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Polymerization of intact β_2-microglobulin in tissue causes amyloidosis in patients on chronic hemodialysis

(amyloid, diagnosis and typing)

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ABSTRACT **Systemic amyloidosis with a predilection for bone and synovium may complicate the course of patients on long-term hemodialysis. This form of amyloidosis can be typed as distinct from other amyloid diseases by using small tissue samples obtained by bone biopsy and at postmortem. Immunoblot analysis of two-dimensional gels of partially solubilized amyloid fibrils established that tissue deposits are composed of monomers, dimers, and higher polymers of β_2-microglobulin (β_2m) and that amyloid P component was also present. Anti-β_2m antiserum recognized fibrils, as shown by immunoelectron microscopy. Purified monomer isolated from dissociated fibrils yielded peptides corresponding to the entire known sequence of β_2m. Virtually all serum β_2m, as well as that present in tissue fluid bathing amyloid fibrils, was monomeric. Hemodialysis-related amyloidosis is an example of a deposition disease occurring in hemodialysis patients. We have shown conclusively that, in this amyloid disease, polymerization of an intact normal serum protein to a fibrillar configuration may occur without proteolysis. We propose the designation Aβ_2m for this form of amyloid fibril subunit protein.**

β_2-Microglobulin (β_2m) is a protein of low molecular mass, first isolated from the urine of patients with tubular proteinuria (1), that has about 30% homology to the immunoglobulin constant region domains (2, 3). Its circulating pool in plasma and other body fluids is derived largely from the shedding of this protein from the surfaces of nucleated cells, where it is noncovalently associated with other molecules, notably class I histocompatibility antigens (4, 5). Serum levels are increased in renal diseases that impede its glomerular filtration or reabsorption and in chronic inflammatory diseases and malignancies, in which its synthesis is increased (6, 7).

Up to recently, it has been assumed that an elevated β_2m level was a secondary phenomenon and that "β_2m is not an etiological factor in any disease" (8). However, partial amino acid sequences have shown β_2m to be the major subunit protein of amyloid fibrils in hemodialysis-related amyloidosis (HRA) (9, 10), and the presence of β_2m in tissue deposits also has been corroborated by immunohistology (11, 12). HRA is a form of systemic amyloidosis with a predilection for synovium and bone that is increasingly being recognized among patients on long-term hemodialysis (13–16); clinical correlates include carpal tunnel syndrome (13–15), erosive arthropathy (15), spondyloarthropathy (16), lytic bone lesions, and pathologic fractures (14, 15, 17). We now report the full amino acid sequence of HRA subunit protein, its identification as intact normal β_2m, and evidence for its polymerization in tissue without apparent degradation to form amyloid fibrils. Moreover, a method is described that should permit the typing of this form of amyloid in small tissue specimens obtained at surgery or by biopsy.

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MATERIALS AND METHODS

Purification of HRA Subunit Protein and Normal β_2m. Purification of HRA subunit protein from bone amyloid obtained postmortem (10) and β_2m from the urine of patients with tubular proteinuria (18) has been described.

RIA of β_2m. β_2m levels were determined by double-antibody RIA (7).

Two-Dimensional Gel Electrophoresis. This was performed with an Anderson ISODALT II system (19) and included Ampholines (pH 3.5–10; LKB) in the first dimension and $NaDodSO_4$/polyacrylamide gradient gels (one-third 10% to two-thirds 20% polyacrylamide) in the second dimension. Bone biopsy material obtained antemortem was kept frozen in aliquots at −70°C. For two-dimensional gel studies, samples were thawed, and erythrocytes were removed by hypotonic lysis and freeze-thawing (three times), pelleted, and washed in phosphate-buffered saline (pH 7.2) until the supernatant was free of protein. The final pellet was dissolved directly in sample buffer containing 0.05 M 2-(*N*-cyclohexylamino)ethanesulfonic acid (Ches), 2% $NaDodSO_4$, 1% dithiothreitol, and 10% (vol/vol) glycerol. Insoluble material was removed by centrifugation (Beckman Microfuge).

