Primary Amyloidosis with Plasmacytic Dyscrasia and a Tetramer of Bence Jones Type Lambda Globulin in the Serum and Urine

By D. M. Parr, W. Pruzanski, J. G. Scott and D. M. Mills

An abnormal protein has been isolated from both the serum and urine of a patient with generalized amyloidosis and plasmacytic dyscrasia. Immunological and biochemical techniques were used to demonstrate that the proteins isolated from each source were identical and were a tetramer of a lambda-type Bence Jones globulin. This appears to be the first reported occurrence of a urinary Bence Jones tetramer. The sedimentation coefficient of the protein was 5.05, and its molecular weight 79,000. Reduction and alkylation, electrophoresis, and ultracentrifugation showed it to consist of four monomers covalently bonded to form two dimers, which were in turn noncovalently bonded to form the tetramer.

Although normal urine contains traces of polymers of free light chains and a polymer of Bence Jones globulin as a minor component has been found in the urine in myeloma, a tetramer of Bence Jones globulin as a sole abnormal protein in the urine has not been encountered until now. Two reported patients with multiple myeloma and a tetramer of Bence Jones globulin in the serum did not excrete it in the urine, presumably because of its high molecular weight. Another patient with primary amyloidosis had Bence Jones proteinemia without proteinuria, but no molecular weight of the protein was reported.

A patient presenting with generalized amyloidosis, plasmacytic dyscrasia, and large amounts of a tetramer of Bence Jones type lambda globulin in the serum and in the urine is reported here. To the best of our knowledge, it is the first such case described. A brief structural analysis, and a comparison of the serum and urinary Bence Jones globulins has been made, and the relationship of generalized amyloidosis to plasmacytic dyscrasia is discussed.

Materials and Methods

Fresh serum and urine were tested immediately or stored at −20°C for further analysis. Total protein was estimated by the biuret method. Electrophoresis was performed

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on cellulose acetate membranes, using barbital buffer, pH 8.6, ionic strength 0.075, and the membranes were scanned with a Beckman Analytrol. The method of Smithies was employed for starch gel electrophoresis in borate buffer, pH 8.6, (0.02 M boric acid, 0.008 M sodium hydroxide) and that of Smithies et al. for electrophoresis in urea-formate buffer, pH 3.6 (0.05 M formic acid, 0.01 M sodium hydroxide, 8 M urea).

Immunelectrophoresis was carried out according to the method of Grabar and Williams. Antisera against whole human serum and against Fc, Fab, IgA, IgG and IgM proteins were obtained from Hyland Laboratories, Los Angeles, and anti-IgD antiserum from Melpar Laboratories, Falls Church, Va. Antisera against Bence Jones type kappa and lambda proteins and against IgE were prepared in our laboratory, in rabbits, by multiple injections of the respective purified human proteins in Freund’s adjuvant.

Radial immunodiffusion analysis was performed according to the method of Mancini et al. Immunoquantitation of IgG, IgA, IgM, and IgD was done using immunoplates from Hyland Laboratories and from Kallestad Laboratories, Minneapolis. "Oz" typing was kindly performed by Dr. Daniel Ein, National Institutes of Health, Bethesda, Md. "St" typing was done employing our own antiserum against "St" Bence Jones globulin according to the technique of Tischendorf and Osserman. "111" typing was kindly performed by Dr. F. W. Tischendorf, Tubingen, Germany.

Cryoproperty was examined by storing fresh samples of serum and urine at 4°C for 48 hours. The presence of pyroglobulins was tested for by gradual warming of the serum up to 56°C. The Bence Jones heat test was performed by the successive gradual warming, boiling, and cooling of urine acidified to pH 4.0.

