Immunoglobulin D Myeloma and Amyloidosis: Immunochemical and Structural Studies of Bence Jones and Amyloid Fibrillar Proteins

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Urinary Bence Jones protein and amyloid fibril protein isolated from the subcutaneous tissue of a patient with IgD myeloma and associated amyloidosis were subjected to physicochemical and immunochemical identification. Peptide maps and amino-terminal tetrapeptide composition obtained from the two proteins were comparable. Immunochemical cross-reactivity between the two proteins, with other lambda-type amyloid and Bence Jones proteins, and with a serum component was demonstrated. The results suggest that the source of the amyloid fibril protein is an intact circulating light polypeptide chain as well as smaller amino-terminal fragments.

The frequent association between amyloidosis and multiple myeloma reported by Magnus-Levy in 1931 has been clarified greatly by recent observations linking immunoglobulin fragments to the genesis of amyloid fibrillar proteins. Magnus-Levy himself and, later, Osserman and co-workers postulated that deposits of amyloid might be directly related to gamma globulin or its subunits or both. Confirmation of this theory, however, awaited development of techniques for concentration and purification of the amyloid constituents. Several lines of data now indicate that, in many cases, amyloid fibrilar protein is derived predominantly from fragments of immunoglobulin molecules: (1) Immunochemical cross-reactivity has been demonstrated between human amyloid fibrils and Bence Jones proteins.

(2) The β-pleated sheet conformation characteristic of amyloid fibrillar proteins is also present or can be thermally induced in the variable region of the immunoglobulin light chain.

(3) Fibrils exhibiting tinctorial, crystallographic, and ultrastructural characteristics of amyloid can be created under physiologic conditions by proteolytic digestion of some human Bence Jones proteins.

(4) Amino acid sequence analysis has demonstrated areas of homology between some amyloid fibrillar proteins and the variable region of immunoglobulin light chains. To date, human amyloid fibrils from six patients, including primary amyloidosis, amyloidosis secondary to rheumatoid arthritis, and amyloidosis associated with immunoglobulin G myeloma, have been examined and found to show such homology.

These cases document the amyloidogenic potential of homogeneous populations...
of single immunoglobulin light chain fragments. We wish to report immunochemic characteristics and physicochemical analyses of Bence Jones and amyloid fibrillar proteins in a patient with immunoglobulin D myeloma and amyloidosis which demonstrate that multiple light chain fragments of different size may cooperate in the formation of amyloid.

CASE REPORT

The patient, a 71-yr-old white male, has been reported previously. Briefly, on June 6, 1970, 1 yr before admission, the patient was admitted to another hospital with renal failure of uncertain etiology and was started on chronic hemodialysis.

On June 11, 1971, he presented with cervical pain and headache, and radiologic examination of the cervical spine revealed fracture of the odontoid process with anterior dislocation of the first vertebra over the second. Physical examination revealed a sallow, lethargic, somewhat wasted male with numerous ecchymoses present over the upper extremities. The tongue was diffusely enlarged with semitranslucent nodules on the ventral and lateral surfaces. A 6 x 8-cm exophytic, fissured mass and adjacent firm subcutaneous nodules were present over the sacrum. The knees and shoulder joints were symmetrically enlarged with painless effusions and synovial thickening. Prominent subcutaneous nodules were present over the extensor surfaces of both forearms. Admission hematocrit value, platelet count, and white blood cell count were 16 volumes/100 ml, 381,000/cu mm, and 10,500/cu mm with a normal differential. Serum protein electrophoresis demonstrated no myeloma protein. Immunoelectrophoresis using antihuman serum revealed absence of IgM and reduction of IgA and IgG. An unidentified protein band was subsequently shown by immunoelectrophoresis to be IgD. Immunoelectrophoresis with specific antisera showed a homogeneous precipitation line to lambda-type light chains (see Fig. 1). Quantification of D-myeloma protein and lambda light chains was 1.61 mg/ml (normal, 0.03 mg/ml) and 11.7 mg/ml (normal, 4.5 ± 1.2 mg/ml), respectively. Urine Bence Jones heat test at pH 4.5 was negative, but immunoelectrophoresis of concentrated urine revealed Bence Jones-type lambda light chains. No anomalous immunoglobulin was detectable in the hemodialysate. Increased plasma cells with occasional multinucleated forms were present on bone marrow examination. Roentgenograms disclosed multiple cystic areas in the phalanges, carpal bones,
acromium, and tibial shaft. Tissue specimens obtained from lingual and sacral masses contained amyloid material.

