Immunoglobulin heavy-chain-associated amyloidosis

(amyloid/heavy-chain disease/immunoglobulin genes/deletion mutant/amino acid sequence)

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ABSTRACT Immunoglobulin- or multiple myeloma-associated amyloidosis has been distinguished by the tissue deposition of Congophilic, fibrillar protein consisting of light chains or light-chain fragments (AL amyloidosis). We now report the isolation and characterization of another form of immunoglobulin-associated amyloid obtained from a patient who had extensive systemic amyloidosis and in whom the amyloid deposits consisted not of light chains but rather of an unusual form of heavy chain. This component, isolated from splenic amyloid extracts, represented an internally deleted IgG1 heavy chain as evidenced by immunochemical, electrophoretic, and amino acid sequence analyses. A comparable immunoglobulin-related monoclonal protein, consisting only of IgG heavy chains, was present in the patient's urine. Based on serologic reactivity with a battery of anti-immunoglobulin antisera, these two immunoglobulin-related components were antigenically identical; however, when compared to normal IgG, both were deficient in Fc-associated γ-chain determinants. The structural abnormality of the amyloid γ-chain protein was further evidenced by SDS/PAGE and immunoblotting analyses: An unusually low molecular mass of ≈22 kDa was found for this material vs. the expected value of ≈55 kDa for a normal γ heavy chain. Despite the lack of certain Fc determinants, the amyloid and urinary heavy-chain proteins expressed the IgG1 subclass allotype marker G1m(a) located on the third constant region (CH3) domain of the internally deleted IgG1 heavy chains. That the amyloid protein contained an intact CH3 domain was established through amino acid sequence analyses of cyanogen bromide fragments and peptides generated by a lysine-specific protease. These studies also revealed that the γ-chain amyloid protein contained the complete heavy-chain variable (VH) domain [including the diversity (DH) and joining (JH) segments] that was contiguous with the CH3 domain. The low molecular mass of the protein resulted from the total absence of the first (CH1), hinge, and second (CH2) heavy-chain constant regions. Such extensive CH deletions and the presence of a complete VH distinguish this amyloid-associated heavy chain from all other heretofore characterized γ-heavy-chain disease proteins. This heavy-chain-related form of immunoglobulin-associated amyloidosis is tentatively designated AH amyloidosis.

Amyloidosis encompasses a diverse clinical and pathological spectrum of human diseases that have in common the vascular or tissue deposition of proteinaceous material characterized by distinctive tinctorial, ultrastructural, and tertiary structural features. Regardless of the chemical composition of the substance deposited, all forms of amyloid protein exhibit birefringence after Congo red staining and a fibrillar composition when viewed by polarizing and electron microscopy, respectively. In addition, a β-pleated structure is evidenced by x-ray crystallography (1, 2).

At least 11 different types of proteins have been identified as amyloid constituents (2). Immunoglobulin-related polypeptides represent a class of proteins that have been implicated in the pathogenesis of one type of amyloidosis that occurs in patients with monoclonal B-cell proliferative disorders of idiopathic or neoplastic origin—i.e., immunoglobulin- or multiple myeloma-associated amyloidosis (2, 3). The products of such cells—monoclonal immunoglobulin components—are commonly detected in patients' serum or urine in the form of complete molecules containing both heavy and light polypeptide chains (i.e., myeloma proteins) or as free light chains (i.e., Bence Jones proteins). In these cases, light chains or, more commonly, light-chain fragments (but not heavy chains) have been found to be the principal amyloid protein constituent (1). Light-chain-associated amyloidosis has been termed AL amyloidosis (1, 2).

Although amyloidosis has been reported to occur in patients with other forms of monoclonal malignant or seemingly benign B-cell proliferative disorders—e.g., γ-heavy-chain disease (4, 5), the presence of immunoglobulin heavy polypeptide chains in amyloid deposits has heretofore, to our knowledge, not been established. We now document that the amyloid deposits in a patient with widespread systemic amyloidosis associated with monoclonal serum and urinary immunoglobulins (including a γ-heavy-chain protein) consisted of an aberrant γ-related heavy chain. Serological and chemical characterization of amyloid extracts established unequivocally that the amyloid protein represented an atypical heavy chain of the IgG1 subclass. Primary structural analyses confirmed the unusual composition of the amyloid protein which contained a large internal deletion and consisted only of the complete variable (VH) and third constant (CH3) domains. Because of the heavy-chain nature of this form of immunoglobulin-associated amyloidosis, we propose that this entity be designated AH amyloidosis.