Antisera. Antiserum to amyloid P component was raised in rabbits as described (20). Rabbit anti-β_2m antiserum was obtained from Accurate Chemical and Scientific Corporation (Westbury, NY).

Immunoblots. Electrophoretic transfers of two-dimensional gels were carried out as described (20). Blots were developed with primary antisera at 1:60 dilution, and ^{125}I-labeled staphylococcal protein A (ICM) was used at 2×10^6 cpm per blot.

Isolation of Tryptic Peptides. Subunit protein "B" (10) was reduced completely in 6 M guanidine/0.6 M Tris·HCl/1 mM EDTA buffer, pH 8.2, containing 5 mM dithiothreitol at 37°C for 1 hr and then alkylated 1 hr with iodo[2-^{14}C]acetic acid (0.7 Ci/mol; 1 Ci = 37 GBq; Amersham) to a concentration of 11 mM. After extensive dialysis against H_2O, the protein was lyophilized. The protein was dissolved (1 mg/ml) in 0.2 M ammonium bicarbonate (pH 8.2) and incubated 4 hr at 37°C with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington) at an enzyme/substrate ratio of 1:50 (wt/wt). Proteolysis was terminated by freeze-drying.

Abbreviations: β_2m, $\beta_2$microglobulin; HRA, hemodialysis-related amyloidosis.

Peptides were isolated by reverse-phase chromatography on a μBondapak C_{18} column (0.78 × 30.0 cm, Waters Associates) with a gradient of 0–66% acetonitrile in 0.1% CF_3COOH (Pierce Chemicals) at pH 2.5 (system A). Some peptides were further purified by HPLC on a second μBondapak C_{18} column (0.38 × 30 cm) with a gradient of 0–66% acetonitrile in 20 mM NH_4OAc at pH 5.6 (system B). Columns were monitored at 210 nm, and peptides containing carboxy[^{14}C]methylcysteine were identified with a liquid scintillation counter (Beckman L5-250).

Amino Acid Analysis and Sequence Studies. These were carried out on individual peptides with a Durrum D-500 automated analyzer and Beckman 890C sequenator as described (10).

Immunoelectron Microscopy. Pelleted amyloid fibrils were embedded in dimethylformamide/low acryl (K4m) and serially developed with anti-β_2m antiserum at 1:50–1:500 dilution and goat anti-rabbit IgG–colloidal gold complexes (10- to 15-nm diameter, Janssen Life Science Products) at 1:20 dilution for 30 min (21). Controls for each run included normal rabbit serum and phosphate-buffered saline.

RESULTS

Amyloid Fibrils in HRA Are Composed of β_2m. Pelleted amyloid obtained postmortem or antemortem by bone biopsy consisted predominantly of unbranching curved amyloid fibrils. It bound Congo red and gave apple-green birefringence typical of amyloid by polarization microscopy. Specific staining of fibrils with anti-β_2m antiserum was seen by immunoelectron microscopy, with occasional contaminating collagen fibers providing a negative internal control (Fig. 1).

Fibrils were solubilized in 5 M guanidine/0.17 M dithiothreitol and filtered on Sephadex G-75, yielding void-volume and "A" and "B" peaks, the last two having molecular masses of 24 and 12 kDa, respectively (10). Additional amounts of "A" and "B" were obtained by gel filtration when the excluded peak was redissolved in 5 M guanidine/0.17 M dithiothreitol for over 1 week.

Amyloid fibrils also were solubilized readily in CHES sample buffer. Two-dimensional gels showed 24- and 12-kDa proteins (Fig. 2*A*) that corresponded to the "A" and "B" fractions from gel filtration (10) and to β_2m isolated from the urine of patients with tubular proteinuria (Fig. 2*B*). Identical spots were seen when either antemortem or postmortem material was solubilized. When run on two-dimensional gels, the pattern was distinct from that in cases of systemic amyloidosis because of immunoglobulin light chain, AA protein, or prealbumin deposition in tissue (22).

