IMMUNOLOGIC STUDIES OF THE MAJOR NONIMMUNOGLOBULIN PROTEIN OF AMYLOID

I. IDENTIFICATION AND PARTIAL CHARACTERIZATION OF A RELATED SERUM COMPONENT

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Recent studies have demonstrated the existence of two chemically distinguishable classes of amyloid fibrils (1–5). One type of fibril is derived most often from amyloid fibrils associated with the primary form of the disease and with multiple myeloma. It has as its major component a fragment of an immunoglobulin light chain (5). The other type of amyloid fibril is generally seen in amyloid from one form of familial amyloidosis (familial Mediterranean fever) (FMF)[1] and secondary amyloidosis (2, 6). The recent delineation of the complete amino acid sequence of the major component of this class of amyloid fibril has shown it to have no homology with any known protein (1–4). This component has been termed A component by Hermodson et al. (2), nonimmunoglobulin acid soluble fraction (ASF) by us (1), and amyloid of unknown origin (AUO) by Ein et al. (3) to distinguish it from the immunoglobulin-related types.

ASF consists of 76 amino acid residues, has a molecular weight of 8,500 daltons and a heterogeneous amino terminal (1). This report describes immunologic studies comparing ASF from different patients, and the prevalence and partial purification of a circulating component in human serum which appears to be antigenically related to ASF.

Methods

Acid Soluble Fraction (ASF).—The preparations of ASF were the same as described earlier (1). They were obtained from tissues of patients with FMF, tuberculosis, Hodgkin's disease, and bronchiectasis. Since ASF was insoluble at physiologic pH, it was treated with 0.1 N NaOH for 2 h and then neutralized with 0.1 N HCl. This degraded material (DASF) was soluble in distilled water, physiologic saline, and other buffers. The DASF from a patient with FMF was labeled with ^{125}I using iodine monochloride (7).[2]

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[1] *Abbreviations used in this paper:* ASF, acid soluble fraction; CFA, complete Freund's adjuvant; DASF, degraded ASF; FMF, familial Mediterranean fever.

[2] We would like to thank Dr. Marcus Rothschild for making this preparation for us.

Immunologic Methods.—Initially two rabbits were injected subcutaneously and in the foot-pads with a mixture of ASF dissolved in 0.02 N HCl and complete Freund's adjuvant (CFA) and two rabbits were injected with a mixture of ASF dissolved in 5 M guanidine and CFA. Because of the weak response from these two groups of rabbits, two more rabbits were injected with a mixture of ASF dissolved in 0.1 N sodium hydroxide and CFA. Initial immunization consisted of 1–2 mg/rabbit followed by booster injections of 0.5–1 mg at weekly or biweekly intervals. Antisera from all three groups of rabbits were used in this study and behaved identically.

Double immunodiffusion was performed in 1% agar according to the method of Ouchterlony (8). Immunoelectrophoresis was performed according to the method of Scheidegger (9).

To test the specificity of the antisera, ^{125}I-labeled DASF was precipitated by a "Sandwich" technique using rabbit anti-ASF and sheep antirabbit gamma globulin. 100 μl of rabbit anti-ASF or 100 μl of rabbit antiperoxidase, as a control, were pipetted into each tube followed by 10 μl of ^{125}I-labeled DASF. The contents of the tubes were mixed and the tubes were incubated for 2 h at 37°C. 300 μl of sheep antirabbit gamma globulin were pipetted into each tube, mixed, incubated at 37°C for 2 h, and then incubated overnight at 4°C. The tubes were then centrifuged for 20 min at 1,000 g and the supernatants removed. The precipitates were washed twice with 2 ml of cold saline and drained dry. The precipitates were then counted in a gamma counter (Nuclear-Chicago, Des Plaines, Ill.).

Partial Purification of ASF Related Component from Normal Human Serum.—Normal human serum was obtained from recently outdated blood from the Bellevue Hospital blood bank and concentrated five times by negative pressure dialysis. Starch zone electrophoresis was performed according to the method of Kunkel (10). Protein determinations were performed using a modified Folin technique (11). Gel filtration was performed with Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in 0.3 M sodium chloride.

RESULTS

In order to prove that the antiserum was monospecific for ASF and not reacting with an unrelated contaminant, a chemically pure preparation of ASF from a patient with FMF which had given a single band on polyacrylamide electrophoresis and yielded an unambiguous amino acid sequence previously (1) was degraded, radioiodinated, and reacted with one of the antisera to ASF. As shown in Table I, the antiserum was able to precipitate over 75% of the TCA precipitable counts.

