ISOLATION AND IDENTIFICATION BY SEQUENCE ANALYSIS OF EXPERIMENTALLY INDUCED GUINEA PIG AMYLOID FIBRILS*

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Since the original observation that mice maintained on a diet of cheese or given daily injections of nutrose (sodium caseinate) are susceptible to amyloid disease (1), the casein model has been used to study the pathogenesis of this disorder. Nevertheless, despite the reproducibility of amyloid induction in mice, guinea pigs, rabbits, and even chickens after multiple casein injections (2), the chemical composition of the amyloid substance in these species is not known nor is it certain whether the changes in their tissues represent a primary or secondary form of amyloidosis.

Amyloid fibrils of human and animal origin appear identical when examined by staining techniques and by high resolution microscopy. Amino acid sequence studies have shown the human primary amyloid fibrils to be homologous with fragments of immunoglobulin light chains (3), while secondary fibrils are composed of a unique 76 residue sequence of nonimmunoglobulin origin, A protein (4, 5). This communication provides the first chemical analysis of casein-induced amyloid fibrils in guinea pigs and demonstrates its similarity to the major nonimmunoglobulin component of human secondary amyloid fibril preparations.

Materials and Methods

Induction of Amyloid.—Amyloidosis was induced in guinea pigs by multiple subcutaneous injections of 10% sodium caseinate. After 6 mo the animals were sacrificed and their spleens removed for further studies.

Isolation of Amyloid Fibrils.—Amyloid fibrils were isolated from eight spleens, AC73-35 by repeated homogenization in saline. The residue was then homogenized in water and the amyloid fibrils recovered by lyophilization of the water suspension and the top layer (6).

Chromatography.—The isolated amyloid fibrils were added to 4 M guanidine, pH 7.5, allowed to stand at 4°C for 24 h, and centrifuged. Approximately one-half of the fibrils remained in the residue. The supernatant solution of amyloid fibrils was chromatographed on a Sepharose 6B column (90 x 2.5 cm, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) using 4 M guanidine, pH 7.5 (Fig. 1). The resulting peaks 1, 2, and 3 were dialyzed against distilled water and recovered by lyophilization. The major retarded peak, peak 4, was recovered by desalting on a P2 column (Bio-Rad Laboratories, Richmond, Calif.) eluted with 0.1 N acetic acid.

Amino acid Analysis.—Amino acid analyses were performed on a JEOL-5AH analyser after hydrolysis in 6 N HCl at 110° C for 20 h. Each phenylthiohydantoin (PTH) residue after

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sequence degradation was converted to its free amino acid by hydrolysis in 57% hydriotic acid (Fisher Scientific Co., Pittsburgh, Pa.) at 125°C for 20 h and the identity confirmed by amino acid analysis (7).

Sequence Studies.—4.3 mg of peak 4 were dissolved in 0.6 ml of 10% acetic acid and sequenced according to the method of Edman and Begg (8) on a Beckman Sequencer Model 890C (Beckman Instruments, Inc., Fullerton, Calif.). The PTH amino acid residues were identified on a Beckman 65 gas chromatograph (9).

RESULTS

At the end of the 6-mo injection period the guinea pig spleens were markedly infiltrated with amyloid. Gel chromatography of the isolated fibrils which dissolved in 4 M guanidine (Fig. 1) revealed a void volume peak and four retarded peaks. The major retarded peak, peak 4, represented 25% of the recovered material. Peak 5 could not be separated from the guanidine on a P2 desalting column.

Amino acid analyses of the starting fibrils, the residue after guanidine extraction, and each of the first three peaks showed a marked similarity in composition (Table I). However, differences were found in the composition of peak 4 which had a greater arginine, aspartic acid, and alanine content and decreased amounts of threonine, proline, cystine, valine, and leucine.

Sequence studies on the guinea pig peak 4 confirmed its homology with the A protein of secondary amyloid with the exception of a 5-residue N-terminal peptide, His-Ala-Lys-Gly-Glu, preceding the usually reported human N-terminal peptide Arg-Ser- (Table II) (4, 5). There was a substitution of isoleucine