Three preparations of purified β_2m isolated from different individuals with tubular proteinuria and subjected to two-dimensional gel electrophoresis showed electrophoretic heterogeneity consisting of two major isoelectricfocusing species in the pH range 5.3–5.7 as has been reported by others (23). In addition, a small amount of dimeric β_2m with an identical pI was also demonstrated (Fig. 2*B*). In contrast, 24- and 12-kDa species seen on two-dimensional gels of solubilized HRA fibrils exhibited greater heterogeneity, consisting of two or three additional spots of higher pI. Also apparent were additional spots at 26 and 48 kDa as well as material of higher molecular mass at the gel interface (Fig. 2*B*).

Identity of the 12-, 24-, and 48-kDa species and some of the higher molecular mass material with β_2m was shown by refractionation of the excluded material from gel filtration and by immunoblot analysis (Fig. 2*C*). Serial development of the same blot with a monospecific antiserum revealed the 26-kDa species to be the monomeric form of amyloid P component, consisting of two closely related spots with a more acidic pI than that of β_2m (Fig. 2*D*). An identical configuration has been noted on two-dimensional gels of several P-component preparations isolated from different types of systemic amyloidosis (24) (data not shown).

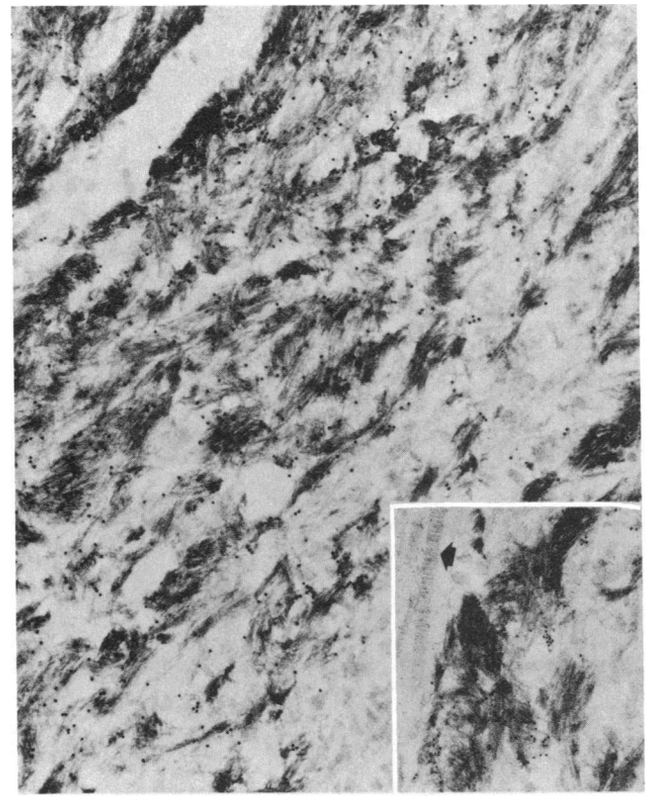

FIG. 1. Electron micrograph of partially purified HRA fibrils developed with anti-β_2m antiserum at a dilution of 1:100 and protein A–colloidal gold. (×42,900.) (*Inset*) Fibril aggregate with adjacent collagen fiber (arrow). (×55,500.)

HRA Subunit Protein Has the Amino Acid Sequence of Normal β_2m. The primary amino acid sequence of the "B" fraction was determined to position 30 by automatic sequence analysis of the whole molecule and revealed an amino-terminal residue of isoleucine with no heterogeneity (10). The remaining sequence was obtained by Edman degradation of individual peptides isolated by tryptic digestion of the completely reduced and ^{14}C-labeled amyloid subunit protein. HPLC yielded 11 tryptic peptides, T1–T11, two of which (T5 and T8) were radioactive, and all of which were initially placed (before sequencing) by homology of amino acid compositions to the corrected (25) published sequence of human β_2m (26). Peptides T5 and T8 have a lysyl–aspartic bond at positions 41–42 and 75–76 and peptide T11 has an arginyl–aspartyl bond at position 97–98 that did not hydrolyze and so prevented formation of three additional tryptic peptides. The sequence of tryptic peptides T5, T6, T7, T8, T9, T10, and T11 was established by automatic analysis, providing the remaining sequence of the molecule to position 99 (Fig. 3).