TABLE I

Precipitation of [125I] DASF by Rabbit Anti-ASF

	CPM precipitated	[125I-]DASF precipitated*
10% TCA‡	205,892	—
Rabbit anti-ASF§	157,948	76.7
Control‖	3,346	1.6
Rabbit anti-ASF minus control	154,602	75.1

* Percent of CPM in TCA precipitate.

‡ [125I]DASF with 0.1 ml rabbit serum precipitated with 10% TCA.

§ [125I]DASF was incubated with rabbit anti-ASF and then with sheep antirabbit gamma-globulin.

‖ Rabbit antiperoxidase was substituted for the rabbit anti-ASF.

Each of the three rabbit antisera to ASF gave a single precipitin line with DASF and also reacted with a component present in some human sera (Fig. 1). The antisera gave lines of identity with DASF from patients with FMF, Hodgkin's disease, bronchiectasis, and tuberculosis, and they also gave lines of identity between these DASF's and the human serum component. On immunoelectrophoresis the DASF moved as a single band towards the anode in the position of the β-globulin fraction of serum.

To further study the relation of the serum component to ASF, absorption studies were carried out with a variety of sera and DASF. It was possible to
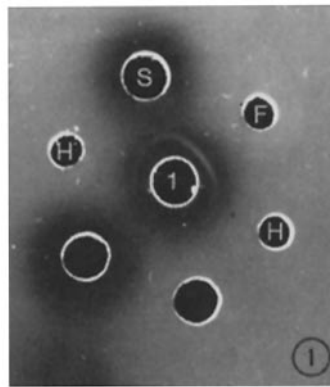

FIG. 1. Double immunodiffusion of anti-ASF vs. human serum and DASF demonstrating reactions of identity. 1, anti-ASF; F, DASF from a patient with FMF; H, DASF from a patient with Hodgkin's disease; S, serum from a patient with tuberculosis but without amyloidosis.

remove all the activity of the antiserum by absorption with ASF, with positive human sera. and even with an equal volume of normal sera that failed to give precipitin lines in agar. However, under similar conditions, five times the volume of pooled cord sera failed to remove the activity of the antiserum.

Since an antigenically-related component was noted in certain normal and pathologic sera, an attempt was then made to determine the frequency of this component related to ASF in normal and pathologic sera. Using specific rabbit anti-ASF sera, 57 normal sera and 89 sera from patients with diseases known to be frequently associated with amyloidosis, were tested by double immunodiffusion. As shown in Table II, the anti-ASF detected a circulating component in only 7% of normal sera but in from 50–80% of sera with certain chronic diseases. Preliminary studies to develop an immunoassay to quantitate this component in serum have proven difficult. However, using the immunoassay small amounts of reactive material have been detected in all of 11 normal sera tested.[3] This, together with the results of the absorption studies suggest that this component may be present in small amounts in all normal sera.

[3] Levin, M., and E. C. Franklin. Unpublished observation.

TABLE II

*Frequency of ASF Related Protein in Normal and Pathologic Sera**

Diagnosis	No. tested	No. +	% +
Normal	57	4	7
Primary and myeloma amyloid	17	9	53
Secondary amyloid	8	7	88
Myeloma	14	7	50
Cirrhosis of the liver	11	6	55
Tuberculosis	20	13	65
Rheumatoid arthritis	10	7	70
Hypo γ-globulinemia	9	6	67
Pooled cord sera	4	0	0
Isolated BIP's	12	0	0

* We would like to thank Doctors H. Kunkel, J. McClement, and D. Zimmon for contributing some of the sera used in this study.

In order to eliminate the possibility that the antisera might be detecting the P component, a protein that has been found adsorbed to the surface of amyloid fibrils (12), purified P component was tested with the anti-ASF, and DASF was reacted with anti-P component by double diffusion analysis.[4] As shown in Fig. 2, the anti-P component did not react with DASF, and the anti-ASF did not react with the P component.

An attempt was made to purify this circulating ASF component from normal human serum. On immunoelectrophoresis (Fig. 3), the reactive component migrated more slowly than albumin. Starch zone electrophoresis of five times concentrated human serum was then performed. The component was found to migrate to the α1-region as determined by double immunodiffusion analysis (Fig. 4). This portion of the starch block was eluted and the protein was concentrated by negative pressure dialysis and subjected to chromatography on a Sephadex G-100 column in 0.3 M NaCl. As shown in Fig. 5, the circulating ASF component was found to elute just before albumin and therefore probably has a molecular weight slightly larger than albumin. Because of the very low yield, further purification has not been possible at this time.

