Amyloid fibril protein related to prealbumin in familial amyloidotic polyneuropathy

(amyloid neuropathy/hereditary amyloidosis)

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ABSTRACT   Amyloid fibrils were concentrated from the kidney, thyroid, and peripheral nerve of six patients with familial amyloidotic polyneuropathy (FAP). The fibril concentrates were solubilized in 6 M guanidine·HCl and fractionated on Sephadex G-100 columns. The elution profile of all FAP amyloid fibril concentrates revealed a protein of apparent M_r of 14,000, designated the FAP protein, that was absent from normal human tissues treated by the same procedure and from fibrils of a primary amyloidosis liver. Antisera against whole denatured fibril concentrates prepared in rabbits reacted with the FAP protein and a component in normal human serum corresponding to prealbumin. It was further established that the FAP protein shared common antigenic determinants with human prealbumin by its reaction of identity with normal prealbumin using commercial antisera against human prealbumin. Amyloid AL or AA proteins could not be identified in FAP fibrils by sensitive immunochemical assay methods. These results suggest that the FAP protein is a unique and significant component of the FAP amyloid fibrils and that it is closely related to the 13,745 M_r prealbumin subunit.

In all forms of amyloid deposits, both localized and systemic, it has become apparent that the characteristics that distinguish this pathologic process, both histologically and clinically, are related to the β-pleated sheet conformation of the fibrillar proteins constituting the major component of these deposits. The unique tinctorial and optical properties of Congo red-stained amyloidotic tissues as well as their resistance to solution and proteolytic digestion are related in whole or in part to the β-fibrillar structure (1). Many proteins under appropriate environmental conditions can be converted into β-fibrils having all the characteristics of the amyloid fibrils present in the human disease process (1). A wide variety of native proteins, therefore, constitute potential precursors of amyloid fibrils in these different disease syndromes.

The chemical nature of the deposits of human systemic amyloidosis has been clarified in recent years by the discovery of two different types of proteins constituting the β-pleated sheet amyloid fibrils in the acquired amyloidosis syndromes. In the acquired "primary" disease the major fibrillar protein is, in the majority of cases, an intact immunoglobulin light polypeptide chain (AL) and/or its amino-terminal variable region fragment, while in the acquired "secondary" process and in familial Mediterranean fever, the major fibrillar protein constituent is most often a nonimmunoglobulin protein, designated protein AA, of unknown cellular origin (2).

To our knowledge no study on the nature of the β-pleated sheet fibril protein present in any heredofamilial amyloidosis syndrome of dominant heritance (3) has been reported. Familial amyloidotic polyneuropathy (FAP), type I or Portugese type (4), is a dominant hereditary disease which affects more than 250 families in the northern area of Portugal (5). In this disease, as in other types of amyloidosis, amyloid deposits can be detected in several organs and tissues such as kidneys, gastrointestinal tract, heart, and thyroid. Characteristically, however, amyloid is not found in the liver or spleen, but is always present in the peripheral nerves (6). Its fibrillar nature has been established, but no analyses of its protein composition has so far been reported. The purpose of this paper is to report our findings on the nature of proteins isolated from amyloid fibrils in FAP.

MATERIALS AND METHODS

Separate preparations of amyloid fibril concentrates were obtained post mortem from the kidneys from each of six patients with FAP (FAP IV, V, VI, VII, VIII, and X) and from the thyroid and peripheral nerve of FAP VI and VII. For comparative studies, amyloid fibrils were also purified from the liver of a primary amyloidosis patient. Kidney from a normal young adult killed in an accident was treated by the same extraction procedure and used as a control. Before use, all tissues had been frozen at $-20°$ for periods varying from 3 days to several years.