Circulating β_2m in HRA Is Monomeric. Circulating serum β_2m levels in two HRA patients were elevated but were not significantly different from those of 12 patients on chronic hemodialysis of comparable duration without occult amyloid (12, 28). Antemortem sera from both of these patients were available, one of which (MAR) provided material for the sequencing studies discussed above. MAR's serum concentration of β_2m was 64 μg/ml, compared to 49 μg/ml in the supernatant after pelleting of amyloid fibrils. A second patient (BRO) had a serum β_2m level of 53 μg/ml (normal level is <3 μg/ml).

Fractionation of both sera by gel filtration showed that >95% of the immunoreactive β_2m was monomeric (Fig. 4 *Top*

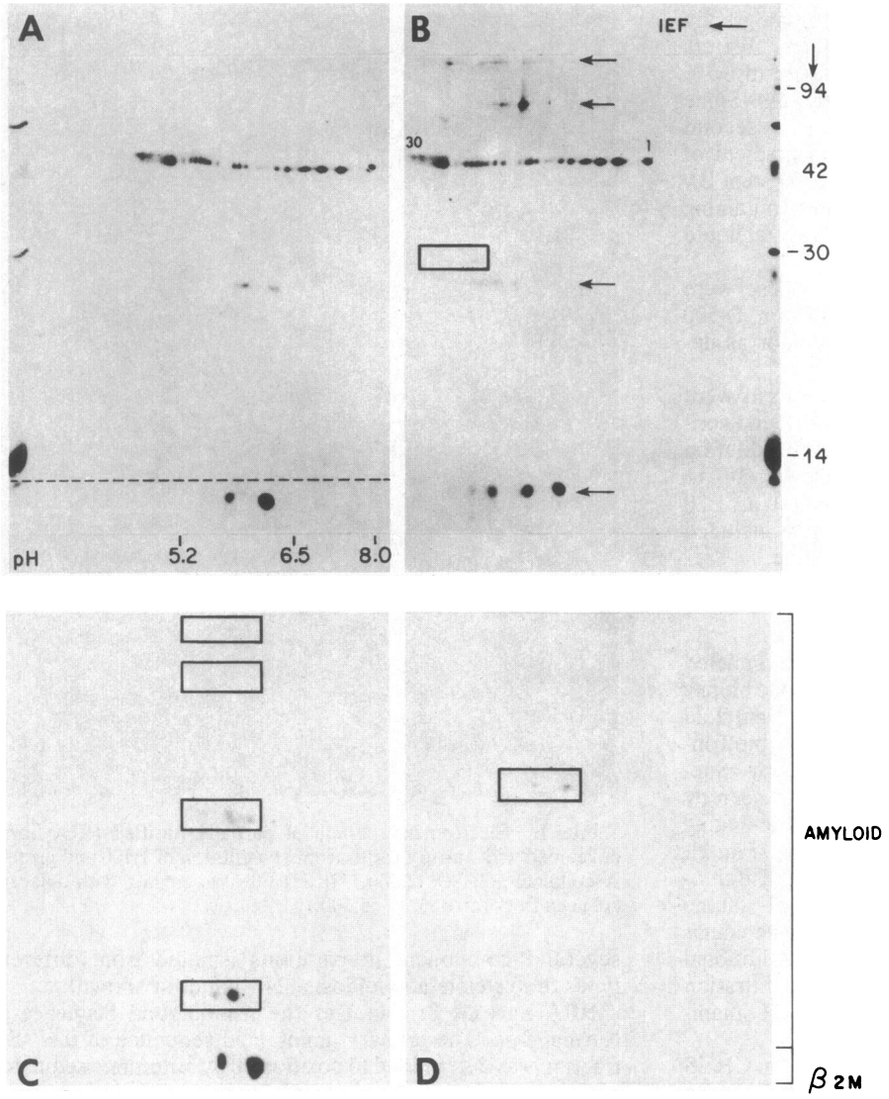

FIG. 2. Two-dimensional gels stained with Coomassie brilliant blue of purified β_2m (10 μg) (*A*) and HRA amyloid purified from bone biopsy processed as described (*B*). pI coordinates were determined by alignment with the 30-spot (1–30) carbamylation train of creatine phosphokinase, included as an internal isoelectricfocusing standard (20). Molecular mass standards (shown $\times 10^{-3}$) include α-lactoalbumin (14.4 kDa), carbonic anhydrase (30 kDa), ovalbumin (42 