Despite adequate calcium balance and vitamin D therapy, there was poor healing radiologically of the cervical fracture. Because of lack of vascular access, hemodialysis was discontinued on hospital day 70.

At autopsy, there was diffuse deposition of amyloid material in many organ systems. Most prominent was involvement of the synovia and para-articular soft tissues of all joints, particularly shoulders, elbows, and knees, with formation of tumor masses up to 10 cm in diameter. There was complete replacement of the odontoid process of the axis by amyloid. Nodular deposits of amyloid were scattered throughout the heart and pericardium, and there was extensive replacement of mucosal and muscular layers of the esophagus, gastric pylorus, and small and large intestines. In the lungs, there was amyloid material in small arterioles and in alveolar septae. The kidneys were contracted bilaterally. Remaining glomeruli and tubules showed no amyloid by light or electron microscopy, although there was involvement of the renal arteries and arterioles. Skeletal muscle, especially the diaphragm, contained amyloid, as did the nodules from the sacrum, eyelids, and forearms.

**MATERIALS AND METHODS**

**Purification of Bence Jones Protein (LBJ)**

Urine obtained from the patient was preserved with toluene and stored frozen. The Bence Jones protein was isolated by precipitation in water saturated with ammonium sulfate at 4°C. This precipitate was allowed to stand overnight at 4°C, then centrifuged for 15 min at 15,000 rpm. The sediment was washed twice with 80% saturated ammonium sulfate and the centrifugation procedure repeated. The resulting sediment was dissolved in saline buffered to pH 7.2 with 0.1 M sodium phosphate (phosphate-buffered saline, PBS), dialyzed against distilled water, and lyophilized.

The protein was further purified by preparative zone electrophoresis in sodium barbital buffer (pH 8.6; ionic strength, 0.06). The main fraction found in the beta region was subjected to gel filtration on a Sephadex G-100 column in PBS, and the fractions containing the major protein peak (approximately 48,000 daltons) were pooled, dialyzed against distilled water, and lyophilized.

**Preparation of Purified Amyloid Protein (LAm)**

Purified amyloid protein was prepared by a previously described method. Fifty grams of sacral amyloid tissue, shown by green polarization birefringence after alkaline Congo red staining to be approximately 90% composed of amyloid deposits, were fragmented, homogenized in an equal volume of saline at 4°C, and centrifuged at 12,000 g for 30 min. The supernatant was discarded. The homogenization and centrifugation procedure was repeated until the resulting supernatant was colorless, then the amyloid-containing fraction of the sediment was separated, again subjected to differential centrifugation, and lyophilized. The amyloid concentrate obtained, which is designated crude LAm, revealed masses of fibrillar material on electron-microscopic examination. The fibril concentrate was denatured under nitrogen at a concentration of 120 mg per 7.5 ml of 6 M guanidine-HCl buffered to pH 8.5 with 0.1 N Tris-HCl containing 50 mM dithiothreitol (DTT). This solubilized preparation was then adjusted to 5 M in guanidine-HCl and subjected to gel filtration chromatography on sequential Sepharose 4B and Sephadex G-100 columns equilibrated with 5 M guanidine-HCl in 1 N acetic acid by methods previously described. A single protein peak was demonstrated on Sephadex G-100 fractionation. The major protein fractions obtained from the G-100 column were pooled, dialyzed extensively against distilled water, and lyophilized. This material is designated LAm.

**Characterization of Amyloid Fibril Protein**

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed on proteins reduced with 1 mM DTT and alkylated with 0.01 M iodoacetamide. Samples and gels were prepared by methods described previously. Molecular weight determinations were performed by disc electrophoresis in SDS.
Peptide maps of tryptic digests of 5 mg of aminoethylated amyloid fibril and Bence Jones proteins were performed according to the method of Katz. Digestion was performed with a trypsin:substrate ratio of 1:100 at pH 8.7 in 0.1 M ammonium bicarbonate buffer.