Purification of Amyloid Fibrils. The method used was a modification of those of Pras *et al.* (7) and Glenner *et al.* (8). The tissues were initially homogenized in 0.1 M phosphate-buffered (pH 7.2) saline in a VirTis 45 homogenizer and centrifuged at 12,500 $\times$ g for 30 min in a refrigerated centrifuge. The supernatant was discarded and the sediment was rehomogenized and centrifuged again for 30 min, the above procedure being repeated five times. The sediment from the last centrifugation was then resuspended in distilled water, homogenized in a Potter–Elvehjem apparatus, and centrifuged at 12,500 $\times$ g for 30 min. Resuspension of the sediment in distilled water, homogenization, and centrifugation was repeated 10 times and the supernatants were retained. These supernatants, containing amyloid fibrils in suspension, were then centrifuged for 2 hr at 75,000 $\times$ g (8). Samples of the resulting sediments were negatively stained with 2% phosphotungstic acid and examined by electron microscopy (Siemens Elmiskop I). The sediments, comprising the amyloid fibril concentrates, were lyophilized.

Solubilization and Fractionation of Amyloid Fibrils. This was done by the method originally described by Glenner *et al.* (9) with slight modification. Eighty milligrams of lyophilized amyloid fibril concentrate was suspended, each time, in 3 ml of 6 M guanidine·HCl (Gdn·HCl) in 0.1 M Tris·HCl (pH 10.2) containing 0.17 M dithiothreitol and the suspension was stirred overnight. One milliliter of 2 M Gdn·HCl in 4 M acetic acid was

then added to bring the final concentration to 5 M Gdn·HCl in 1 M acetic acid. Undissolved material was removed by centrifugation at 105,000 × g for 1 hr, and the clear supernatant was applied to a 1.5 × 90 cm Sephadex G-100 column equilibrated with 5 M Gdn·HCl in 1 M acetic acid. Fractions of 2.5 ml were collected and recorded at 280 nm. Pooled fractions thus obtained were exhaustively dialyzed against distilled water and lyophilized.

Denaturation of Amyloid Fibrils. Ten-milligram portions of lyophilized fibril preparations were suspended in 1 ml of 6 M Gdn·HCl in 0.1 M Tris·HCl (pH 10.2) containing 56 mM dithiothreitol and the mixture was stirred overnight. Undissolved material was removed by centrifugation at 105,000 × g for 1 hr, and the supernatant was dialyzed against distilled water and lyophilized. The resulting material, containing denatured, unfractionated fibril proteins, was used for analysis in sodium dodecyl sulfate (NaDodSO₄)/polyacrylamide gel electrophoresis and for the preparation of antisera.