MATERIALS AND METHODS

Clinicopathological Features. Patient ART, a 65-yr-old female, was found at the time of cholecystectomy to have hepatic amyloid deposition. For 3 mo before surgery, she had experienced progressive abdominal pain, weight loss, and jaundice. The abnormal liver function and hepatobiliary obstruction evidenced clinically were attributed to radiographically demonstrable cholelithiasis; her renal function was also markedly impaired. At surgery, it was noted that the patient's liver appeared cirrhotic and enlarged. Tissue obtained via a wedge liver biopsy revealed a diffuse sinusoidal infiltration of eosinophilic, homogeneous material. Exami-

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Abbreviations: amyloidosis AL and AH, light-chain- and heavy-chain-associated amyloidosis, respectively; VH and CH, variable and constant regions of immunoglobulin heavy chains, respectively; FR and CDR, framework and complementarity-determining regions, respectively; DH and JH, diversity and joining segments, respectively; AP, amyloid P.
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nation of Congo red-stained sections under polarized light showed that this material exhibited the characteristic birefringence of amyloid. Postoperatively, the patient's condition worsened, and she died 1 week later of hepatic and renal failure. Subsequent examination of serum and urine specimens revealed multiple monoclonal immunoglobulin-related components. In addition to the hepatic amyloid, tissue obtained at autopsy showed extensive systemic deposition of amyloid in the heart, kidney, and spleen.

Immunoglobulin Identification and Characterization. Serum and urine specimens from patient ART were examined by agarose gel immunofixation analysis using a panel of anti-γ, -α, and -μ heavy-chain and anti-κ and -λ light-chain antisera (Paragon System, Beckman). The methods used to prepare papain-derived Fc and Fab fragments from normal human IgG (Cohn fraction II γ globulin, Sigma) and antisera specific for these and other human immunoglobulin components were as described (6, 7). Immunodiffusion (Ouchterlony) analyses were performed in 2% agar gels containing 3% polyethylene glycol 6000. The monoclonal immunoglobulin components present in the serum and urine of patient ART were isolated by zone electrophoresis (8) on blocks of a polyvinyl chloride/polyvinyl acetate copolymer (Pevikon-870, Mercer Chemical, Amityville, NY). Isolated proteins were examined under reducing conditions by SDS/PAGE employing a discontinuous buffer system and 12.5% homogeneous polyacrylamide gels (PhastSystem, Pharmacia LKB). Immunoblotting was performed by electrotransfer of the proteins to nitrocellulose membranes followed by application of specific anti-immunoglobulin antisera. The serological determination of Gm allotypes (a), (x), and (g) was performed by Ralph C. Williams, Jr. (University of Florida, College of Medicine, Gainsville, FL) using commercial agglutinators and anti-Rh coats known to be specific for each of the Gm systems tested (Allotype Testing); appropriate positive and negative controls were included in each assay.

Extraction and Isolation of Amyloid Protein. By using described methods (9, 10), amyloid fibrils were extracted from the spleen of patient ART and lyophilized. The presence of amyloid P component (AP) in the saline citrate extract was determined by immunodiffusion analyses by using a specific anti-P antiserum (Dako, Carpinteria, CA). The amyloid-containing water extract was dissolved in 5 M guanidine hydrochloride/1 M acetic acid, and the protein was isolated by gel filtration through a Superose HR 12 column equilibrated with the same solvent (FPLC, Pharmacia LKB).

Complete Reduction and Pyridylethylation. Twenty milligrams of the purified amyloid protein was dissolved in 2 ml of 6 M guanidine·HCl (Pierce) adjusted to pH 8.0 with 0.25 M Tris·HCl. The protein was reduced by the addition of 20 μl of 2-mercaptoethanol, incubated for 2 hr at room temperature under an inert (N₂) atmosphere, and then pyridylethylated (11) by adding 30 μl of 4-vinylpyridine (Aldrich). Thirty minutes later, the pH of the reaction mixture was reduced to 3.0 by the addition of glacial acetic acid. After dialysis against distilled water the protein solution was lyophilized.

Chemical Cleavage. Nine milligrams of the reduced and pyridylethylated amyloid protein was dissolved in 2 ml of 70% formic acid to which 10 mg of cyanogen bromide (Pierce) was added—an amount calculated to be in 20-fold excess over the molar ratio of methionyl residues present in the protein (12). After 24-hr incubation at room temperature under N₂ in complete darkness, the solution was diluted to 30 ml with distilled water and lyophilized.