DISCUSSION

The present study presents evidence that ASF, the major nonimmuno-globulin related component of certain amyloids can be made to be immunogenic, and that an antiserum that was raised against a homogeneous and pure ASF protein from a patient with FMF reacted identically with DASF from four different patients.

In attempting to understand the possible origin of ASF it is important to

[4] We would like to thank Doctors Alan Cohen and Martha Skinner for generously supplying us with purified P component and anti-P component.
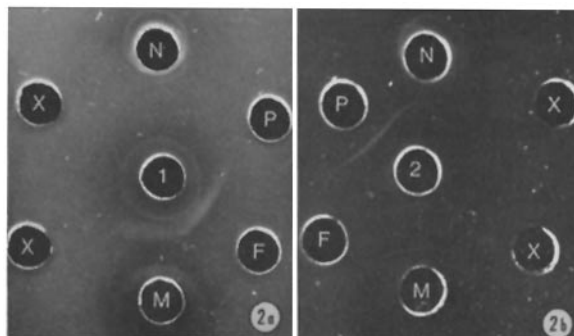
FIG. 2. Double immunodiffusion of anti-ASF well no. 1, and anti-P component well no. 2 vs. DASF, P component and human sera. F, DASF from a patient with FMF; M, serum from a patient with multiple myeloma but without amyloidosis; N, normal human serum; P, purified P component; X, normal saline. Both patterns are from the same plate. Fig. a was photographed after 24 h of incubation while Fig. b was left to incubate for 48 h because the precipitin lines developed more slowly. After 48 h the normal serum also had a faint precipitin line against anti-ASF.
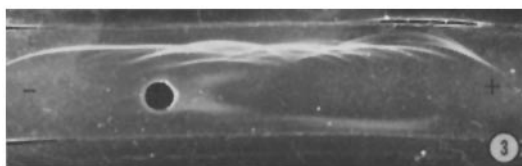

FIG. 3. Immunoelectrophoresis of a serum from a patient with secondary amyloidosis. The antigen well contains the serum, the upper trough contains horse antiwhole human serum, and the lower trough contains anti-ASF. The anode is on the right.

note that the antiserum also reacts with a component in human serum. Although the antiserum detects precipitin lines with only 7 % of normal sera, the other normal sera probably have the circulating component in lesser amounts since preliminary studies using a radioimmunoassay appear to detect reactive material in all normal sera.[3] Pooled cord sera, however, seemingly did not have detectable amounts of reactive material as determined by the radio immunoassay[3] and failed to remove the activity of the antiserum under the conditions of this study. These findings suggest that this component is not synthesized in utero and that the component does not cross the placenta.

Precipitin tests, using this antiserum to ASF, detected an ASF related component in only 7 % of normal sera but detected elevated amounts of the circulating ASF component in from 50–80 % of patients with certain chronic diseases, some of which are often associated with amyloidosis. Furthermore, seven out of eight sera from patients with secondary amyloidosis and six of nine hypogammaglobulinemic sera gave precipitin lines with our antiserum.
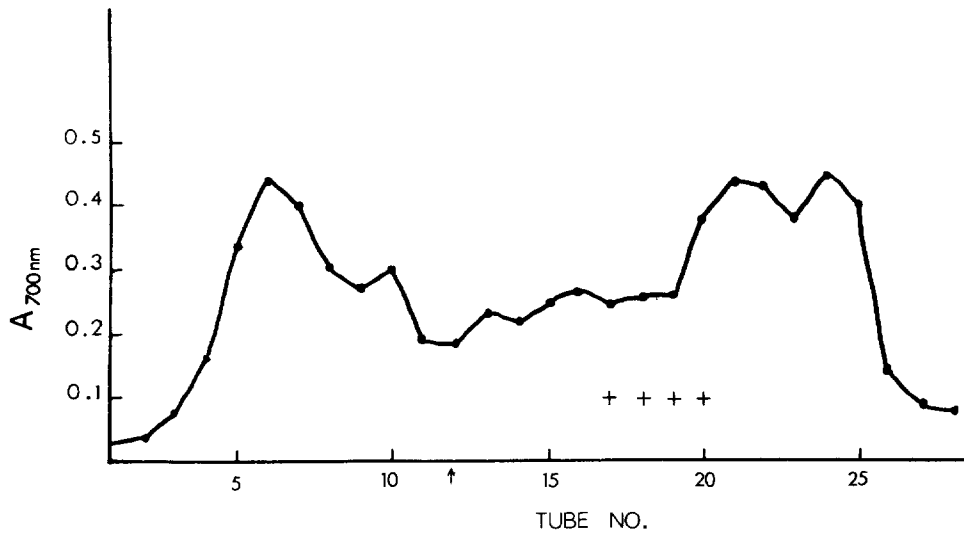
Fig. 4. Starch zone electrophoresis of five times concentrated normal human serum. The +
marks signify the tubes that gave precipitin lines with anti-ASF. The arrow represents the
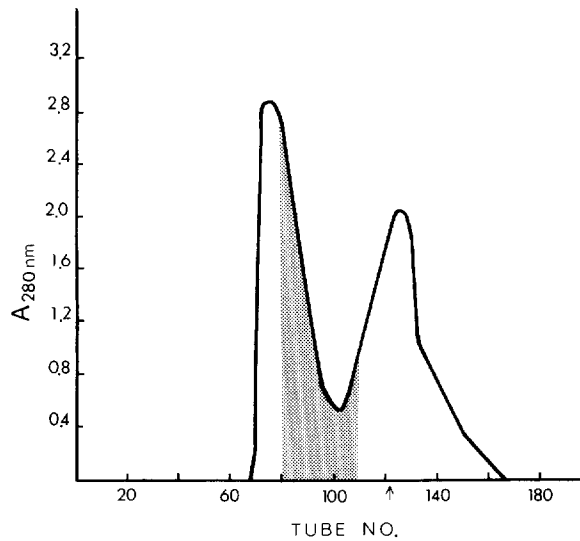sample origin, and the anode is on the right.