Preparation of Antisera. FAP VI and FAP VII fibril concentrates were used, after denaturation, for the production of antisera in rabbits. Between 0.6 and 2 mg of lyophilized denatured fibrils were suspended in 0.3–0.5 ml of phosphate-buffered saline and emulsified in an equal volume of complete Freund's adjuvant. The rabbit inoculations were initially made at 2-week intervals. One week after the third inoculation, about 50 ml of blood was drawn by puncture of an ear vein. The inoculations proceeded at 4-week intervals, blood being collected 1 week after each inoculation. Antisera against whole denatured fibril preparations were thus obtained and used in double immunodiffusion and immunoelectrophoresis. Radioimmunoassay (10) for protein AA was performed by Jean D. Sipe, National Institutes of Health, Bethesda, MD, and immunoassay for light chain determinants was performed with antisera against amyloid protein (11). Commercial anti-human antisera to IgG, IgA,
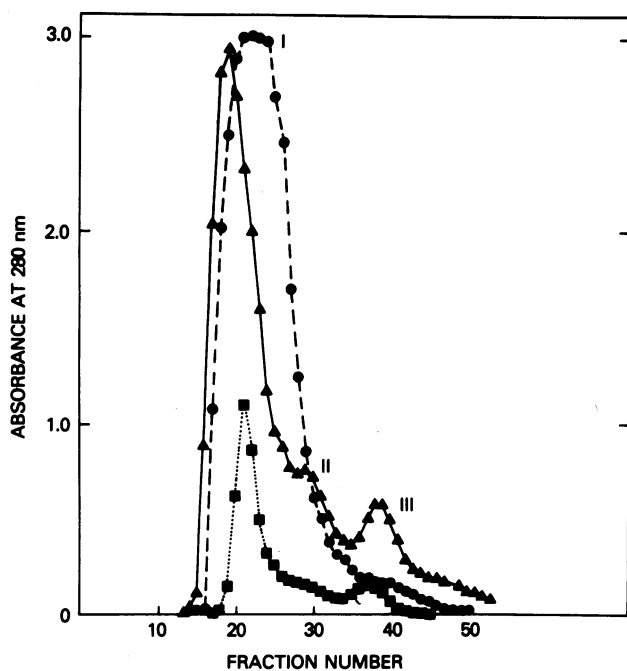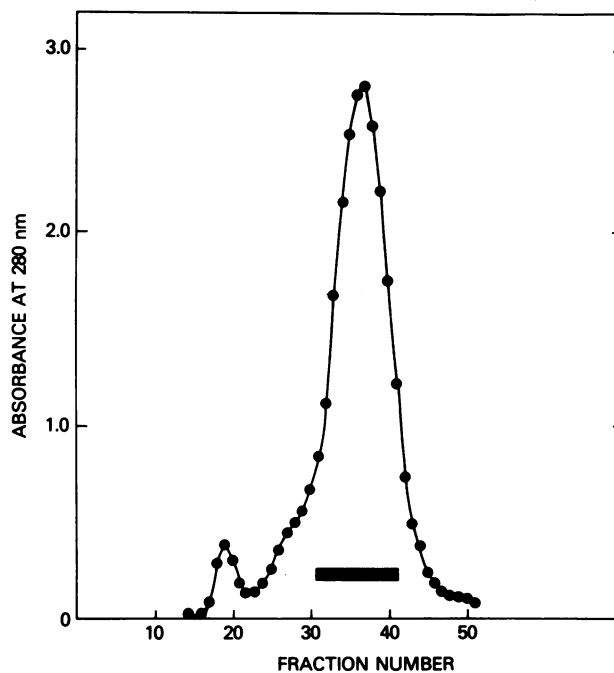

FIG. 2.   Rechromatography of pooled peak III of 13 fractionations of FAP VII fibril preparation in the same Sephadex G-100 system as in Fig. 1. The major fractions of the 14,000 M_r protein peak were pooled.

IgM, IgD, free κ chain, free λ chain, β-lipoprotein, GC-globulin, albumin, retinol-binding protein, $α_1$-antitrypsin, C-reactive protein, $α_2$-macroglobulin, $β_1$ A-C globulin, haptoglobin, ceruloplasmin, and orosomucoid were obtained from Behringwerke and Hyland.

NaDodSO₄/Polyacrylamide Gel Electrophoresis. Protein material (20–80 μg) obtained after denaturation of the fibril

FIG. 1.   Pattern of elution on a Sephadex G-100 column (1.5 × 90 cm) equilibrated with 5 M Gdn·HCl of FAP VII amyloid fibril preparations isolated from kidney and thyroid. A peak (III) was present in all FAP fibril preparations but not in normal kidney treated by the same extraction procedure. ●, Normal kidney; ▲, FAP kidney; ■, FAP thyroid.
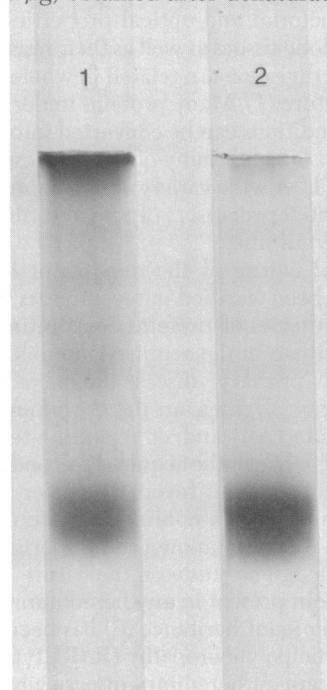

