IMMUNOLOGIC STUDIES OF WATER-SOLUBLE HUMAN AMYLOID FIBRILS

Comparative Studies of Eight Amyloid Preparations*

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Several attempts have been made in recent years to study the antigenic constituents of amyloid and purified amyloid fibrils (1–5). Depending on the state of purity of the antigens used for immunization, one or more serum proteins have been identified in all such preparations. Among these are γ-globulins, lipoproteins, certain complement components, and a number of $\alpha 1$ and $\alpha 2$ globulins. The purest of these preparations induced the formation of antibodies to only one substance, the "P component," which is an $\alpha 1$ globulin with a sedimentation coefficient between 7 and 19S present in normal serum but not in nonamyloid tissues (6). The "P component" appears to correspond to the pentagonal structures, also known as rods or doughnuts, reported by Glenner et al (7). The "P component" does not constitute a major constituent of amyloid since in preparations of amyloid fibrils containing large amounts of the component, only a small fraction of the protein was precipitable by the antiserum (5, 6).

The recent demonstration that the major constituent of amyloid is a fibrillar protein (8) may be the reason why amyloid has proven to be poorly immunogenic and why it has been difficult to obtain antibodies specifically reactive with amyloid (3, 5). The availability of purified water-soluble amyloid fibrils from nine patients with amyloidosis (9, 10) has permitted a series of studies designed not only to characterize the major antigenic constituents of amyloid, but also to determine whether these are unique to amyloid or whether they are present also in normal serum or tissues. In addition, it has been possible to compare amyloid fibrils isolated from different subjects and, in two instances, from different tissues from the same subject. The results of these studies permit the following tentative conclusions. Firstly, the major antigenic constituent of amyloid fibrils

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is a protein unique to amyloid and not present in normal serum, in normal tissues, or in serum from patients with amyloidosis. In its native fibrillar form, this material appears to be poorly immunogenic or possibly even nonimmunogenic. However, after degradation with sodium hydroxide, the material from most amyloid preparations induced the formation of antibodies in rabbits (11). Secondly, different preparations of amyloid show varying degrees of antigenic cross-reaction, while each appears to contain additional antigenic determinants that distinguish it from the others (12). Taken together with the physical and chemical characterization in the accompanying article (10), these results suggest that amyloids from different individuals are a series of closely related, but not identical substances. Because of the complexity of these proteins, a larger number of antisera and amyloid preparations will have to be tested to permit definite conclusions regarding the existence of well defined classes and subclasses of amyloid.

Materials and Methods

Soluble Amyloid and Degraded Amyloid (DAM).—Preparations of amyloid were the same as were used in the preceding article (10). DAM was prepared from each of these by treatment with 0.1 M NaOH, followed by prolonged dialysis against distilled water.

Immunization.—In the initial phases of this study, four rabbits were immunized subcutaneously with a mixture of amyloid 1 in Freund's adjuvant at weekly or biweekly intervals. Numerous bleedings tested by complement fixation against amyloid and DAM during a 9 month period failed to demonstrate antibody. By precipitin analysis, two of these antisera gave a faint precipitin line on immunoelectrophoresis with about one-half of the available preparations of aged amyloid, but never with fresh preparations of amyloid or DAM. The reactive antigenic component had a mobility slower than γ-globulin, but defied further identification. Because of the weakness of these antisera and the ill-defined nature of the material reacting with them on immunoelectrophoresis, we used DAM as the immunizing antigen in subsequent experiments in the hope of producing stronger antisera reactive with amyloid and DAM. It seems possible that amyloid is poorly immunogenic because of the general observation that fibrous proteins retaining their native structure often elicit a poor antibody response (3, 5).

At least two rabbits were immunized by the same procedure with each of eight DAM preparations. DAM 1, 2, 5, 6, 9, and 10 proved to be excellent antigens and induced the formation of significant amounts of antibody within 2–3 months. DAM 3 and 8 have failed to induce an immune response after 9- and 5-month courses of immunization respectively.