M-components were isolated from the whole serum, and also from a precipitate of the urine obtained at 45 per cent saturation with ammonium sulfate. Samples of serum and of the urinary precipitate were dialyzed for at least 72 hours against several changes of 0.01 M phosphate buffer pH 8.3, and then applied to a column of DEAE-cellulose (Whatman, DE 32), 1 × 25 cm, equilibrated with the same buffer. An elution gradient, with concentration increasing up to 0.3 M sodium dihydrogen phosphate, was employed. The eluted proteins were further purified by passage through a column of Sephadex G-100, 1 × 100 cm, in 0.2 M sodium chloride. The purity of the eluted M-components was verified by electro- and immunelectrophoresis employing a variety of antisera and also by ultracentrifugation.

Sedimentation velocity measurements were made at 20° ± 0.05°C in a Spinco

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Moles/22,000 g Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>11.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.1</td>
</tr>
<tr>
<td>Aspartic acid*</td>
<td>15.8</td>
</tr>
<tr>
<td>Threonine</td>
<td>22.1</td>
</tr>
<tr>
<td>Serine</td>
<td>27.0</td>
</tr>
<tr>
<td>Glutamic acid*</td>
<td>24.4</td>
</tr>
<tr>
<td>Proline</td>
<td>17.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>13.5</td>
</tr>
<tr>
<td>Alanine</td>
<td>14.0</td>
</tr>
<tr>
<td>Half cystine</td>
<td>4.3</td>
</tr>
<tr>
<td>Valine</td>
<td>18.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.8</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.8</td>
</tr>
<tr>
<td>Leucine</td>
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</tr>
<tr>
<td>Tyrosine</td>
<td>8.3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.2</td>
</tr>
</tbody>
</table>

* Aspartic and glutamic acids include asparagine and glutamine, respectively.
model E ultracentrifuge, using a 2° single sector cell with the schlieren optical system and a rotor speed of 59,780 rpm. A Nikon shadowgraph was used to estimate the positions of the boundaries on photographs taken at 8-minute intervals. The molecular weight was determined by the sedimentation equilibrium method of van Holde and Baldwin at 9000 rpm at a temperature of 20.0° ± 0.05°C using a double sector cell and ultraviolet scanner. A value of 0.73 ml/g was used for the partial specific volume, calculated from the amino acid composition (Table 1) by the method of Cohn and Edsall. The solvent used for both sedimentation velocity and equilibrium studies was 0.18 M sodium chloride, 0.04 M boric acid, 0.02 M sodium hydroxide.

Disulfide bonds were reduced and alkylated in 1 per cent solutions of the purified M-components, isolated from serum and urine, and dialysed against 0.5 M Tris HCl, pH 8.2. The dialyzed proteins were reduced by dithiothreitol at 0.03 M and left to stand for 3 hours at room temperature. They were alkylated by the addition of sufficient iodoacetamide to make a final concentration of 0.09 M, and kept at room temperature for 1 hour prior to examination by electrophoresis in urea-formate starch gel.

The amino acid composition of the urinary M-component was determined by analysis in a Spinco Model 120C analyzer after 24-hour hydrolysis at 110°C under vacuum in constant boiling HCl.

