

NEW, THIRD CLASS OF AMYLOID FIBRIL PROTEIN*

BY G. HUSBY, J. B. NATVIG, AND K. SLETTEN

(From the Institute of Immunology and Rheumatology, Rikshospitalet University Hospital, Oslo, Norway, and the Institute of Biochemistry, University of Oslo, Oslo, Norway)

(Received for publication 12 November 1973)

Evidence has recently been obtained for two different classes of amyloid fibril, one consisting of immunoglobulin light-chain fragments (1), the other having a nonimmunoglobulin protein (protein AS) as a major component (2-4). A serum protein antigenically closely related to protein AS has been found in increased amounts in many patients with different types of amyloidosis (5, 6), and may be a circulating precursor of the fibrils.¹

We here describe a third and new class of amyloid subunit, protein AR, derived from a patient with primary amyloidosis. This amyloid protein was different from any known amyloid proteins and also from immunoglobulin fragments. A component related to amyloid protein AR was detected in the serum of the patient.

Materials and Methods

Preparation of Amyloid Fibrils.—Amyloid fibrils were isolated from the spleen of the patient A. R., a 57 year old male who died only 4-5 mo after the onset of symptoms of generalized primary amyloidosis. There was no evidence for M-components or light-chain disease. After removal of soluble proteins, extraction of fibrils was carried out with distilled water (3, 7). For solubilization, the extracted fibrils were treated with 0.1 N NaOH (alkali-degraded amyloid), or with 6 M guanidine/0.1 M dithiothreitol (DTT) (3). Amyloid preparations from patients with primary (J. R.) (4) or secondary amyloidosis (T. H.) (3, 4, 8), and from two patients with amyloidosis associated with Waldenström's macroglobulinaemia (J. B. and E. F.) (4, 6), were also included for comparison. All the crude amyloid fibril preparations used in the immunologic experiments were degraded with 0.1 N NaOH.

Sera and Serum Proteins.—Since regular serum from the patient A. R. was not available, blood squeezed from the vessels of the liver was used. 60 sera from patients with different clinical types of amyloidosis were tested in addition to pooled and individual normal sera, and isolated serum proteins and fragments (4).

Antisera.—Antiamyloid antisera were obtained by immunizing rabbits with alkali-degraded amyloid fibrils. Antisera to human serum and various serum proteins were also used (3, 4).

Gel Filtration.—For the isolation of subfragments, amyloid fibrils treated with NaOH or with guanidine/DTT were gel filtered on Sephadex G-100 columns (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) equilibrated with 5 M guanidine—1 N acetic acid, followed by gel filtration on Sephadex G-25 medium equilibrated with 10% formic acid (3).

RESULTS

Antigenic Properties of Amyloid A.R. and Its Subfragments.—When amyloid A. R. was reacted with an antiserum to secondary amyloid T. H., only one

*Supported by the Norwegian Medical Research Council.

¹Husby, G., and J. B. Natvig. 1974. A serum component related to nonimmunoglobulin amyloid protein AS, a possible precursor of the fibrils. *J. Clin. Invest.* In press.

precipitation line fusing with that of the high molecular weight component of amyloid T. H. (void volume material at Sephadex G-100 [3]) was observed (Fig. 1 A). No reaction was obtained with two strong antisera to protein AS (antiamyloid J. B. and antiamyloid T. H.) (Fig. 1 A). However, when amyloid A. R. was reacted with antiamyloid A. R., a distinct precipitation line was obtained (Fig. 1 C). No other amyloid preparation or their subfragments studied, altogether 21 amyloid preparations (4), gave any precipitation line with antiamyloid A. R., and did not even inhibit the precipitation reaction between amyloid A. R. and antiamyloid A. R. (Fig. 1 C).

These findings strongly indicate that amyloid A. R. lacked the nonimmunoglobulin protein AS which has been found in nearly all amyloid preparations studied. In addition, amyloid A. R. appeared to contain antigenic material different from all the other amyloid preparations. When antiamyloid A. R. was tested against normal human serum, immunoglobulins and fragments thereof, and a panel of monoclonal IgG, IgA, and IgM proteins, and 12 different Bence-Jones proteins, no precipitation line was obtained. Furthermore, no precipitation reaction was obtained between amyloid A. R. and antisera to IgG, IgA, IgM, $F(ab')_2$, light chains, and whole human serum. Immunoelectrophoresis revealed an electrophoretic mobility of amyloid A. R. slightly anodic to the application point (Fig. 1 D).