Immunologic Methods.—All antisera were inactivated at 56° for 30 min and absorbed with normal human serum and either normal spleen or liver homogenates. Antibody activity was tested for in the following ways:

1. Qualitative determinations of antibody were done by immunoelectrophoresis (13) or by double diffusion in agar using Ouchterlony's method (14).

2. Quantitative precipitin analyses were done according to the method of Heidelberger (15). In tests involving precipitation, only DAM could be used as the test antigen because of the insolubility of amyloid in solvents containing even trace amounts of salt (9). Since amyloid proved to be reactive with these antisera, it was possible to use it for absorption studies designed to compare the antigenic relationship of amyloid and DAM by quantitative analysis. Absorption of the antisera was carried out by the addition of amyloid in distilled water to the

antisera. In general, amounts up to 50 times the amount of DAM were employed. It was not important to avoid the region of antigen excess where one commonly encounters solubilization of complexes, since the antigen used for absorption was insoluble under the conditions of the experiment and thus behaved as an insoluble immunoadsorbent. A large flocculent precipitate formed immediately after the addition of amyloid and was removed in the Spinco Model L ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Fullerton, Calif.) by centrifugation at 10,000 rpm for 30 min. An equal amount of distilled water was added to the unabsorbed antiserum used for comparison. A control to detect possible nonspecific removal of antibody γ-globulin from the antiserum was carried out by the addition of amyloid to an antiserum to human γ-globulin.

3. Complement fixation was done according to the method of Mayer (16), using approximately 40–60 $C'H_{50}$ units of guinea pig complement. Since not all tests with each antiserum were done simultaneously, the results are expressed as per cent of complement fixed, for ease of comparison. Because antigens need not be in solution for complement-fixation tests, both amyloid and DAM could be used as antigens and tested directly.

Radioactive Labeling.—To increase the sensitivity of the precipitin tests with some of the early bleedings from the rabbits immunized with DAM 1 and to get a clearer idea of the fraction of DAM precipitated by the antisera, we labeled DAM 1 with ^{125}I, using iodine monochloride (17).[1] The amyloid from which this DAM was made appeared homogeneous on ultracentrifugal analysis. By this criterion, it was probably at least 90% pure. The amount of radioactivity precipitated was measured in a pulse height analyzer.