Endopeptidase Cleavage. Ten milligrams of the reduced and pyridylethylated amyloid protein was suspended in 2 ml of 0.1% methylmorpholine acetate buffer, pH 8.0. Two units of the lysyl residue-specific endopeptidase prepared from *Lysobacter enzymogenes* (Endoproteinase Lys-C, Boehringer Mannheim) dissolved in 100 μl of the methylmorpholine

acetate buffer were added, and the reaction mixture was incubated at 37°C for 24 hr. The sample was then freeze-dried using a SpeedVac sample concentrator (Savant Instruments).

HPLC. Peptides obtained after chemical or enzymatic cleavage were isolated by HPLC using a 210 × 4.6 mm column containing a reversed-phase carrier (Aquapore, RP 300 C₈, Brownlee Lab) with a 0.1% trifluoroacetic acid-to-70% acetonitrile/water (vol/vol) linear gradient at a flow rate of 1 ml/min. The absorbance was read at 220 nm, and the fractions were collected manually.

Amino Acid Analyses. The HPLC-separated peptides were hydrolyzed under reduced pressure for 24 hr in constantly boiling 6 M HCl. Derivatization with phenylisothiocyanate (13) was performed automatically with an ABI 420A derivatizer (Applied Biosystems), and the amino acid composition of the resulting phenylthiocarbonyl amino acids was determined by using an ABI 130A amino acid analyzer.

Sequence Analyses. Automated amino acid sequence analyses by Edman degradation were performed with an ABI 477A gas-phase protein sequencer connected on-line to an ABI 120A phenylthiohydantoin analyzer.

RESULTS

Characterization of the Serum and Urinary Monoclonal Immunoglobulin ART. The serum of patient ART contained two electrophoretically distinct monoclonal IgGκ proteins, and these, plus a third immunoglobulin component consisting only of IgG heavy chains, were found in the patient's urine (Fig. 1). The three proteins were isolated and characterized,
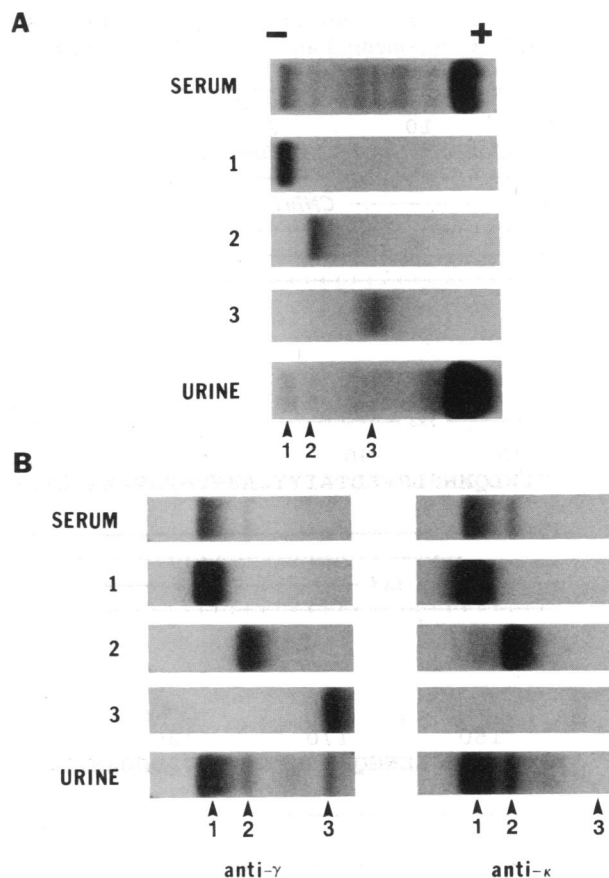

FIG. 1. Electrophoretic and immunofixation analyses of serum, urine, and three isolated monoclonal immunoglobulin components 1, 2, and 3 from patient ART. (*A*) Agarose gel electropherogram; protein stain. (*B*) Immunofixation analyses with specific anti-heavy chain (anti-γ) and anti-light chain (anti-κ) antisera, as indicated.