Gel filtration of degraded amyloid A. R. yielded two main components (Fig. 2). One subfragment (protein AR) was eluted corresponding to a mol wt of approximately 16,000 daltons with a yield of 66% of the total protein eluted. The other main component (25%), the void volume material, had a mol wt higher than 70,000. No protein AS was eluted even when NaOH was used for degradation of amyloid A. R. before gel filtration.

Double immunodiffusion of antiamyloid A. R. against the subfragments of amyloid A. R. showed that material from the protein peak with mol wt 16,000 daltons (protein AR) gave a precipitation line fusing with that obtained with crude amyloid A. R. The component of amyloid A. R. eluted in the void volume was, however, not precipitated (Fig. 1 C). However, antiamyloid T. H. reacted with the void volume material of amyloid A. R., giving a precipitation line of identity with that of the void volume material of amyloid T. H. (Fig. 1 A).

Amyloid E. F. also appeared to be different from preparations previously studied. Amyloid E. F. was precipitated by antiamyloid T. H., giving a line of identity with that of the void volume components of amyloid T. H. and A. R. In contrast, no precipitation reaction related to protein AS or protein AR was obtained with amyloid E. F. (Table I). However, both crude amyloid E. F. and a 20,000 daltons subfragment constituting 67% of the total protein of amyloid E. F. were precipitated by an antiserum to λ -light chains, giving a reaction of partly identity with three different λ -Bence-Jones proteins (Table I). No reaction was seen with anti- κ -light chains.