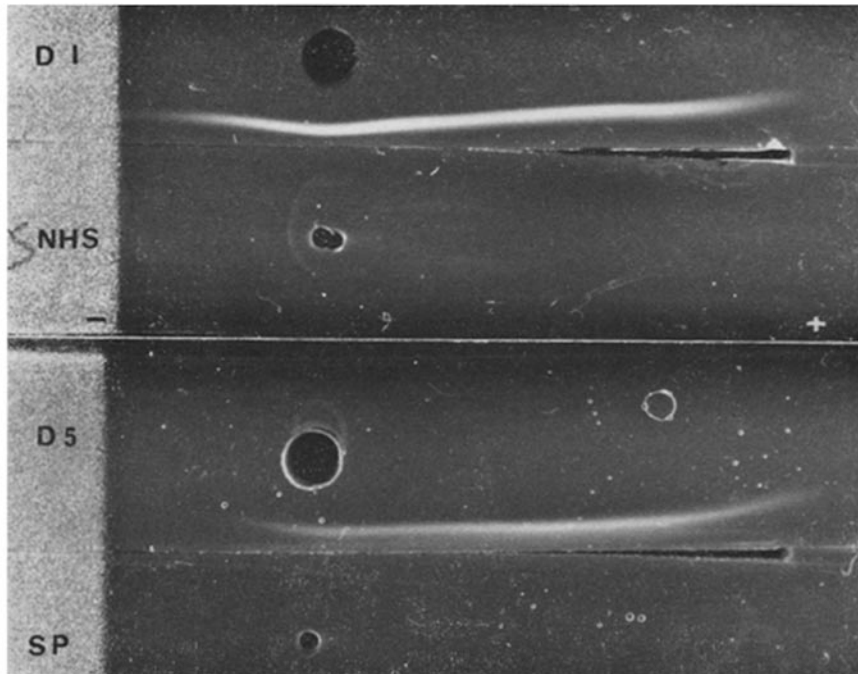
RESULTS

Nature of Antisera and Antigens.—Figure 1 *a* shows the immunoelectrophoretic pattern of DAM 1 and DAM 5 and their homologous antisera. Similar patterns were also noted with each of the other preparations on immunoelectrophoresis. In the fresh state, each preparation migrated slightly to the anodal side of the point of application. After prolonged storage in the cold, however, the mobility of several of these preparations changed and the major component migrated in the region of the slow γ-globulins. The lower half of the slides indicates that these antisera, even prior to absorption, failed to react with either normal human serum or an extract of spleen. Since some of the antisera gave a weak reaction with either a tissue extract or normal human serum, all antisera were absorbed with a mixture of NHS and a saline extract of the tissue from which the amyloid was extracted prior to use.

Figure 1 *b* shows an Ouchterlony plate, indicating that a representative antiserum gives a single precipitin line with DAM but fails to react with NHS, spleen, γG globulin, Fab, and Fc fragments. While six of eight antisera produced precipitin lines with the homologous antigen, none gave a precipitin line with any other DAM preparations. This appears to be due to the lack of sensitivity of the method, since significant cross-reaction was noted by quantitative precipitation and complement-fixation tests.

Since the possibility remained that the reaction was due to a minor contaminant associated with the fibrils rather than to a reaction between antibody

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



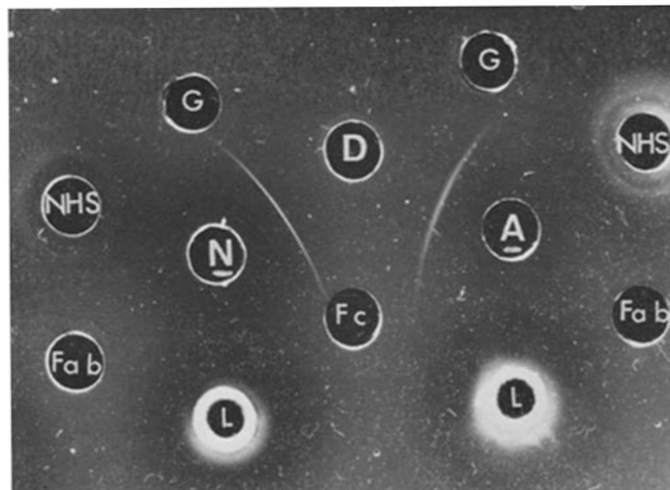


FIG. 1 *a*. Immunoelectrophoretic patterns of DAM 1 and DAM 5 (D-1 and D-5), normal human serum (NHS), and normal spleen extract (SP). Top, antiserum 210 to DAM 1; bottom, antiserum 231 to DAM 5. 1 *b*. Ouchterlony analyses, comparing reactions of DAM 2 (D), NHS, liver extract (L), γG globulin (G), Fc fragment (Fc), and Fab fragment (Fab) with Antiserum 212 neat (N) and absorbed with an excess of liver extract and serum (A) in the center wells.

and fibrils, 1 ml of each of the antisera was absorbed with a powder prepared by lyophilization of 1 ml NHS, 1 ml of sodium hydroxide–treated serum, and an aqueous extract prepared from 400 mg dry tissue, half of which was also treated with 0.1 M sodium hydroxide. As shown in Figure 1 *b*, there was no diminution in the precipitin line after such exhaustive absorption. Similarly, the amyloid fibrils and DAM preparations failed to react with antisera to albumin, γ-globulins, $\alpha 1$ and $\alpha 2$ globulins, and C-reactive protein.