kDa), and phosphorylase b (94 kDa). Arrows indicate the positions of monomer, dimer, and higher polymers of β_2m; the box indicates that of amyloid P component. (*C* and *D*) A single immunoblot, a composite of the bottom part of *A* cut at the dashed line (labeled "β_2m") and the entire two-dimensional gel of *B* (labeled "Amyloid"). This blot was developed serially with antisera to β_2m (*C*) and to amyloid P component (*D*), and "was erased" between exposures (i.e., the first antibody was removed) with 50 nM sodium phosphate pH = 7.5/10 M urea/0.1 M 2-mercaptoethanol at 60°C for 30 min (20).

and *Middle*). Similarly, all β_2m in the supernatant fraction was found in a narrow peak corresponding to a molecular mass of about 12 kDa (Fig. 4 *Bottom*). Consequently, there was no aggregation or complexing of β_2m under physiologic

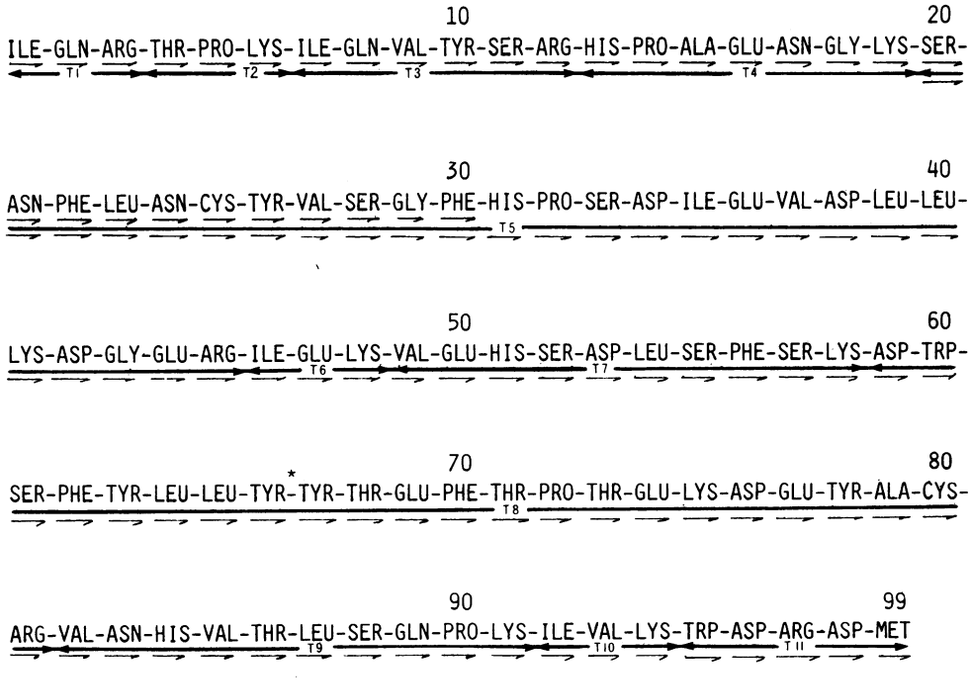

FIG. 3. Complete amino acid sequence of the HRA subunit protein (10). Direct sequencing (25, 26) and availability of the cDNA clone (27) for β_2m show 99 residues and not 100 as originally reported because of the absence of serine at position 67 (shown by an asterisk). T 1–11, tryptic peptides; →, positions determined by automated analysis.