Amino-terminal group analysis of both LAm and LBJ proteins was performed using the dinitrophenylation (DNFB) technique. The amino-terminal chymotryptic tetrapeptide was analyzed as previously described using 4 mg of LAm and 5 mg LBJ following digestion with alpha chymotrypsin (Worthington Biochemical Corp., Freehold, N.J.) at an enzyme to protein concentration of 1:100 for 5 hr at 37°C in 0.1 M ammonium bicarbonate, pH 8.5.

Preparation of Antibodies

Antibodies to native LBJ and crude LAm, the latter denatured with 6 M guanidine-HCl in 0.1 N Tris-HCl at pH 8.5 containing 50 mM DTT, were prepared as follows: A suspension of 1.5 mg of antigen was made in 1.5 ml of PBS and emulsified with an equal volume of complete Freund's adjuvant (Bacto-adjuvant complete, H H 37 Ra, Difco Laboratories, Detroit, Mich.). The emulsion was injected into the four footpads of New Zealand white rabbits. Booster injections were given subcutaneously into the back with 1.0 mg of protein prepared in the same way; boosters were initially given at 2-3 wk and monthly thereafter. Blood was taken about 10 days after the initial booster and at various subsequent intervals. Antibody activity against LBJ and LAm appeared 1 and 3 mo after initial inoculation, respectively.

Immunodiffusion was carried out on glass plates overlaid with 1% Agarose (Seakem Agarose, Bausch & Lomb) in barbital buffer (ionic strength, 0.03; pH 8.6) at room temperature overnight and then washed in PBS before photography. Lambda and kappa Bence Jones proteins obtained through the courtesy of Dr. W. D. Terry and Dr. F. W. Tischendorf and amyloid fibril proteins previously characterized by were used as standard antigens. Antigens were used at concentrations of 0.2-1.0 mg per ml PBS. Antisera to LBJ (0.5 ml) was absorbed with three different lyophilized lambda Bence Jones proteins (15 mg each).

RESULTS

Chemical and Physical Analysis

The purified amyloid material represented 41% of the dry weight of the original starting material. SDS-polyacrylamide gel electrophoresis of this purified material revealed three distinct protein bands with molecular weights of 23,000 daltons, 17,500 daltons, and 12,500 daltons, respectively (see Fig. 2). Corresponding protein bands were observed in the crude amyloid preparation, indicating that the major protein components of the crude preparation were retained during purification. The Bence Jones protein isolated from the patient gave a single distinct band with molecular weight of 23,000 daltons, corresponding to the amyloid protein band of highest molecular weight.

Structural similarity was also demonstrated by peptide maps of the purified amyloid fibril and Bence Jones proteins (Fig. 3). Except for two extra peptides in the amyloid protein and minor variations in staining intensity, the maps were identical.

The amino-terminal amino acid analysis indicated that the amino termini of both LBJ and LAm proteins were unreactive to DNFB, suggesting the presence of pyrrolid-2-one-5-carboxylic acid (Glp) as the amino-terminal amino acid. The amino-terminal acid chymotryptic tetrapeptide was unreactive to ninhydrin and demonstrated identical Glu-Ser-Ala-Leu composition for both proteins (Table 1). This composition is characteristic of lambda-type light chains of subgroup II or IV.
Fig. 2. SDS disc gel electrophoretic comparison of crude amyloid fibril concentrate, crude LAm (Lc), purified amyloid fibril proteins, LAm (La) and patient's BJ protein, LBJ (Lb). Arrow denotes light chain monomer. Anode is at bottom.

Fig. 3. Peptide map comparison of shared tryptic peptides of amyloid fibril protein and Bence Jones protein of patient (clear circles) and extra peptides present in amyloid protein only (black circles). PR is phenol red marker and origin is at X. Arrow designates chromatographic dimension.
Table 1. Chymotryptic Tetrapeptide Composition*

<table>
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<th>LBJ</th>
<th>LAm</th>
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<tr>
<td>Glu</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Ser</td>
<td>0.86</td>
<td>1.00</td>
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<tr>
<td>Ala</td>
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<tr>
<td>Leu</td>
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<td>Rif†</td>
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<td>0.60</td>
</tr>
<tr>
<td>Yield‡</td>
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<td>21.9%</td>
</tr>
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*Molar ratio of amino acids of acidic tetrapeptide compared to Glu as 1.00.
†Electrophoretic mobility relative to Glp-Ala.
‡Calculated as per cent of moles recovered per mole starting material.