To further characterize the antigen and to determine what fraction precipitated with the antiserum, we precipitated the ^{125}I-labeled preparation of DAM 1 with the homologous antiserum. 87% was precipitable with 10% TCA, and 55% was precipitated by the antiserum at equivalence. Thus, more than 63% of the TCA-precipitable antigen was precipitated by the antiserum. It is not possible to determine whether the nonprecipitable fraction represents a nonantigenic contaminant or the denaturation of some of the protein during the iodination or fractionation procedures, or if both factors were involved. Similar studies were not carried out with the other DAM preparations used.

Antigenic Relationships by Precipitin Analysis of Different DAM Preparations and between DAM and Amyloid.—The stronger of each pair of antisera was selected for studies to quantitate the relationship of different DAM preparations to each other and, in most instances, to the native amyloid preparations. Two approaches were used: quantitative precipitation and complement fixation.

The results of quantitative precipitin analyses with four of the five antisera examined in detail are illustrated in Fig. 2. Because of lack of materials during certain phases of the study, it was not possible to compare each DAM preparation with each of the antisera studied.

As expected, each antiserum reacted optimally with the antigen used for immunization; to a variable degree each reacted with most, but not always all, of the other preparations studied. None of the antisera gave a precipitate with NHS or a saline extract of liver, spleen, or kidney over a very wide range of concentration. Antisera 231, 236, and 242 showed significant degress of cross-reaction with each of the DAM preparations tested. Antiserum 213 failed to react with one of eight preparations tested, while antiserum 210 appeared to have a more limited specificity since it failed to react with five of eight DAM preparations by precipitin analyses. We further confirmed these studies with antiserum 213 by absorbing aliquots with DAM 1, 2, and 3, and then by testing the supernatants with DAM 2, the antigen used for immunization. In accord with the precipitin results, DAM 2 removed all the antibody, DAM 1 about 33%, and DAM 3 only negligible amounts.

Because of the insolubility of amyloid in physiological saline, it was not possible to test the reactivity of the native protein with these antisera by precipitin analyses. Consequently, absorption experiments were designed to determine the effect of the absorption of the antisera with homologous amyloid in

the region of antigen excess on subsequent ability of the antisera to precipitate DAM. Control experiments demonstrated that the addition of amyloid to an antiserum to γG globulin did not affect the precipitation of γG globulin