FIG. 3.   NaDodSO₄/polyacrylamide gel electrophoresis of: (gel 1) whole denatured FAP amyloid fibril preparation; (gel 2) pooled peak III fractions from Sephadex G-100 rechromatography (Fig. 2). The peak III protein has a molecular weight of about 14,000.
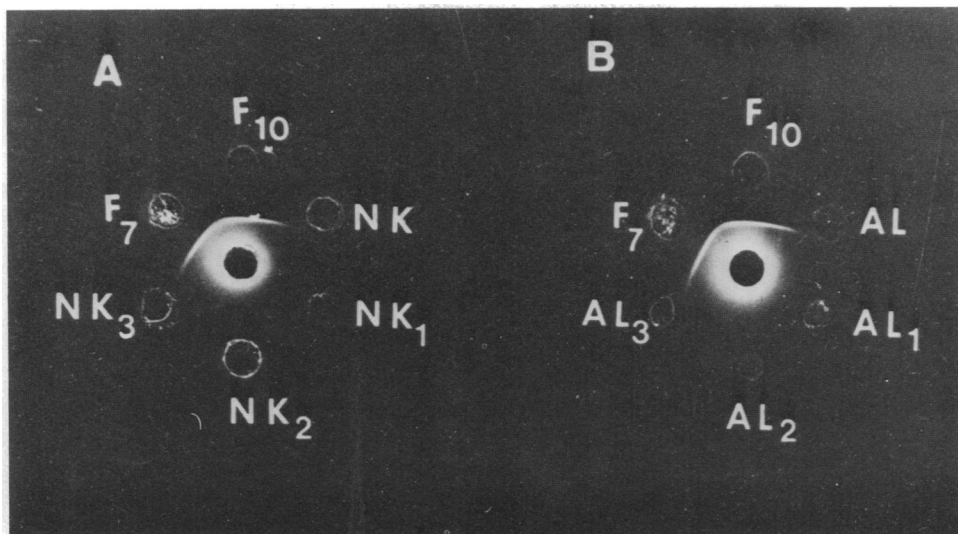
FIG. 4.   (A) Double immunodiffusion of denatured amyloid fibril proteins F_7 and F_{10} tested against normal kidney (NK) and three fractions $NK_{1,2,3}$ corresponding to fractions I, II, III of the Sephadex G-100 column shown in Fig. 1. (B) Similar to A tested with amyloid liver (AL) and three fractions $AL_{1,2,3}$ corresponding to fractions I, II, and III of the Sephadex G-100 column. Center wells in A and B contain antiserum against FAP VII amyloid fibrils.

preparations and fractionation on Sephadex G-100 columns was subjected to electrophoresis in 10% polyacrylamide gels containing 1% NaDodSO₄ and stained for protein with Coomassie blue for molecular weight determinations (12). Ovalbumin, chymotrypsin, and ribonuclease were used as standards for the estimation of molecular weights, with 0.1% dithiothreitol being incorporated in all samples prior to electrophoresis.

RESULTS

Isolation and concentration of amyloid fibrils was particularly difficult in tissue preparations from patients with FAP, since two of the organs from which fibrils have been isolated in significant amounts in acquired systemic amyloidosis (liver and spleen) are always spared from amyloid deposition. In the other organs the deposition is always sparse when compared to the acquired types of amyloidosis. Only from the kidney were we able to extract amyloid fibrils in sufficient quantity to permit protein analysis. Even so, a rough estimate indicated that in the six FAP kidneys studied by us the total amount of amyloid was always less than 5% of the organ's total weight. All amyloid fibril preparations obtained by differential centrifugation were to some extent contaminated by amorphous material, and every attempt to obtain a uniformly homogeneous fibril preparation resulted in heavy losses of an already scarce material. We were forced, therefore, to use preparations that could be seen to be contaminated by small amounts of both collagen fibers and cellular debris.