Fig. 4. Immunodiffusion demonstrating similar individual specific determinants of BJ protein (LBJ), a component in the patient's serum (LSe), and the amyloid protein of the same patient (LAm). (A) Antiserum to patient's Bence Jones protein (anti-LBJ) precipitates with LBJ and gives a partial cross-reaction with five different lambda Bence Jones proteins, demonstrating the presence of antibodies against common light chain determinants and idiotypic determinants of LBJ. (B) After absorption of this antiserum with three different lambda-BJ proteins, the antiserum retains activity only against LBJ. (C) Demonstration of a common idiotypic in the patient's serum (LSe), Bence Jones protein (LBJ), and amyloid fibril protein (LAm) after absorption as above.
Immunochemical Analysis

Unabsorbed antiserum to LBJ, when tested in immunodiffusion against its homologous antigen, LBJ, and five different lambda Bence Jones proteins, demonstrated a line of partial identity between LBJ and all five lambda Bence Jones proteins tested (Fig. 4A). The reaction between the five lambda Bence Jones proteins and anti-LBJ disappeared with absorption of anti-LBJ with the three lambda Bence Jones proteins, but the reaction with LBJ persisted (Fig. 4B). When tested against absorbed anti-LBJ, a precipitin line of identity occurred with LBJ, LAm, and the patient’s serum (Fig. 4C). No reaction could be demonstrated between anti-LBJ, either absorbed or unabsorbed, and any of five different kappa Bence Jones proteins.

Studies with antiserum to LAm revealed reaction of partial identity between LAm and four previously reported lambda-type amyloid proteins, but no demonstrable reaction against three kappa-type amyloid proteins. When an antiserum to a previously characterized lambda-type amyloid protein (VI) was tested against LAm and three other lambda-type amyloid proteins, a line of identity could be demonstrated. Antiserums to previously characterized kappa-type amyloid proteins (IX, VIII) failed to react with the LAm protein.

Finally, LAm was examined for the presence of nonimmunoglobulin material using antiserum to an amyloid protein (IV) of unknown origin. No reaction could be demonstrated between LAm and the antiserum.

DISCUSSION

A large group of amyloid fibril proteins have been shown to possess as their major protein component some portion of an immunoglobulin light polypeptide chain. Acrylamide-gel electrophoresis of purified amyloid fibril protein obtained from this patient demonstrates three major protein components which are shown to share certain structural properties with a lambda-type Bence Jones light chain isolated from the patient’s urine. The evidence linking the amyloid components and urinary light chain is as follows: (1) Peptide maps are nearly identical for both proteins. For reasons to be discussed, we attribute minor differences in the maps to variations in the carboxy-terminal tryptic peptides obtained from the two smaller amyloid protein components and not to the presence of a nonimmunoglobulin molecule. (2) The amino termini of both proteins were unreactive to DNFB. The amino acid compositions of the N-terminal chymotryptic tetrapeptides obtained from the two proteins are identical to each other and to the amino-terminal chymotryptic tetrapeptides of lambda-type light chains of subgroup V\(\lambda_{11}\) or V\(\lambda_{14}\). (3) Immunochemical studies demonstrate shared idiotypic determinants between the two proteins. Immunochemical analysis further demonstrates the presence in the patient’s serum of a protein having idiotypic determinants in common with both LBJ and LAm. These results confirm the characterization of LAm as a lambda-type amyloid fibril protein exhibiting common structural determinants with the Bence Jones protein that was present in the patient’s circulation and urine.

In most instances of immunoglobulin-related amyloid, the protein component of the amyloid is homogeneous and consists of either an intact light polypeptide chain or an amino-terminal fragment of a light chain. The
Fig. 5. Schematic diagram of the three components of the amyloid fibril protein (LAm) illustrating the means by which amyloid tryptic peptides structurally identical with but electrophoretically different from corresponding Bence Jones (LBJ) tryptic peptides might arise (see text). The peptides unique to amyloid are circled. Hypothetical sites of proteolytic cleavage by trypsin are indicated by arrows. Calculated molecular weights are indicated to the right of each protein.