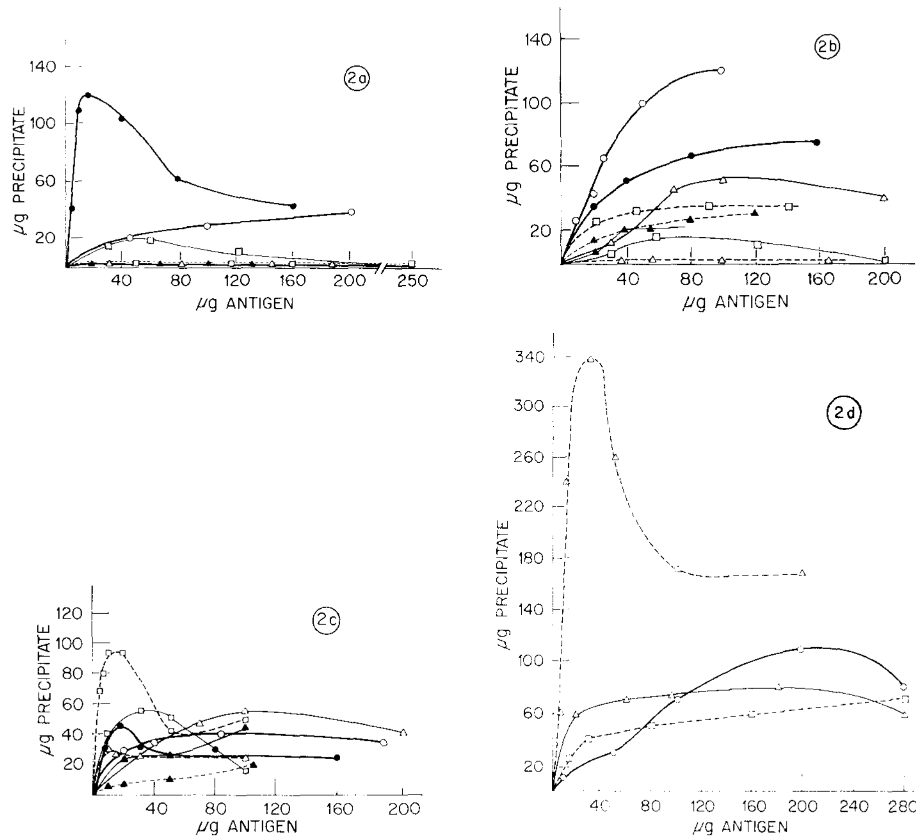


FIG. 2. Precipitin analyses with various DAM preparations and: *a*, Antiserum 210 to DAM 1; *b*, Antiserum 212 to DAM 2; *c*, Antiserum 231 to DAM 5; *d*, Antiserum 242 to DAM 9.

Antigens used in Figs. 2 and 3: DAM 1 ●——●; DAM 2 ○——○; DAM 3 ▲----▲; DAM 5 □----□; DAM 6 ▲——▲; DAM 8 △——△; DAM 9 △----△; DAM 10 □——□.

and, therefore, did not remove an unrelated antibody. As shown in Table I, addition of amyloid 2 to antiserum 213 completely removed all antibody reactive with DAM 2, thus showing that amyloid contained all of the antigenic determinants present in DAM. Addition of amyloid 1 to antiserum 210 and amyloid 6 to anti-DAM 236 resulted in a 55–75% decrease in the amount precipitated by the homologous DAM, a result which indicates a significant, but

not complete, cross-reaction. In contrast, addition of amyloid 9 to antiserum 242 removed less than 20% of the antibody. In the case of Amyloid 5, insufficient amounts of material were available to carry out absorption studies. The results of these experiments are summarzed in Table I and are compared to similar comparative studies of DAM and amyloid by complement fixation.

Antigenic Relationships by Complement Fixation between Different DAM Preparations and between DAM and Amyloid.—Complement fixation was done to confirm and extend the results of the precipitin analyses. It was also done

TABLE I

Cross Reaction of Amyloid and DAM as Measured by the Absorption of Precipitating Antibody and by Complement Fixation

Amyloid No.	Antiserum	Residual* Precipitate	C′ Fixation‡	
			% Fixed	$\frac{\mu g\ AM}{\mu g\ DAM}$ at peak
		%		
1	210	25; 45§	25	10
2	213	0	100	5
6	236	30	100	6
9	242	80	AC‖	—

$$* \% \text{ Residual precipitate} = \frac{\mu g \text{ ppt with absorbed AS}}{\mu g \text{ ppt with neat AS}} \times 100.$$

$$‡ \% \text{ Fixed} = \frac{C'H_{50} \text{ fixed at peak by AM}}{C'H_{50} \text{ fixed at peak by DAM}} \times 100.$$

§ 25% was the result obtained with the unlabeled preparation of DAM; 45% residual was estimated by measuring the ^{125}I-labeled preparation.

‖ AC, anticomplementary.

because the complement-fixation reaction can be carried out with insoluble antigens. Thus, since the interaction of the antisera and amyloid could be directly measured by this assay, it was possible to compare, by complement fixation, the cross-reaction between DAM and the amyloid from which it was prepared.

Only one of the reagents (amyloid 9) proved to be anticomplimentary at the concentrations used. Fig. 3 shows the comparison of different preparations of DAM with the same four antisera to DAM shown in Fig. 2. In a qualitative sense, the degree of cross-reaction noted by complement fixation was similar to that observed by precipitin analyses. However, minor inconsistencies were noted in several instances and cannot be explained at this time. Among these are the greater reaction by complement fixation than by precipitation between DAM 5 and 8 and antiserum 242; and the weak reaction between DAM 9 and antiserum 231, and DAM 6, 8, and 9 and antiserum 210 respectively in the face of

negative precipitin reactions. It seems possible that some of these discrepancies may reflect differences in the sensitivity of the two assays.