FIG. 2. SDS/PAGE (reducing gel) and immunoblot analyses of amyloid protein ART and normal IgG. (*Left*) Coomassie blue stain. The position of the molecular mass markers (kDa) and the heavy (H) and light (L) chains of normal IgG are as indicated. (*Right*) Immunoblot with an anti-Fc antiserum. KDa, kDa.

and their reactivity was compared by immunodiffusion analyses to that of normal IgG and its papain-derived Fc and Fab fragments. The serum immunoglobulin components, designated 1 and 2, represented "complete" and "incomplete" IgG molecules, respectively. Based on serologic reactivity using a battery of anti-immunoglobulin antisera, component 2 lacked certain Fc- and Fab-associated γ-chain determinants as compared to component 1 and normal IgG; both components contained κI-type light chains as demonstrated by their reaction with anti-κ and anti-κI subgroup-specific antisera. The third (urinary) monoclonal immunoglobulin component, designated 3, was a heavy-chain fragment consisting solely of incomplete γ chains, as evidenced in immunodiffusion analyses by its reaction of partial identity when compared to normal Fc$_\gamma$ using an anti-Fc$_\gamma$ antiserum and by its lack of reactivity with anti-light-chain and anti-Fab antisera.

The results of Gm allotyping provided evidence of the IgG1 subclass nature of the monoclonal serum and urinary IgG proteins ART. Components 1 and 2, as well as component 3,

expressed the IgG1-associated allotype G1m(a). Another IgG1-associated Gm allotype, G1m(x), was serologically detected on components 1 and 2 but not on component 3. All three components lacked the IgG3 subclass-associated allotype G3m(g).

Characterization of Amyloid Protein ART. Electron microscopic examination of hepatic tissue and the amyloid water extract from the spleen of patient ART revealed the ultrastructural features typical of amyloid fibrils (1). Gel filtration of the water extract yielded a major protein peak representing ≈70% of the applied sample. The presence of the amyloid P component, AP, characteristically found in amyloid deposits (1) was serologically detected in the saline citrate protein extract (10) of splenic tissue.

The γ heavy-chain nature of the fibrillar amyloid protein isolated from the water extract was evidenced serologically by using specific anti-human-immunoglobulin antisera. These analyses also showed that the amyloid protein was antigenically identical to the urinary component 3—i.e., it consisted of an "incomplete" γ heavy chain. Amyloid protein ART was deficient in Fc-associated determinants as demonstrated by its reaction of partial identity when compared with the Fc fragment derived from normal IgG. As found for the urinary heavy-chain component 3, amyloid protein ART expressed the IgG1-associated Gm allotype G1m(a) but not G1m(x).

Further evidence for the absence of light chains and the unusual heavy-chain nature of amyloid protein ART was obtained by SDS/PAGE and immunoblotting analyses. Under reducing conditions, protein ART consisted of a single species with a molecular mass comparable to the ≈22-kDa light polypeptide chain of normal IgG (Fig. 2). However, the 22-kDa amyloid protein reacted not with anti-light-chain

FIG. 3. Primary structure of amyloid protein ART. The residues marked by arrows were determined by automated amino acid sequencing of the reduced and pyridylethylated intact protein (*Upper*) and of peptides generated by cyanogen bromide (CNBr) cleavage (*Middle*) and from enzymatic digestion with the lysine-specific (Ly) endoproteinase (*Lower*). The junction between the heavy chain V$_H$ and C$_H$3 domains is as indicated (amino acids are indicated by the single-letter code; ( ), residues not sequenced).

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antisera but rather with an anti-γ-heavy-chain (anti-Fc) antiserum. In contrast, the ≈55-kDa heavy chains and ≈22-kDa light chains of normal IgG gave the expected reaction with anti-Fc and anti-light-chain antisera, respectively.

Primary Structure of Amyloid Protein ART. Automated sequence analyses of the intact amyloid protein yielded an unambiguous 32-residue amino-terminal sequence characteristic of the amino-terminal portion of a human immunoglobulin V_H; no light-chain-associated residues were identified. That the amyloid protein represented a heavy chain of the V_H subgroup V_HIII was evidenced by the presence of an "unblocked" amino-terminal residue and by the virtual sequence identity between the initial 30 residues of protein ART and those that comprise the first V_H framework region (FR1) characteristic for V_HIII heavy chains (14). The V_H sequence of the amino-terminal 32 residues of IgG component 1 was identical to that of the amyloid protein [in addition, the light chains of IgG component 1 had a 23-amino-terminal residue FR1 sequence typical of a κI chain (14)]. Sequence analyses on components 2 and 3 were precluded due to the limited amounts of material for study.

With the exception of 10 residues from positions 67 to 76, the remainder of the amino acid sequence of the 228-residue amyloid protein ART was established through analyses of the chemically and enzymatically produced peptides (Fig. 3). The correct ordering of the peptides was facilitated by their overlapping nature and the availability of published V_H and C_H sequence data on human IgG proteins (14).