The fibril concentrates were only partially solubilized in pH 10.2 buffered 6 M Gdn·HCl even after the addition of the reducing agent dithiothreitol and overnight stirring. After undissolved material was removed by centrifugation and the supernatants were applied to the Sephadex G-100 columns, a typical pattern of elution was obtained for all the FAP fibril preparations isolated from the kidney (Fig. 1). Most of the material was eluted in the void volume, but two other peaks were always present. Thyroid fibril preparations showed the same pattern of elution. Normal kidney, however, showed a different pattern, the third peak being invariably absent (Fig. 1). Preparations of primary amyloidosis fibrils, however, produced a large third peak.

Aliquots from each of the protein peaks were analyzed by

NaDodSO₄/polyacrylamide gel electrophoresis. It was found that the void volume fraction (I) contained several high molecular weight components and that the second peak (II) was primarily composed of a protein of about 30,000 M_r. A protein of about 14,000 M_r was eluted in the third peak (III). This last protein was obtained from all preparations of FAP amyloid fibrils and was purified further. Pooled third peaks of 13 fractionations of the FAP VII fibril preparations were rechromatographed on the same Sephadex G-100 system. The resulting elution profile is seen in Fig. 2. The main protein peak was homogeneous on NaDodSO₄/polyacrylamide gel electrophoresis (Fig. 3); this purified 14,000 M_r protein, isolated from the FAP VII fibril concentrate, was designated F_7. A similar procedure was used after third peaks of fractionations of FAP VI and X fibril preparations were pooled.

A series of double immunodiffusion experiments showed that immunization with denatured FAP fibrils produced antibodies in rabbits directed against one protein present in all denatured FAP fibril preparations (Figs. 4 and 5). It was found that these antisera reacted to give a precipitin line of identity with all the preparations of denatured FAP amyloid fibrils isolated from different organs of different cases, but not with primary amyloid fibrils or preparations from normal kidney (Figs. 4 A and B and 5A). Other experiments showed that the precipitin line resulted from an antigen present only in the third peak and not in the void volume or second peak fractions. It was thus possible to prove that the rabbits produced antibodies that reacted with a protein present in all preparations of FAP amyloid fibrils, but not in normal kidney or in amyloid fibrils isolated from a patient with primary amyloidosis. Repeated experiments led to the conclusion that this 14,000 M_r protein, designated the FAP protein, was characteristic of all FAP amyloid fibril preparations.