CASE REPORT

H. M., a 58-year-old man, was healthy until July 1968, when he noted a pain in the left shoulder and scapula, and paresthesiae over the left thumb and index finger. Atrophy of the left deltoid muscle was noted. An X-ray showed osteoarthritic changes of the lower four cervical vertebrae. In October 1968, anemia (Hb-10.5 g per cent) and heavy proteinuria were found. There was also shortness of breath on exertion, and swelling of the ankles. The patient was hospitalized. There was no lymphadenopathy and no hepato- or splenomegaly. There was also no enlargement of the tongue, or skin changes. Ejection systolic murmur, grade 2/6, was heard in the pulmonary area. Hemoglobin was 11.0 g per cent, reticulocytes 1.8 per cent, white cells 6000/cu mm with normal differential count, and platelets 170,000/cu mm. Sedimentation rate was 51 mm in the first hour. Blood urea nitrogen was 16 mg per cent, uric acid 2.6 mg per cent, calcium 9.0 mg per cent, alkaline phosphatase 2.1 Bodansky units. Urinalysis showed heavy proteinuria. A chest X ray and electrocardiogram were normal. A skeletal survey showed cervical osteoarthritis. Bone marrow was replaced with lymphocytoid, plasmacytoid, and large reticulum-like cells. A renal biopsy showed equivocal evidence of amyloidosis and some glomerular mesangial thickening. Gradually, the patient developed aching in temporal muscles while chewing. His voice was normal on awakening but deteriorated to a hoarse croak during the day. A weakness in the gluteal muscles appeared on walking distances of less than 100 yards. Electromyography suggested a myopathic pattern, especially in the deltoid and biceps muscles. The patient was given a course of prednisone (30 mg) and chlorambucil (2-4 mg a day) for 3 weeks, but without significant benefit. In March 1969, hemoglobin had dropped to 8.4 g per cent and a blood film showed a leukoerythroblastic picture. Congestive heart failure with massive edema of the legs developed. Digoxin, diuretics, and a blood transfusion were administered in addition to increasing doses of steroids, chlorambucil, and melphalan. He experienced attacks of convulsive movements with loss of consciousness, and died suddenly on May 30, 1969. At autopsy, numerous petechiae and hemorrhages were noted in the skin. There was marked pitting edema over the lower extremities. The heart weighed 544 g. An ill-defined hemorrhagic zone of 2 × 1½ cm was noted in the left posterior myocardium and small gray foci were seen in the interventricular septum. The liver weighed 2070 g and the spleen 164 g. No lymph node enlargement was found. The marrow of sternum, ribs, iliac crest, and vertebrae had a uniform, solid gray-white appearance with no bony expansion or destruction. The cut surfaces were firm and dry, and it was difficult to express marrow from ribs or sternum. This diffuse marrow infiltrate was clearly different from the nodular soft gray and hemorrhagic lesions usually seen in multiple myeloma. The skull was normal.
The microscope sections from the bone marrow showed dense infiltrate of plasmacytes, lymphocytes, and lymphocytoid cells. The trabecular bone was clearly defined and there was no evidence of destruction of the cortex, or of adjacent soft tissue involvement. Deposits of amyloid were present in the walls of arteries and veins of the bone marrow. In the myocardium, diffuse fibrosis was seen, together with marked amyloid infiltration. Renal tubular cells were swollen and proteinaceous material was seen in the lumen. Amyloidosis of the afferent arterioles and intralobular vessels and in the glomeruli was noted. Amyloid was also found in the walls of arteries and veins in the heart, portal area of the liver, thyroid, spleen, periadrenal fat, and prostate. The vessels of the brain were intact. In all the tissues, deposits of amyloid stained weakly positive with Congo red, but were strongly positive when examined with alkaline Congo red and with methyl violet. All deposits showed positive birefringence when examined by a polarizing light microscope.

**Immunochemical Studies**

Total protein concentration in the serum was 7.15 g per cent, with albumin 2.78 g per cent, alpha-1 globulin 0.4 g per cent, alpha-2 globulin 3.18 g per cent, beta globulin 0.51 g per cent, and gamma globulin 0.28 g per cent. Cellulose acetate electrophoresis showed an abnormal spike of alpha-2 mobility (Fig. 1). Starch gel electrophoresis in borate buffer, pH 8.6, showed an abnormal band migrating between the bands of albumin and transferrin. Immunoelectrophoresis of the serum showed an abnormal precipitation line in the alpha-1–alpha-2 region, which was elicited only with anti-Bence Jones type lambda antiserum. No abnormal precipitation lines of similar or other mobility were observed using antisera against Fab, Fc, IgG, IgA, IgM, IgD, IgE, or Bence Jones type kappa. Immunoquantitation of the serum showed IgG 205 mg per cent, (normal 1137 ± 200 mg per cent), IgA 52 mg per cent (normal 231 ± 73 mg per cent), IgM 23 mg per cent (normal 103 ± 36 mg per cent)
and IgD less than 2.0 mg per cent (normal 0.3–40 mg per cent). No increase in IgE was found in undiluted serum when examined by Ouchterlony radial immunodiffusion. The Sia test was negative, and no cryo- or pyroglobulins were found. Serum lysozyme was 9.1 μg/ml (normal 5–15 μg/ml).