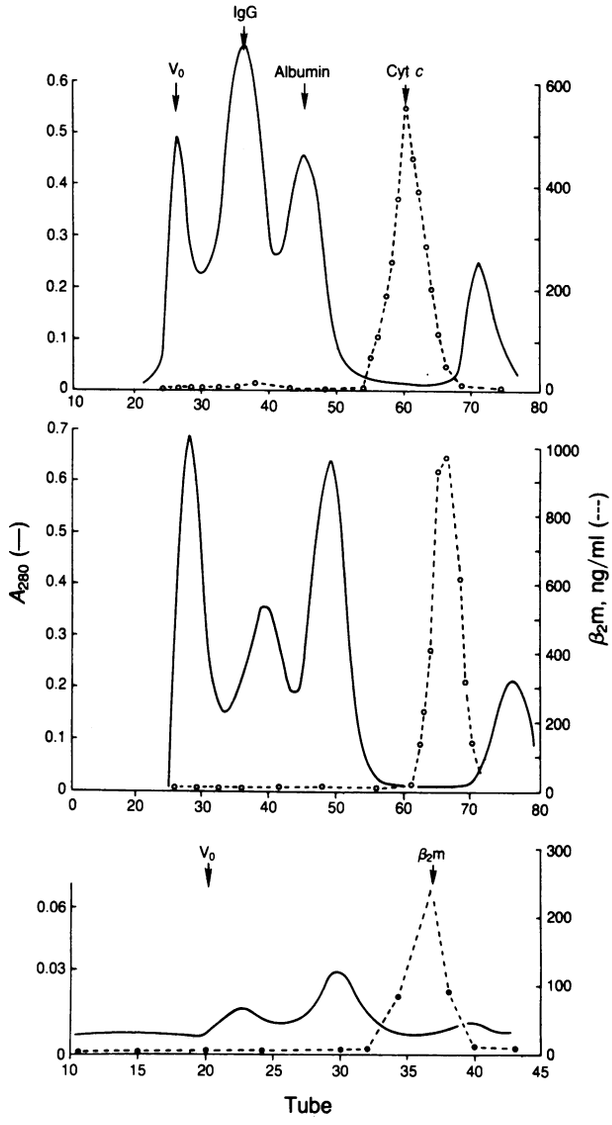

FIG. 4. Fractionation of sera (300 μl) and supernatant fluid (90 μl) from patients with HRA. (*Top* and *Bottom*) Serum of patient MAR shown in Figs. 1–4 and supernatant fluid obtained after pelleting of amyloid fibrils as described. (*Middle*) Serum from patient BRO, found to have carpal tunnel involvement and β_2m amyloidosis immunohistologically (12). Sera in *Top* and *Middle* were fractionated on a Sephadex G-200 column (LKB, 1.5 × 120 cm) in phosphate-buffered saline (pH 7.4) (2 ml per tube) at 4°C and a flow rate of 3 ml/hr; serum in *Bottom* was chromatographed on a Fractogel TSK HW 55-S column (LKB, 0.9 × 85 cm) in 0.1 M $NaHCO_3$ (pH = 8.0) (3 ml per tube) at 4°C and a flow rate of 5 ml/hr. Protein concentrations of fractions (—) were monitored by A_{280}, and β_2m levels (---) were determined by double-antibody RIA (6). Molecular mass markers determined by column calibration are indicated. Cyt *c*, cytochrome *c*; V_0, void volume.

conditions either in blood or in the extracellular fluid in bone in which amyloid fibrils were found.