Fig. 5. Gel filtration of the fraction containing the ASF related component obtained by
starch zone electrophoresis (Fig. 4). The ASF related component was chromatographed on a
Sephadex G-100 column (190 × 3.5 cm) in 0.3 M NaCl and 6 ml/tube was collected. Approximately every 15 tubes were pooled, and the shaded area represents the two pools that gave
precipitin lines with anti-ASF. The arrow indicates the area where albumin eluted as determined by immunodiffusion.

While the serum component has not been fully characterized it is possible to describe some of its properties. It migrates in the α1-globulin region and appears to be slightly larger than human albumin. It is not related to the P component, another α1-globulin known to be associated with amyloid deposits. While its precise nature remains unknown, this circulating ASF component may be either a substance that cross-reacts with ASF, a protein that binds ASF and reacts with the antisera by virtue of its carrier properties, or a precursor of ASF. The possibility that the antisera are directed against a minor serum protein contaminant associated with the ASF must of course be considered. This seems unlikely in view of the fact that the antisera which precipitate more than 75 % of the DASF fraction which by other criteria was shown to be chemically pure (1), give a reaction of identity between DASF and the α1-globulin serum component.

The finding of light chain fragments in primary and multiple myeloma associated amyloidosis (5) and the in vitro synthesis of amyloid-like fibrils from light chains with enzyme digestion (13) suggests the possibility that ASF too, may be the result of proteolytic digestion of a circulating precursor which can assume the properties of amyloid fibrils. In this regard, it is interesting to note that the amino terminus of the ASF molecule is heterogeneous, possibly as a result of degradation. Although an ASF protein of 76 amino acids has been found in humans (1) and a similar protein containing 76 amino acids has been found in monkeys (2), a possible degradative origin is further supported by the finding of an ASF-like molecule containing only the first 45 amino acid residues in one patient (3) and a larger molecule with additional residues at the amino terminus in the duck.[5] This, plus the high incidence of positive reactions with sera from patients with secondary amyloidosis and certain chronic diseases, suggest that the circulating ASF component may be a precursor of the ASF found in amyloid fibrils and that the ASF may be a proteolytic product. Further studies into the origin and biological functions of this circulating component may lead to an understanding of the pathogenesis of secondary and FMF associated amyloidosis.

SUMMARY

Antisera have been prepared against the major nonimmunoglobulin component of secondary and familial Mediterranean fever (FMF) associated amyloid which has been called A component or acid soluble fraction (ASF). The antisera were shown to be monospecific for ASF by precipitation of ^{125}I-labeled antigen and gave a reaction of identity with four different ASF preparations. The antisera were able to detect a circulating component in human serum that migrated in the α1-globulin region. This circulating component gave a line of identity with degraded ASF by double immunodiffusion. 57 normal sera and 89

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[5] Earl P. Benditt. Personal communication.

sera from patients with diseases known to be frequently associated with amyloidosis were tested by immunodiffusion for the circulating ASF component. 7% of normal sera and 50–80% of the pathologic sera had elevated amounts of this component. Absorption studies showed that all normal sera probably have small amounts of this component while cord sera do not have detectable amounts. This component was partially purified and was shown to be slightly larger than albumin. The relation of the circulating component to the acid soluble fraction of amyloid is discussed.

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