The urinary protein concentration was 0.66 g per cent, accounting for 14.2 g of protein excreted in 24 hours. The concentration of albumin was 0.416 g per cent, alpha-1 globulin 0.049 g per cent, alpha-2 globulin ( + M-component) 0.152 g per cent and beta globulin 0.043 g per cent. No band corresponding to gamma globulin was observed on cellulose acetate electrophoresis. Immuniquantitation of the urinary proteins showed an IgG excretion of 4.2 mg per cent (90 mg/24 hr) and IgA 1.2 mg per cent (26 mg/24 hr). No IgM or IgD were excreted. The Bence Jones heat test was positive at 57°C. On both cellulose acetate and starch gel electrophoresis the urinary M-component migrated with mobility identical to that found in the serum. Immunoelectrophoresis of the urine showed an abnormal precipitation line of alpha-1–alpha-2 mobility produced only with anti-Bence Jones type lambda antiserum. Purified serum and urinary Bence Jones globulins were found to be of Oζ (−), St (−), I I I (−) type. Urinary lysozyme was 2.42 μg/ml (normal 0–2.0 μg/ml).

**Special Studies**

M-components were separated from the serum and from the urinary precipitate by ion-exchange chromatography. The elution patterns are shown in Fig. 2. The peaks from both preparations were pooled as shown and concentrated in a Diaflo ultrafiltration cell (Amicon Corp., Lexington, Mass.), after which the main peaks were further purified by gel filtration. The fractions thus
obtained were compared to the whole serum and urinary protein by means of starch gel electrophoresis in borate buffer pH 8.6 (Fig. 3).

It was noted that the serum and urinary M-components were eluted from the Sephadex G-100 column in a smaller total volume than would have been anticipated for monomeric or dimeric forms of light chains. The gel filtration of these proteins was therefore repeated, comparing the elution patterns to

![Starch gel electrophoretic pattern at pH 8.6 of serum (S), urinary protein precipitate (UP), and fractions from DEAE-cellulose chromatography (A, B, C, and D, corresponding to the peaks so labeled in Fig. 2); peaks B and D after purification by gel filtration.](image)

**Fig. 3.**—Starch gel electrophoretic pattern at pH 8.6 of serum (S), urinary protein precipitate (UP), and fractions from DEAE-cellulose chromatography (A, B, C, and D, corresponding to the peaks so labeled in Fig. 2); peaks B and D after purification by gel filtration.

![Gel filtration of serum and urinary M-components on Sephadex G-100. (A) Solvent 0.2 M sodium chloride. (B) Solvent 6 M urea—0.1 M formate.](image)

**Fig. 4.**—Gel filtration of serum and urinary M-components on Sephadex G-100. (A) Solvent 0.2 M sodium chloride. (B) Solvent 6 M urea—0.1 M formate.
that of a known dimer of Bence Jones type lambda globulin isolated from the urine of a myeloma patient. These elution patterns are illustrated in Fig. 4A, which shows that the M-components were each eluted in about two thirds of the volume required to eluate the control dimer. Fig. 4B shows the results obtained when a dissociating solvent, 6 M urea–0.1 M formate, was used, with all other variables unchanged. Under these conditions, the M-components were not eluted until the same volume of eluant had been collected that was required to elute the control dimer. The second, smaller, absorption peak was due to the presence of a small amount of monomeric material, produced from the dimers by dissociation.

Urinary and serum proteins eluted from these columns were concentrated, and examined by starch gel electrophoresis, with the results illustrated in Fig. 5. In the borate gel (Fig. 5A) the proteins eluted in 0.2 M sodium chloride both showed a single band in a position identical to that occupied by the abnormal band in the whole serum. The same proteins, eluted in the dissociating solvent, showed only traces of material in that position, whereas the major part had migrated further towards the anode.