DISCUSSION

Our study provides a direct demonstration that an intact normal serum protein may undergo polymerization in tissue to form fibrils with the tinctorial and ultrastructural characteristics of amyloid. By establishing the entire sequence of the subunit protein isolated from tissue deposits, we show that proteolytic digestion is not associated with, or a prerequisite for, fibril formation in systemic amyloidosis occurring in patients on chronic hemodialysis. This is in contradistinction to the AA (secondary) and immunoglobulin light chain forms of amyloid disease in which proteolytic digestion of serum precursor proteins is the rule (29, 30). β_2m appears to be one of a growing number of low molecular weight proteins, including such diverse molecules as substance P (31) and insulin (32), that are capable of adopting a fibrillar configuration. Furthermore, many of these fibrils have been found to have a predominantly β-pleated sheet configuration by x-ray diffraction analysis (33)—a common feature of amyloid occurring in diverse pathologic states ranging from plasma-cell dyscrasias to Alzheimer's disease (29, 30, 33). The >50% β-pleated sheet configuration, recently established with the bovine homolog (34), would appear to make β_2m especially suited for amyloidogenesis.

Immunoblot analysis of solubilized frozen tissue investigated by two-dimensional gel electrophoresis is a powerful method for typing amyloid in small biopsy specimens (22). In HRA, this has permitted us to show the identity of antemortem β_2m subunits with autopsy material subjected to purification, dissociation, fractionation, and amino acid sequencing. Furthermore, immunoblots confirm that fibrils are composed of dimers, tetramers, and higher polymers of β_2m, only partially dissociated in buffer used for two-dimensional gels. The observed heterogeneity is consistent with observations reported by others for β_2m in urine (23) and colostrum (35), but its significance is unclear. Since human β_2m is coded for by a single gene (27) and does not contain carbohydrate (1), one may assume that it reflects posttranslational changes or modifications (e.g., deamidation) introduced during purification.

Although our studies of the "B" fraction clearly provide the entire reported sequence of normal β_2m, we cannot exclude the presence of abnormal or partially fragmented molecules in the higher molecular mass fractions. This possibility is unlikely, however, as repeated treatment of high molecular mass material obtained by gel filtration continued to yield monomeric β_2m with an identical HPLC profile.

Alloantigen activity of high molecular mass fractions of rodent sera have been reported due to complexing of β_2m to other molecules, including plasma proteins (36, 37). Similar complexes of β_2m still bound to the heavy chain of class I histocompatability antigens have been identified in tissue-culture media of lymphoid cells (18, 38). Once shed, however, the heavy chain undergoes rapid degradation, and <2% of human β_2m in serum and urine remains associated with soluble HLA antigens (39). Copurification of fibrils and P component is consistent with the presence of the latter in association with other forms of amyloidosis (40). Although P component binds to amyloid fibrils *in vitro* (41), there is little reason at present to assume a facilitating role in fibril formation.

The striking predilection of HRA for synovium and bone (13–17) suggests the importance of tissue-specific factors. Although β_2m appears to be a suitable substrate for tissue transglutaminases (42), ready dissociation of fibrils in standard chaotropic agents excludes polymerization due to covalent cross-linking. Alternative possibilities to explain polymerization include nucleation reactions, binding to other molecules of ground substance, or local conditions of concentration and ionic strength, which have been shown to induce a fibrillar configuration by soluble β_2m (43). A facilitating role for other nonproteinaceous substances that may accumulate in certain tissues cannot be excluded. An example of the last alternative might be furnished by aluminum salts, which are significantly increased in dialysis patients developing encephalopathy (44) or arthropathy (45). Aluminum has also been reported as a significant constituent of amyloid deposits and neurofibrillary pathology in Alzheimer's dementia (46).

β_2m is one of a group of low molecular mass proteins that are not eliminated by conventional cuprophan dialysis membranes (47)—i.e., so-called "middle molecules." It is the first

to be linked to specific clinical syndromes occurring thus far exclusively in hemodialysis patients (13–17). The exact incidence of this syndrome in such patients is unknown. However carpal tunnel syndrome may complicate the clinical course of 2% to 31% of patients on long-term hemodialysis, and in some series >70% of these patients have been found to have amyloid deposits at surgery for this condition (14). Because of the increasing importance of HRA as a clinical entity, the potential value of the periodic use of high-permeability membranes or intermittent hemofiltration (48) needs to be examined both for its prevention and as therapy of established disease.

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