Protein AR-Related Component in Serum.—Antiamyloid A. R. was also

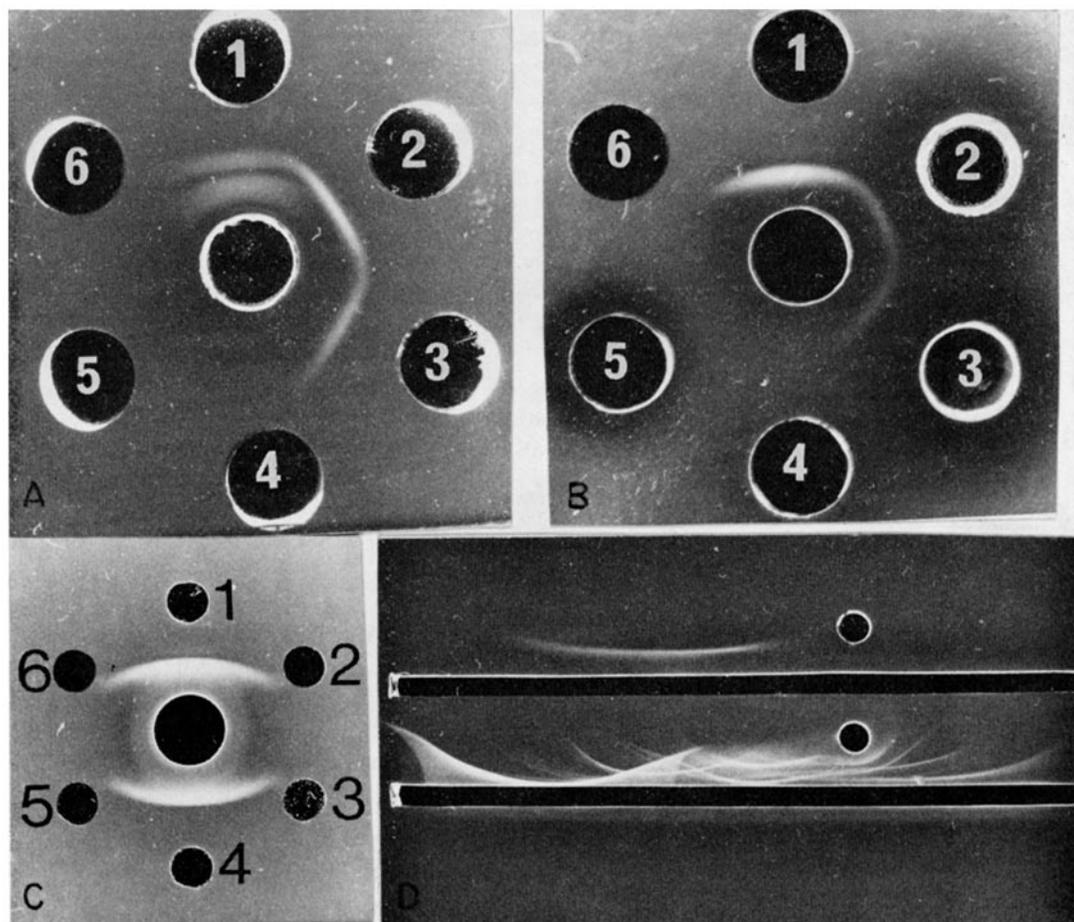


FIG. 1. (A) Anti-amyloid T. H. in central well tested against amyloid T. H. (well 1); amyloid A. R. (2); the high molecular weight subfragment (the void volume material—Sephadex G-100) of amyloid A. R. (3) protein AR subfragment of amyloid A. R. (4); and saline (5, 6). The precipitation line of amyloid T. H. (1) closer to the antiserum well is due to protein AS, while the other precipitation line fusing with that of amyloid A. R. (2) corresponds to the void volume material of amyloid T. H. (B) Anti-amyloid A. R. in central well tested against amyloid A. R. (well 1); serum of patient A. R. (2); protein AR subfragment of amyloid A. R. (3); normal serum (5); and saline. (4, 6). (C) Anti-amyloid A. R. in central well tested against amyloid A. R. (wells 1, 4); amyloid T. H. (2) protein AS of amyloid T. H. (3); amyloid J. B. (5); and the void volume material of amyloid A. R. (6). (D) Immunoelectrophoresis of amyloid A. R. (upper well) and normal serum (lower well). Anti-amyloid A. R. in upper throw and anti-whole serum in lower throw. Anode to the left.

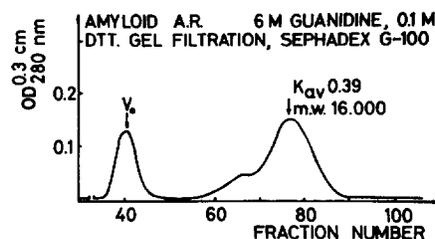


FIG. 2. Gel filtration of amyloid A. R. on a 3.2×96.5 -cm Sephadex G-100 column with $V_0 = 240$ ml. Elution rate 8.1 ml/h. Fraction volume: 5.42 ml. Elution buffer: 5 M guanidine in 1 N acetic acid (Temperature 23°). Sample volume 13.5 ml. $OD_{280\text{ nm}}^{1\text{ cm}} = 20$. The yield of material with K_{av} 0.39 (protein AR) was 66%, determined by estimation of the areas of the peaks.

TABLE I
Antigenicity of Various Amyloid Subfragments from Different Types of Amyloid Fibril

Patient	V_0 material	Protein AS	Light chains		Protein AR	Amyloid E.B. and its subfragments*
			κ	λ		
A. R. Primary amyloidosis‡	+	-	-	-	+	-
J. R. Primary amyloidosis§						
T. H. Secondary amyloidosis§	+	+	-	-	-	-
J. B. Waldenström's macroglobulinaemia§						
E. F. Waldenström's macroglobulinaemia	+	-	-	+	-	-
E. B. Myelomatosis, amyloid tumour*	-	-	-	-	-	+

* Amyloid E. B., kindly provided by Dr. E. F. Osserman, has previously been shown to be antigenically unique (3, 4).

‡ Amyloid material kindly provided by Dr. F. Sauger.

§ Previously studied in (3, 4). These three preparations showed the same pattern as indicated for T. H.

|| Amyloid material kindly provided by Dr. E. C. Franklin.

tested against pooled and single normal sera and 60 sera containing the protein AS-related component. No precipitation reaction was obtained with any of these sera. However, the serum from the patient A. R. showed a precipitation reaction of identity with crude amyloid A. R. as well as with its subunit protein AR (Fig. 1 B). The protein AS-related component could not be detected in this serum.

Additional Studies to Identify Amyloid Subunit Protein AR.—Partial *N*-terminal amino acid sequence analysis by Edman degradation (3) of the protein AR subfragment of amyloid A. R. yielded only one amino acid in each step, indicating that this component was a homogenous protein. The 11 *N*-terminal amino acids were Asp-Phe-Met-Leu-Thr-Gln-Pro-His-Val-Ser-Glu. This sequence is completely different from the corresponding amino acids of the non-

immunoglobulin amyloid protein AS (8), and cannot be identified with any known *N*-terminal sequences of light-chain variable subgroups or with any known sequence in another part of immunoglobulin chains² (9, 10). The most remarkable characteristics of the amino acid composition of protein AR³ were the high content of proline (7.74%) and the low content of the basic amino acids lysine (3.59%) and arginine (2.70%)

DISCUSSION

The subfragment comprising 66% of the proteins of amyloid fibrils A. R. was different from any known amyloid protein. Amyloid A. R. lacked the non-immunoglobulin protein AS, but consisted instead of another completely different subunit, which we have called protein AR. Strongly reacting antisera to this protein AR did not precipitate with any other amyloid preparations or subfragments thereof, or with immunoglobulins or their chains and fragments. The immunologic studies thus showed the presence of a new, uncommon amyloid fibril protein.

