fibril can consist of the V region of the light chain, the V region plus part of the constant region, the intact light chain, or a combination of various molecular forms.[11] The latter seems to be the case in this patient, as revealed by the biosynthetic studies[2] and further supported by the study of extracted fibrils. The presence of similarly sized short fragments of λ light chain in both the synthesized and deposited protein suggests that aberrant synthesis may play a pathogenetic role in the present case of AL amyloid. Alternatively, the short fragment seen in the synthetic studies could be a consequence of immediate degradation. Whether the synthesized and deposited short fragments have the same primary structure remains to be elucidated.

The counterparts of the deposited proteins are also usually found in the monoclonal serum immunoglobulin or urinary Bence-Jones proteins which are manifestations of B-cell–related disorders. In several cases the amino acid sequences of the fibril and the monoclonal protein in urine or serum have been determined and found to be identical.[12] Similar studies comparing the biosynthetic product and the protein deposited have not been done thus far.

Why amyloid forms in only some monoclonal gammopathies is unknown. Several observations suggest that certain types of light chains are more "amyloidogenic" than others. First, AL-λ amyloid is more frequent than AL-κ amyloid, as determined by immunohistochemical methods,[3] and monoclonal λ is more frequent than monoclonal κ M-protein in patients with AL-amyloid.[1] In contrast, κ light chain is predominant among normal immunoglobulins and in nonamyloidotic immunoglobulin deposition disease.[13] Second, certain V region subgroups that are normally uncommon are overrepresented in AL-λ amyloid. There is preferential association of the V λ_{VI} (λ V region subgroup VI) with amyloid which is three to five times higher than the expected incidence in the normal population. On the other hand, patients with monoclonal gammopathies have no predominance of a particular V region subgroup.[1]

More than one half of all isolated AL amyloid proteins have a blocked amino terminus, as demonstrated in CHE, and have been suspected to be a λ type with a pyrrolidone–carboxylic acid terminus, ie, Subgroups I, II, and V.[14] However, because of limited data, no common structural features are as yet apparent among lambda amyloid fibrils.

In cases where the amyloid protein is small, ie, composed of a fragment of the V region, it may present restricted antigenicity with conventional anti-λ or anti-κ antisera which are raised against constant region (such as those used for immunohistochemical studies) and lead to inconclusive (or even negative) results. For reasons that are not clear but may relate to the concentration of fibrils or differences in sensitivities of the techniques employed, the tissue amyloid in immunofluorescence studies was not reactive with standard anti-light chain antisera, including those used for immunoblotting, whereas the extracted fibrils in immunoblot preparations were.

Recently the serologic typing of light chain components in patients with amyloid has been made possible by the availability of antisera to the chemically defined subgroups of human κ and λ chains.[1] Their use in tissue sections has not been explored. At the present time the biochemical analysis of the extracted amyloid fibrils remains the most reliable method of determining the precise subgroups of AL amyloid.

Of additional interest in this patient is the develop-

ment of renal cell carcinoma. Several cases of generalized amyloidosis, both AA and AL, associated with renal cell carcinoma have been reported.[9,15] It has been suggested that the neoplasm itself is pathogenetically related to the amyloid; the presence of amyloidosis, antedating by approximately 10 years evidence of the renal neoplasm, makes this association unlikely in the present case.

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