The samples eluted in the urea-formate were also examined by starch gel electrophoresis in urea-formate (Fig. 5B). The isolated M-components under these conditions had almost the same mobility as the main band of the dimer Bence Jones protein used as a control. The faster-moving band seen in the control protein was due to the presence of monomers, produced by the dissociating effect of the urea in the gel. The control protein in the gel illustrated had not been subjected to gel filtration in urea.

The results, so far, suggested that the serum and urinary M-components of the patient are identical tetramers of Bence Jones globulin, and to confirm
this ultracentrifugation studies were carried out. Sedimentation velocity analyses of the purified serum and urinary components at concentrations of approximately 7 mg/ml showed $S_{20,w}$ values of 5.0 in each case. The molecular weight of the urinary protein, as determined by the sedimentation equilibrium method, was found to be 79,000. These results were consistent with the presumption that the patient’s M-components are tetramers of Bence Jones globulin.

The formation of dimers, but not monomers, in the presence of a dissociating solvent suggested that the monomers may be covalently bonded into dimers. The isolated M-component therefore was reduced and alkylated in the presence of 8 M urea, and the product of this reaction examined by starch gel electrophoresis in urea-formate. The mobility of this product was intermediate between that of the dimer of a known lambda Bence Jones globulin and the monomer formed from this dimer by the action of the urea in the gel. The probable explanation of this phenomenon is that, since reduction and alkylation at high urea concentration leads to the breaking of intra- as well as interchain disulfide bridges, the resulting polypeptide chains have a less compact structure, which causes their passage through the gel to be slowed down. The experiment was repeated at 0, 2, 4, and 8 M urea, with the results illustrated in Fig. 6. It was observed that the product of reduction and alkylation in the absence of urea, which would retain intact intrachain disulfide bridges, had the same electrophoretic mobility in urea-formate starch gel as the

**Table 2.—Sedimentation Coefficients ($S_{20,w}$)***

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent Systems</th>
<th>Buffer</th>
<th>Buffer/8 M Urea</th>
<th>Buffer, Urea Dialyzed Out</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact M-component (M)</td>
<td></td>
<td>5.0</td>
<td>1.6</td>
<td>3.4</td>
</tr>
<tr>
<td>M, partially reduced and alkylated</td>
<td></td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M, completely reduced and alkylated</td>
<td></td>
<td>0.9</td>
<td></td>
<td>Aggregated</td>
</tr>
<tr>
<td>Control Bence Jones dimer (^{16})</td>
<td></td>
<td>3.5</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Light chain monomer (^{16})</td>
<td></td>
<td>3.5</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Bence Jones monomer (^{4})</td>
<td></td>
<td></td>
<td>2.3</td>
<td></td>
</tr>
</tbody>
</table>

* Corrected for viscosity and density of solvents.
monomers formed by dissociation of the known dimer of Bence Jones globulin used as a control, which also would have unbroken intrachain disulfide bonds.

Further sedimentation velocity measurements were carried out on dimers formed by dialysis of intact tetramers against buffer containing 8 M urea, and on both partially and completely reduced and alkylated monomers, also in the presence of 8 M urea. All samples were of the same protein concentration after dialysis. After centrifugation, the solutions were removed from the cells, dialyzed against three changes of buffer containing no urea, and sedimented again. The sedimentation coefficients thus obtained are given in Table 2, and confirm that the effect of urea is to dissociate the initial tetramer into dimers, whereas monomers are formed in urea solutions only after previous reduction and alkylation. It is of interest that the dissociating effect of urea appeared to be irreversible; after removal of the urea by extensive dialysis the original Bence Jones globulin had a sedimentation coefficient closely corresponding to that of a dimeric Bence Jones protein, whereas the sedimentation coefficient of the reduced and alkylated material, also after dialysis to remove the urea, agreed with that of a monomer obtained from a serum Bence Jones tetramer by Caggiano et al. The tetramer here reported thus consists of four monomers, covalently bonded to form two dimers, which in turn are noncovalently bonded to form the tetramer.