Attempts were made to determine the presence of this protein or any related precursor in the blood of FAP patients. The rabbit antiserum against FAP detected (Fig. 5B) a protein present not only in the serum of FAP patients, but also in the serum and cerebrospinal fluid of normal subjects which was antigenically identical to FAP protein. Commercial antisera against human prealbumin were then tested against the FAP protein; they reacted with all preparations of FAP protein (Fig.
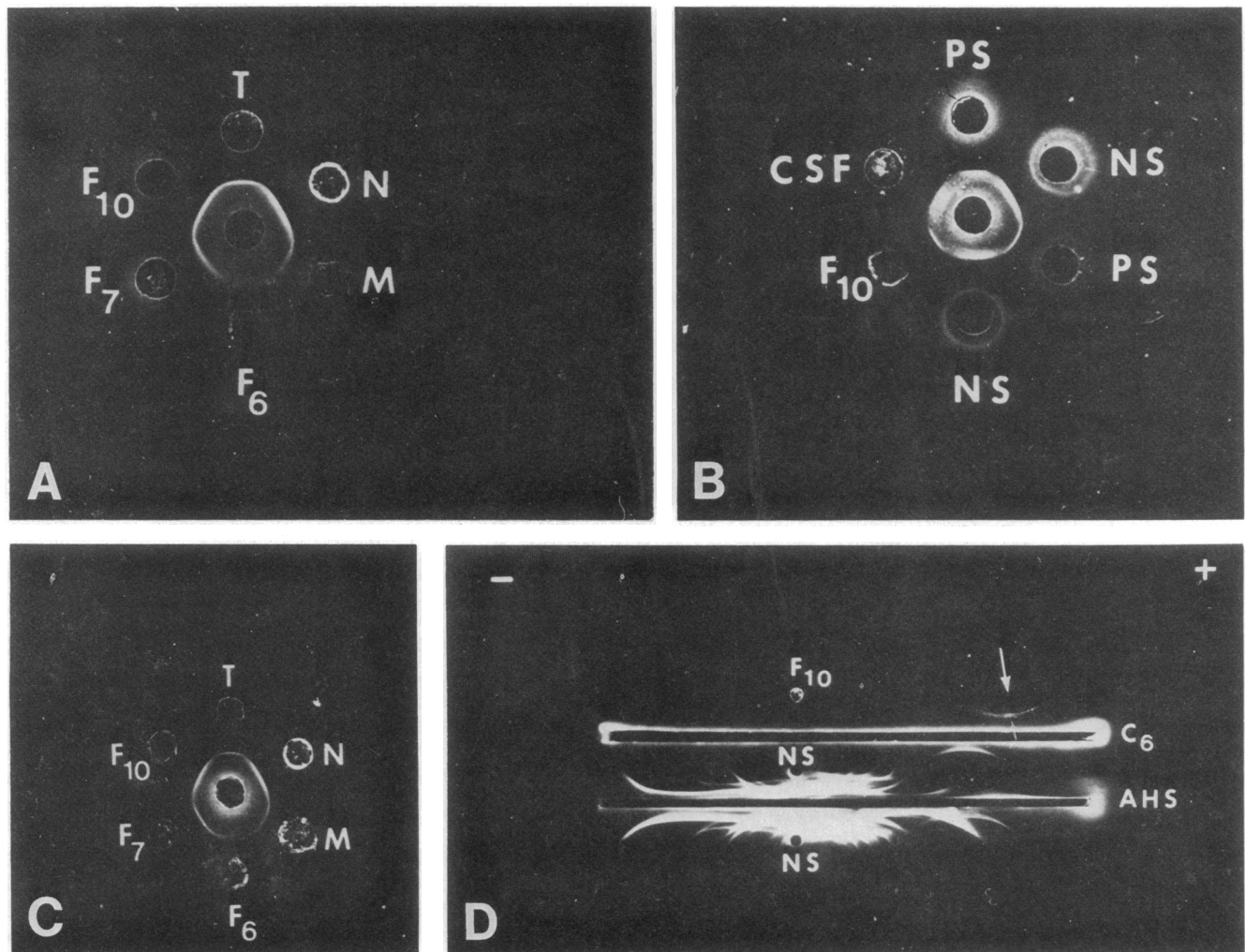
FIG. 5.   (A) Double immunodiffusion of denatured amyloid fibril preparations isolated from thyroid (T), peripheral nerve (N), and meninges (M) from FAP VII, and peak III proteins from the kidney of FAP VI (F_6), FAP VII (F_7), and FAP X (F_{10}). Center wells in A and B contain antiserum against FAP VII amyloid fibrils. (B) FAP patient's serum (PS); normal human serum (NS); peak III protein from FAP X (F_{10}); normal human cerebrospinal fluid (CSF). (C) Commercial antiserum against human prealbumin (center well) reacts with a line of identity with amyloid fibril preparations as in A. (D) Immunoelectrophoresis of peak III protein from FAP X (F_{10}) and normal human serum (NS) tested against antiserum to FAP (C_6) and to normal human serum (AHS). The antiserum against FAP reacts with a serum component of electrophoretic mobility identical to that of prealbumin. The FAP protein has a higher mobility than prealbumin (arrow).

5C). All immunodiffusion experiments could be reproduced with commercial antisera against human prealbumin, which gave results indistinguishable from the rabbit antisera prepared from the denatured FAP fibrils. It was then established that the FAP protein had antigenic determinants identical to those of human prealbumin. Immunoelectrophoretic analysis, using FAP antisera, detected in normal and FAP sera a component having an electrophoretic mobility identical to that of prealbumin. The FAP protein, however, had a mobility slightly greater than that of prealbumin (Fig. 5D). Commercial specific antisera against 18 other human serum proteins, including immunoglobulin fragments and albumin, did not react with any of the FAP protein preparations.