This was further supported by the chemical data. Although some amino acid residues in the *N*-terminal part of the polypeptide chains were somewhat similar to κ -light chains, other residues were completely different from both known light chain and protein AS sequences (8–10).

Furthermore, the serum from the patient A. R. contained a protein antigenically related to the amyloid protein AR. This protein could not be detected in any of our other sera from patients with amyloidosis, but has recently been found in a few cases within a larger panel of sera from patients prone to get amyloidosis.⁴ On the other hand, serum from the patient A. R. did not contain the protein AS-related component which was present in most of the other cases of amyloidosis tested (6, footnote 1). We therefore, suggest that protein AR is a major component of a third class of amyloid fibril. Despite the fact that protein AR was not detected in any other amyloid preparation, the void volume material of amyloid A. R. was antigenically similar to that of nearly all amyloid preparations studied. The most pronounced antigenic differences among the various amyloids studied therefore appear to be related to the smaller subfragments. Many amyloid preparations (e.g., amyloid T. H., J. R., and J. B.) contained protein AS, while amyloid A. R. contained protein AR with apparently unique antigenicity. Amyloid E. F. seemed to be a representative of fibrils closely related to light-chain fragments (1). This indicates that different proteins can, perhaps in interaction with the void volume material, form highly insoluble fibrils with similar ultrastructural and optical features. Further studies will probably yield a larger number of amyloid-forming proteins.

² Capra, D. 1973, personal communication.

³ Husby, G., J. B. Natvig, and K. Sletten, unpublished observations.

⁴ Husby, G., J. B. Natvig, and M. Harboe. 1974. *Scand. J. Immunol.* In press.

SUMMARY

An unusual protein AR was isolated from the amyloid fibril preparation derived from a patient with primary amyloidosis. Protein AR was unique in its antigenicity, and revealed no structural identity with any known amyloid proteins or with immunoglobulin chains or fragments. Thus a new third class of amyloid fibril proteins besides the immunoglobulin light-chain variable region fragments and the nonimmunoglobulin protein AS, has been characterized. A component antigenically related to protein AR was found in the serum of the patient.

REFERENCES

1. Glenner, G. G., W. Terry, M. Harada, C. Isersky, and D. Page. 1971. Amyloid fibril proteins: proof of homology with immunoglobulin light chains by sequence analysis. *Science*. **172**:1150.
2. Benditt, E. P., N. Eriksen, M. A. Hermodson, and L. H. Ericsson. 1971. The major proteins of human and monkey amyloid substance: Common properties including unusual N-terminal amino acid sequences. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **19**:169.
3. Husby, G., K. Sletten, T. E. Michaelsen, and J. B. Natvig. 1972. Antigenic and chemical characterization of non-immunoglobulin amyloid fibril proteins. *Scand. J. Immunol.* **1**:393.
4. Husby, G., K. Sletten, T. E. Michaelsen, and J. B. Natvig. 1973. Amyloid fibril protein subunit, protein AS, distribution in tissue and serum in different clinical types of amyloidosis including that associated with myelomatosis and Waldenström's macroglobulinaemia. *Scand. J. Immunol.* **2**:395.
5. Levin, M., M. Pras, and E. C. Franklin. 1973. Immunologic studies of the major nonimmunoglobulin protein of amyloid. *J. Exp. Med.* **138**:373.
6. Husby, G., J. B. Natvig, T. E. Michaelsen, K. Sletten, and H. Höst. 1973. Unique amyloid protein subunit common to different types of amyloid fibril. *Nature (Lond.)*. **244**:362.
7. Pras, M., M. Schubert, D. Zucker-Franklin, A. Rimon, and E. C. Franklin. 1968. The characterization of soluble amyloid prepared in water. *J. Clin. Invest.* **47**:924.
8. Sletten, K., and G. Husby. 1974. The complete amino acid sequence of non-immunoglobulin amyloid fibril protein AS in rheumatoid arthritis. *Eur. J. Biochem.* **41**:117.
9. Smith, G. P., L. Hood, and W. M. Fitch. 1971. Antibody diversity. *Ann. Rev. Biochem.* **40**:969.
10. Putnam, F. W., E. J. Whitley, Jr., C. Paul, and J. N. Davidson. 1973. Amino acid sequence of a K Bence Jones protein from a case of primary amyloidosis. *Biochemistry*. **12**:3763.