DISCUSSION

The patient's disease may be classified as widespread amyloidosis, with plasmacytic dyscrasia manifested by a diffuse infiltration of the bone marrow by plasma cells at various stages of maturity, and the presence of Bence Jones protein in the serum and urine. This diagnosis, rather than that of multiple myeloma, is favored by the absence of osteolytic lesions or osteoporosis, the absence of destruction of the cortical bone, and by the dense and homogeneous infiltration of the bone marrow, which contrasted clearly with the tumorlike soft masses of myeloma.

Similarities between amyloidosis and multiple myeloma, such as the frequently diminished level of normal immunoglobulins, and the frequent presence of M-components in serum and/or urine have led Osserman to speculate that there may be a relationship between the gamma globulin system and amyloidosis. Pick has shown by immunofluorescence studies that although amorphous masses of amyloid could not be labeled, an altered tissue surrounding these masses showed a prominent fluorescence with labeled antisera against Bence Jones globulins of both types, IgG, complement, and albumin, with fluorescence most evident against Bence Jones globulin of the homologous type. It was also shown that fluorescein-labeled Bence Jones globulin has affinity to amyloid, and this did produce fluorescence of the amyloid masses. Sellin, using different techniques, could not confirm a relationship between Bence Jones globulins and amyloid.

Approximately 60 of 75 patients with primary amyloidosis have had serum M-components that were identified immunoelectrophoretically. IgG was found in 50 per cent, Bence Jones globulin in 25 per cent, and the rest were almost equally divided between IgA and IgM types. Of considerable inter-
est is the observation that, of 55 typed urinary Bence Jones globulins in primary amyloidosis, only 24 were of type kappa, contrasting with the usually observed 2:1 ratio, in multiple myeloma, of kappa:lambda. Nor is this ratio maintained in cases of myeloma in which only Bence Jones proteins are produced, since in three combined series of light chain disease, 33 kappa and 32 lambda Bence Jones globulins have been found.32-34

Bernier and Putnam2 have noted that the urine of patients with myeloma may contain various polymeric forms of Bence Jones globulin. One of four patients studied excreted small amounts of polymer with a sedimentation coefficient of 5.5 S, in addition to the major component of lower S value. Berggard1 reported that 10-20 per cent of free light chains in normal human urine and in tubular proteinuria, were in tetrameric form, having a molecular weight of 88,000 and S\(_{20,\text{w}}\) = 5.3, but suggested that these tetramers were formed in the urine.

Three occurrences of probable or verified Bence Jones tetramers in the plasma have been reported, all of which were of lambda type, and none of which were present with Bence Jones proteinuria. Molecular weights in two examples were 84,000 and 88,000 and S\(_{20,\text{w}}\) values were 4.9 and 5.4. One of these was a patient with primary generalized amyloidosis,5 another was a patient with multiple myeloma,3 and the third a case of plasma cell leukemia, where an IgG/L serum M-component was also present.4 In the latter two cases, as in the present report, the tetramer consisted of two dimers, bound together noncovalently, each dimer being formed by disulfide-linked monomers. The authors of the third report suggested that all four monomers may be linked to produce a circular molecule.

To the best of our knowledge, our patient is the first reported case of primary generalized amyloidosis with a tetramer of Bence Jones type lambda globulin in the blood and in the urine. It seems also to be the first case where such a tetramer appeared in the urine as a sole abnormal component. The kidneys in our patient showed amyloidosis of the vessels and of the glomeruli, but there was no azotemia. It seems that the free passage of Bence Jones globulin into the urine was due to abnormal glomerular filtration, but a large amount of this protein was also retained in the blood.

ACKNOWLEDGMENTS

We wish to thank Dr. R. Gunton of the Department of Medicine, Victoria Hospital, London, Ont., for making available the serum and urine used in this study. We are also grateful to Dr. C. E. Connell for much helpful discussion, to Mr. David Kells for assistance with the ultracentrifugation, and to Mrs. Ilona Csermely and Mrs. S. Saito for general technical assistance.

REFERENCES


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