DISCUSSION

FAP is a genetically determined neuropathic disease affecting families, many of which are related (13), in a restricted area of northern Portugal. The disease apparently results from a single mutation which occurred a few centuries ago (14). The clinical picture, though quite characteristic, shows some variation from patient to patient. One feature of the disease, however, occurs

in every one of the several hundred patients in our study, i.e., the presence of amyloid deposits. In view of these characteristics, we initiated the present investigation in the belief that the essential protein component of the FAP amyloid fibrils might be identical in every case. This view was substantiated by the finding of a common, unique, and significant protein component in different FAP fibril preparations. It cannot be stated, at this moment, that this is the only or even the major protein component of these amyloid fibrils. We believe that the great majority of the void volume and second protein peak result from contamination, but we cannot exclude the possibility that one or more of these proteins is a component of the amyloid fibrils. On the other hand, it is highly improbable that the FAP protein could result from contamination with serum or tissue proteins. First, the protein was detected in several amyloid-containing organs of different patients but not in a normal kidney or in a liver of a patient with primary amyloidosis. Second, it was extracted in relatively large amounts, whereas not even traces of 18 other serum proteins could be found in any fibril preparations. Furthermore, no light chain (AL) or AA amyloid proteins could be detected in our preparations by means of different types of antisera (10, 11). We conclude,

therefore, that in FAP a unique amyloid fibril protein occurs and that this protein is immunochemically related to human prealbumin.

Human prealbumin is a protein of 54,980 M_r composed of four identical subunits. Each subunit contains 127 amino acids and has a calculated M_r of 13,745 (15). This approximates the apparent molecular weight of the FAP protein and suggests that this protein corresponds to the prealbumin subunit. The difference in electrophoretic mobility of both the normal and patient's serum prealbumin and the FAP protein may be related to the monomeric nature of the latter. Prealbumin forms a complex with retinol-binding protein, which is a transport protein for vitamin A (16) and is involved in the plasma transport of thyroxine (17). These are its only known physiological functions, and, though prealbumin concentration in plasma is sometimes reduced in various conditions, such as hepatic disease, it has never been specifically associated with any pathological process. This report indicates that a prealbumin-related protein might be directly involved in the pathogenic mechanism of a degenerative disease and provides a definition of an amyloid fibril protein in a heredofamilial amyloidosis syndrome of dominant heritance. It is highly likely that chemical differences exist between the normal prealbumin subunit and the FAP protein and that these differences will be revealed by analysis of the amino acid composition and sequence. Preliminary data reveal that half the amino acids comprising the FAP protein share the same number of residues with prealbumin, while the remaining amino acids vary between these two proteins by only one to three residues per molecule (K. A. Sobiech and G. G. Glenner, personal communication). It is conceivable, therefore, that in FAP a genetic mutation may have occurred leading to the production of an abnormal prealbumin which is abnormally degraded, bound, and/or precipitated in the connective tissues as amyloid fibrils. Furthermore, we would like to call attention to two additional points relating to pathogenesis in FAP. One is that about 50% of the native prealbumin molecule possesses a β structure with little or no α helix (18). This is the characteristic major conformation of all types of amyloid fibrils, as revealed by x-ray diffraction studies (1). The other point is that prealbumin exists in the cerebrospinal fluid in relatively high amounts, about 8% of the total proteins as compared to 0.5% in the serum. This might indicate that prealbumin is, at least in part, synthesized within the blood–brain barrier and that it might play some role in neural metabolism. The predilection of amyloid in FAP to neural tissues could thus be explained. These considerations suggest new lines of investigation into the elucidation of the pathogenesis of this heredofamilial amyloidosis syndrome.

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