Cyanogen bromide cleavage of the amyloid protein yielded four peptide fragments, a number consonant with the presence of three methionyl residues as determined from the amino acid composition. The first two cyanogen bromide fragments, CNBr1 and CNBr2, consisted of 34 and 49 residues, respectively. Their sequences included FR1 (positions 1–30), the first complementarity-determining region (CDR1, positions 31–35), FR2 (positions 36–49), CDR2 (positions 50–65), and a portion of FR3 (positions 66–83). Thirty-nine of the 127-residue CNBr3 fragment encompassed the end of FR3 (positions 84–98), CDR3 (positions 99–111), and FR4 (positions (112–122). The CNBr3 fragment also included the diversity (D_H) and joining (J_H) segments, spanning positions 99–122 at the carboxyl-terminal portion of the V_H. The remaining sequence of CNBr3 (positions 123–210) was identical to the first 88 residues of the C_H3 domain of proteins representative of the IgG1 subclass (14). The 18-residue fourth cyanogen bromide fragment, CNBr4 (positions 211–228), contained the carboxyl-terminal portion of the C_H3.

Cleavage with the lysine-specific protease yielded eight peptides, the sequences of which confirmed and extended the sequence data obtained from analyses of the intact protein and of the four cyanogen bromide fragments. The total absence of the first C_H domain (C_H1), hinge region, and second C_H domain (C_H2) was confirmed conclusively through analyses of the 43-residue peptide Ly4. Peptide Ly4 included the carboxyl-terminal 24 residues of the V_H domain (positions 99–122) and the first 20 residues of the C_H3 domain (positions 123–142). Consonant with the serologically determined Gm allotype data, this peptide also contained the G1m(a) allotype-associated aspartyl and leucyl residues (15) at positions 138 and 140, respectively [positions 356 and 358 in the EU protein index numbering system (14) for a complete IgG1 heavy chain]. Although peptide Ly7 contained the G1m(x) allotype-associated glycyl residue (15) at position 213 (corresponding to position 431 in the C_H3 domain of IgG1 heavy chains), this determinant was not serologically detectable.

DISCUSSION

Immunochemical and amino acid sequence analyses of the amyloid protein ART revealed that this component represented an unusual IgG1 heavy chain with an extensive internal deletion. Amyloid protein ART consisted of 228 amino acid residues, the first 122 of which included an entire V_H region and the next 106 residues, the complete C_H3 domain. Totally lacking were the residues corresponding to the C_H1, hinge, and C_H2 regions.

The absence of IgG heavy-chain-associated structure was readily apparent serologically by comparison of the reactivity of amyloid protein ART to that of the Fc fragment derived from normal IgG. Determination of the amino acid sequence of amyloid protein ART provided definitive evidence of its two-domain (V_H-C_H3) primary structure. The amino-terminal 122 residues encompassed an entire V_H region and included segments encoded by the V_H (positions 1–98), D_H (positions 99–111), and J_H (positions 112–122) genes. The sequence of the first 98 residues, which included V_H segments FR1, CDR1, FR2, CDR2, and FR3, represented the product of a V_HIII gene. By sequence homology, amyloid protein ART was most closely related (≈85%) to the V_HIII protein TUR and to those proteins encoded by the rearranged V_HIII genes *333 Cl* and *VH 26 Cl* (14). Despite the large number of human D_H genes (14, 16), the variability in residue number and sequence introduced by base insertions (N regions) and the recombinatorial process (17–19), we concluded that the 12-residue portion of amyloid protein ART from positions 99–111 represented a D_H gene product. Further, the unusual Ala-Ala-Ala sequence at positions 105, 106, and 107 has been found in the CDR3 (D_H) regions of two other human immunoglobulin proteins, TEI and BUT (14). The remaining 11-residue FR4 portion of protein ART (positions 112–122) represented the product of either the J_H *1*, *-4*, or *-5* gene (14). Because of variability in the amino-terminal portion of the J segment that results from recombinatorial and other events (18, 19), the specific J_H-encoding gene could not be identified from the amino acid sequence data. The primary structure of the carboxyl-terminal 106 residues of amyloid protein ART was completely identical to that of the C_H3 domain encoded by the IgG1 C_H3 gene. Amyloid protein ART also contained the specific C_H3 aspartyl and leucyl residues (positions 138 and 140) associated with the serologically defined allotype of this gene, G1m(a), found on proteins of the IgG1 subclass (15). Despite the presence of the G1m(x)-associated C_H3 glycyl residue (position 213), the amyloid protein (and urinary-heavy-chain component 3) failed to express this allotype. The serologic detection of the G1m(x) on IgG components 1 and 2 implies the importance of conformational stability on the expression of this particular allotype.