Demonstration by acrylamide-gel electrophoresis after reduction and alkylation of three major protein components, all with antigenic determinants that are comparable to those of the Bence Jones protein, suggests the presence in this particular case of multiple amino-terminal light chain fragments. Molecular weight determination of the three components of LAm reveals values consistent with those expected for an intact light immunoglobulin chain (~22,500), an amino-terminal fragment slightly larger than a light chain variable region (~11,000), and an amino-terminal fragment of intermediary size. The finding on peptide maps of two peptides unique to the isolated amyloid is compatible with this suggestion and may arise in the following manner. During tryptic digestion, most of the individual peptides obtained from any one of the amyloid components will be structurally identical with and electrophoretically indistinguishable from the corresponding peptides obtained from the other amyloid components and from the Bence Jones protein. However, the peptide obtained from the carboxy-terminal portion of either of the two smaller amyloid proteins will be structurally identical with the corresponding Bence Jones peptide only at the amino terminus but will lack residues at the carboxy terminus which are present in the Bence Jones peptide (see Fig. 5). Thus, the carboxy-terminal amyloid tryptic peptides will differ in electrophoretic mobility from the corresponding Bence Jones peptides. The observation of two peptides which are unique to amyloid is expected, since each of the smaller amyloid components will contribute one of the extra peptides.

The finding of one or more amino-terminal segments of a light chain in addition to the intact light chain has been noted previously in two other amyloid fibril proteins (VIIIa and VIIIb, IIIa and IIIb). Tan and Epstein have recently shown that lysosomal enzymic digestion of intact light chains frequently produces polymeric precipitates composed of the whole light chain and either the variable or constant region halves. More recently, they have demonstrated that these precipitates have the tinctorial and electron-microscopic characteristics of amyloid fibrils. Therefore, it may be postulated that, during proteolytic digestion, cleaved variable region fragments may coprecipitate with an intact light chain to form amyloid fibrils. We believe this to have occurred in the
present case. The almost constant finding of an amino-terminal variable region fragment in both naturally occurring and artificially created amyloid proteins of immunoglobulin origin indicates that the variable region of the light chain plays a special role in the genesis of amyloid fibrils.

Another group of amyloid fibril proteins, particularly those isolated in cases of secondary amyloidosis and some familial forms of amyloidosis, have been shown to possess as their major component a protein of unknown origin (AA). While these amyloid fibrils may also contain immunoglobulin material, the converse, that is, the presence of protein AA in amyloid fibrils of immunoglobulin origin, is a subject of current debate. The failure to demonstrate, by sensitive immunochemical techniques, nonimmunoglobulin material in the present case is in keeping with our previous observations.

Several distinguishing features of IgD myeloma are illustrated by this case. Hobbs and Corbett have stressed the frequent presence of clinically recognizable extraosseous plasmacytomas in IgD myeloma. In this case, extraosseous tumors were composed of amyloid. Periarticular and soft tissue amyloid masses were also observed in a patient with IgD myeloma reported by Friman et al.

It is conceivable that the periarticular amyloid collections observed in this case and that described by Friman are extraosseous plasmacytomas in which the immunoglobulin proteins produced by plasma cells are transformed into amyloid fibrils. The means by which this transformation occurs in vivo is uncertain, but it might occur by proteolysis within cellular lysosomal compartments by a mechanism similar to that shown to occur in vitro. Similar mechanisms have been postulated to explain the presence of localized nodular pulmonary amyloidosis.

In addition, the finding that the patient’s myeloma protein contained light chains of the lambda, rather than kappa, class is typical of IgD myeloma. Again, this may have special significance in this case, since there appears to be a relationship between amyloidosis and the paraprotein light chain associated with amyloidosis. The ratio of kappa to lambda monoclonal light chains in paraproteins associated with myeloma-related amyloidosis approaches 3:5, which is in striking contrast to the ratio of 3:2 observed in paraproteins of myeloma uncomplicated by amyloidosis. For this reason, it has been suggested that lambda-type light chains are more “amyloidogenic” than kappa-type light chains, a characteristic that may be related to conformational differences in the two light chain molecules.

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