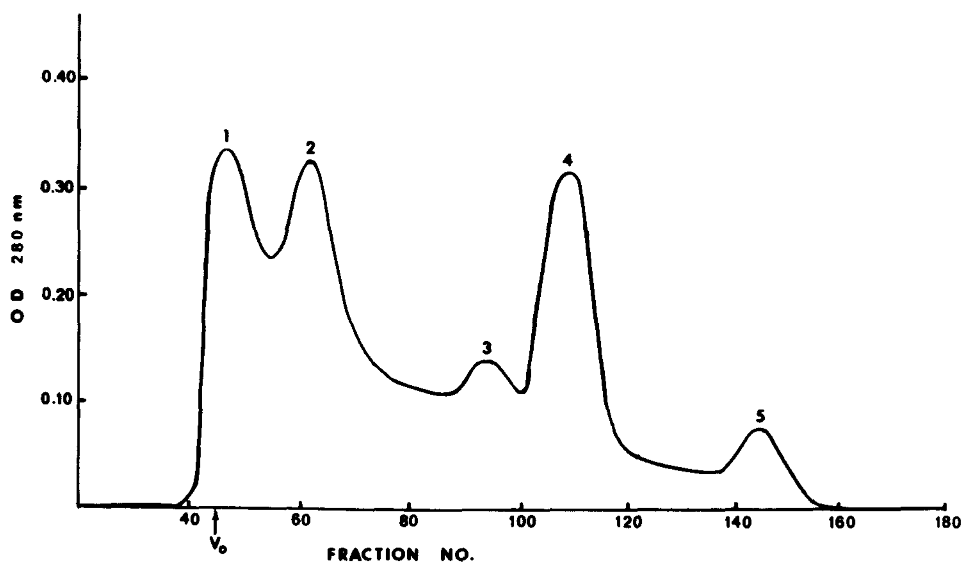

FIG. 1. Gel chromatography of guinea pig amyloid fibrils through Sepharose 6B (2.5 cm x 90 cm) eluted with 4 M guanidine HCl, pH 7.5, and collected in 3-ml fractions.

TABLE I

Amino Acid Analysis of Amyloid Fibrils from Guinea Pigs (Residues/100 Residues)

	Amyloid fibrils	Residue after guanidine extraction	Sepharose 6B chromatography			
			Peak 1	Peak 2	Peak 3	Peak 4
Lysine	6.3	5.2	5.1	5.3	5.9	5.0
Histidine	2.0	2.3	2.5	2.4	2.2	2.0
Arginine	5.9	5.4	5.2	5.5	6.3	8.3
Hydroxyproline	trace	1.0	0	0	0	0
Aspartic acid	8.2	8.4	8.4	8.6	8.2	10.6
Threonine	4.6	4.7	5.2	5.1	4.8	2.7
Serine	5.7	5.4	7.0	6.4	5.6	5.4
Glutamic acid	11.4	11.2	11.7	12.4	12.7	11.9
Proline	5.4	5.8	6.6	5.5	5.2	3.5
Glycine	10.6	10.8	11.1	11.1	7.7	10.8
Alanine	10.9	9.2	7.5	8.3	9.9	15.6
Cystine	1.2	1.4	1.9	1.4	2.7	0.4
Valine	5.7	6.3	6.0	6.1	6.2	3.6
Methionine	2.3	2.1	1.8	1.9	2.1	3.2
Isoleucine	4.3	4.6	4.2	4.0	4.4	2.4
Leucine	8.2	8.8	7.9	8.2	8.8	5.5
Tyrosine	3.3	3.1	3.3	3.1	3.4	4.0
Phenylalanine	4.0	4.4	4.7	4.5	3.8	5.0

TABLE II

Comparison of Experimental Amyloid in Guinea Pigs with Human A protein, Monkey A protein, and Human P component

	1 2 3 4 5
Guinea Pig A protein	His-Ala-Lys-Gly-Glu-Arg-Ser-Ile-Phe-Ser-
Human A protein (5)	Arg-Ser-Phe-Phe-Ser
Monkey A protein (4)	Arg-Ser-Trp-Phe-Ser-
Human P component (11)	His-Ala-Asp-Leu-( )-Thr-Lys-Val-Phe-Val-
	6 7 8 9 10 11 12 13 14 15
Guinea Pig A	Phe-Leu-Lys-Glu-Ser/Ala-Gly-( )-Gly-Pro-( )-
Human A	Phe-Leu-Gly-Glu-Ala-Phe-Asp-Gly-Ala-Arg-
Monkey A	Phe-Leu-Gly-Glu-Ala-Tyr-Asp-Gly-Ala-Arg-
Human P	Phe-( )-( )-Ser-Glu-Val-Val-( )-Ser-Val-
	16 17 18 19 20
Guinea Pig A	Asp-Met-Leu-( )-Ser/Ala-
Human A	Asp-Met-Trp-Arg-Ala-
Monkey A	Asp-Met-Trp-Arg-Ala
Human P	Val-Ser-Ile-

for phenylalanine at position 3, lysine for glycine at position 8, glycine for phenylalanine at position 11, proline for alanine at position 14, and leucine for tryptophan at position 18.