The unusual γ-heavy-chain structure of amyloid protein ART differed from that of all other reported γ-heavy-chain disease proteins (20). In contrast to the intact two-domain V_H-C_H3 structure of amyloid protein ART, proteins associated with γ-heavy-chain disease have been characterized by extensive V_H-related internal deletions and, in some cases, the total absence of V_H, D_H, and J_H segments. These proteins were also found to lack the entire C_H1 domain but did contain

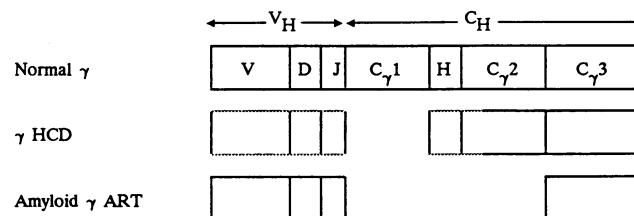|							
	←—V_H—→		←————— C_H —————→				
Normal γ	V	D	J	C_γ1	H	C_γ2	C_γ3
γ HCD							
Amyloid γ ART							

FIG. 4. Comparison of normal γ-heavy-chain V_H and C_H structure with that of γ-heavy-chain disease (HCD) proteins and that of the γ-heavy-chain-associated amyloid protein ART. The dashed lines indicate partial or total absence of V_H and C_H structure or, in some cases, lack of sequence data (20).

all or most of the hinge plus the entire C_H2 and C_H3 regions (Fig. 4). The partial or, in some cases, total absence of V_H-, D_H-, and J_H-encoded residues and lack of C_H1 and other C_H-region-associated structures in the γ-heavy-chain disease proteins have been attributed to aberrant biosynthesis as well as to the proteolytic mechanisms occurring posttranslationally (20). Studies at the genomic level have demonstrated in one such protein, IgG3 OMM (20), two noncontiguous deletions: The first involved virtually all of the V_H and the second eliminated the acceptor splice site at the 5' end of the C_H1 domain. The protein transcript resulted from the splice of the remaining J_H to the hinge-region exon. In another heavy-chain-disease protein, IgG1 RIV (21), the alteration or destruction of DNA splice sites produced a shortened mRNA in which the leader peptide exon was spliced directly to the hinge exon.

In the case of amyloid protein ART, we attribute the complete absence of the C_H1, hinge, and C_H2 regions to a mutational event that retained the 3' heptamer J_H and 5' nonamer C_H3 recognition sequences and resulted in the direct splice of the 3' end of the J_H gene to the 5' end of the C_H3 gene (19). As postulated for patient OMM, who had a "normal" light-chain-containing monoclonal IgG3 serum protein plus the IgG3-heavy-chain protein (22), the mutation present in amyloid protein ART presumably occurred after the heavy-chain $\mu \rightarrow \gamma$ switch (19), as evidenced by the normal structure of the monoclonal serum IgG component 1. The identical sequence of the first 32 V_H residues in both component 1 and the amyloid heavy chain implies that the two proteins are the products of the same V_HIII gene. It is not known whether the clonal events responsible for formations of component 1 (and 2) preceded or occurred simultaneously with the mutation responsible for the internally deleted heavy chain.

Heretofore, the exclusive light-chain nature of immunoglobulin- or myeloma-associated amyloidosis has been demonstrated through extraction and chemical characterization of amyloid tissue deposits. Whether the amyloidosis that has been noted clinically in several patients with γ-heavy-chain disease (4, 5) resulted from light- or heavy-chain deposition had not been established. Analyses of the protein contained in the amyloid deposits of patient ART established unequivocally its heavy-chain nature. Presumably, these deposits resulted from the precipitation in tissue of the heavy-chain protein (component 3) that was detected in the patient's urine. Although the exceptional two-domain heavy-chain composition of amyloid protein ART is comparable in molecular size to that of a light chain, the structural basis for the amyloidogenicity of heavy-chain- or light-chain-associated amyloid proteins remains to be determined. The ability to produce *in vitro* amyloid fibrils from a heavy-chain-disease protein (23) as well as from monoclonal light chains (1) and to localize within these components specific amyloidogenic peptides (24, 25) should provide further insight into the pathogenesis of immunoglobulin-associated amyloidosis.

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