Antiserum prepared in rabbits to the guanidine-denatured guinea pig amyloid fibrils gave a single precipitin line when tested by double diffusion in agar against the guanidine-denatured fibrils or the isolated peak 4 material (Fig. 2). The antiserum also detected a cross-reacting substance in amyloidotic guinea

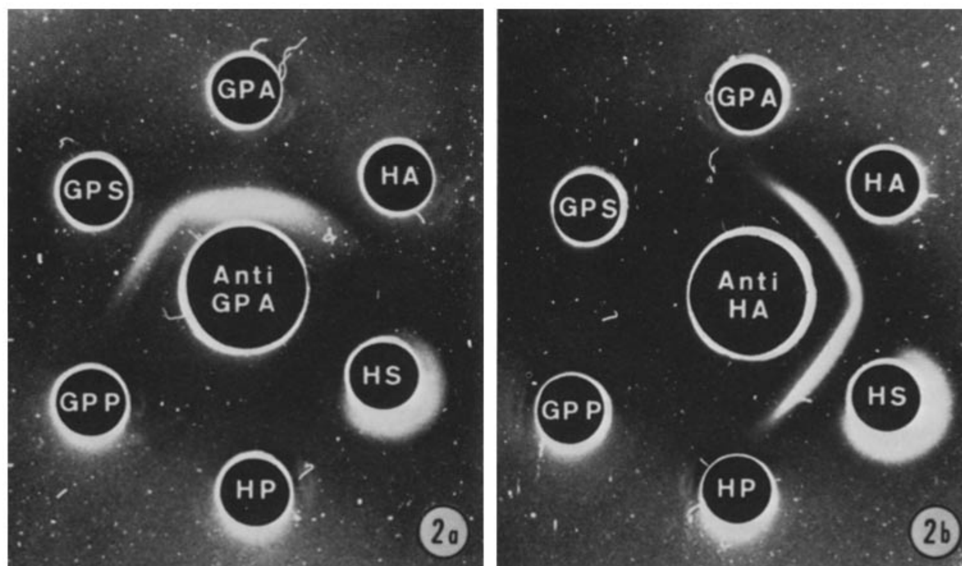

FIG. 2(*a*). Double-diffusion analysis of antiserum to denatured amyloid fibrils of guinea pig origin (anti-GP A) vs. guinea pig P-component (GP P), amyloidotic serum (GP S), and isolated A protein (GP A) showing complete identity between guinea pig A protein and a component in guinea pig serum. No reaction is noted with human A protein (H A), serum (H S) or P component (H P). (*b*) Double-diffusion analysis of antiserum to A protein of human origin vs. the same antigens as in 2 *a*. No reactivity with guinea pig proteins is seen.

pig serum which showed complete identify to the isolated peak 4 protein. The antiserum to guinea pig A protein gave no reaction with human A protein or amyloidotic human serum. Conversely, antiserum to human A protein did not react with guinea pig A protein nor with amyloidotic guinea pig serum. The presence of weak precipitin lines against antiserum to guanidine-denatured guinea pig fibrils identified trace amounts of A protein in the chromatographed peaks 1, 2, and 3.

DISCUSSION

In this study, sequence analysis of experimentally induced guinea pig amyloid fibrils has revealed the secondary (A protein, nonimmunoglobulin) nature of these fibrils when compared to the known sequences of the human A protein (Table II). Amyloid associated with chronic granulomatous disease in monkeys has also been shown to contain the unique A protein sequence (Table II) (4, 10).

Deletions in the first one, two, or three amino acids, Arg-Ser-Phe, of A protein have been noted in the human A protein sequence. The current study demonstrates an additional N-terminal peptide (His-Ala-Lys-Gly-Glu) in the guinea pig. While the significance of this finding is not known, it has been suggested that A protein might be derived by proteolysis of a larger protein because of

individual N-terminal variations. Also, the demonstration of a guinea pig serum factor immunologically identical with the A protein is consistent with a circulating precursor of the amyloid fibrils.

When the N-terminal sequence of A protein from human and monkey amyloid are compared, substitutions are noted in two positions, 3 and 11. As might be expected phylogenetically, more substitutions, a total of five, are found when the guinea pig and human A protein are compared. In terms of the nucleotide interchange for the codon of the amino acids two of these, positions 3 and 14, represent first base changes and three of these, positions 8, 11, and 18, represent second base changes. Equal amounts of serine and alanine at positions 10 and 20 is thought to be individual variations found in this study since the sample consisted of pooled guinea pig spleens.

We have previously noted the sequence beginning His-Ala in the amyloid-associated protein, human P component (Table II) (11), but this sequence to 23 residues is not homologous with A protein. P component is a minor constituent of amyloid tissues, and is antigenically identical to a plasma alpha globulin found in both normal and amyloidotic subjects (12). Neither human nor guinea pig P components react with either human or guinea pig antisera to A protein (Fig. 2). In conclusion, the present study not only provides a definitive N-terminal sequence analysis of experimental amyloid fibrils, but chemically verifies their relationship to secondary amyloid.

SUMMARY

Amyloidosis was produced experimentally in guinea pigs by multiple casein injections. Amyloid fibrils were isolated and fractionated and a protein obtained that had an amino acid composition comparable with A protein, a unique non-immunoglobulin constituent of secondary amyloid deposits. N-terminal sequence analysis demonstrated a sequence homologous with that of A proteins from human and monkey preparations but preceded by a 5-residue peptide which had an N-terminal histidine. A definite species specificity in A protein from human and guinea pig was identified on immunologic analysis.

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