The results of complement-fixation reaction between the antisera and intact amyloid also corresponded closely to those obtained by the inhibition of precipi-

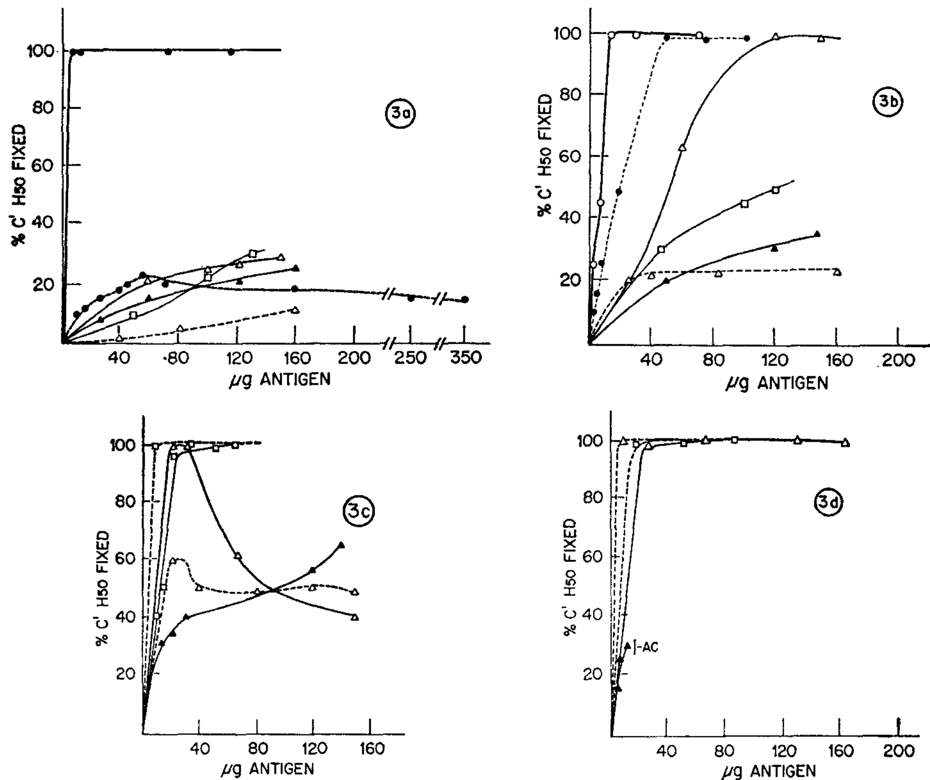


FIG. 3. Complement fixation with various DAM preparations and several amyloid preparations. *a* Antiserum 210 to DAM 1; *b* Antiserum 212 to DAM 2; *c* Antiserum 231 to DAM 5; *d* Antiserum 242 to DAM 9. Symbols for DAMs—Same as in Fig. 2. Symbols for AM 1 ●—● in Fig. 3 *a*; AM 2 ●—● in Fig. 3 *b*; AM 9 ▲——AC in Fig. 3 *d*.

tation by the addition of intact amyloid to the antisera (Table I). In the case of amyloid 1, much larger amounts of amyloid than DAM were needed for every point on the curve, and the antigen never fixed more than 30% of the amount of complement fixed by DAM 1. In contrast, amyloid 2 and amyloid 6 fixed as much complement as did the corresponding DAM. However, five to six times the amount of antigen were required to accomplish this. Similar studies could not be done with amyloid 5 for lack of material and with amyloid 9 because it proved to be anticomplementary.

In only two patients was amyloid from different tissues available for comparative studies. In both instances, two cross-reacting antisera revealed some differences in the reactivity of amyloids prepared from the spleen and kidney from the same subject. However, the amyloid from the spleen and kidney of the same individual bore a closer resemblance to each other than they did to the amyloid prepared from the same organs of the other subject. Additional studies with pure preparations will be required to permit definitive conclusions on the identity or lack of identity of amyloid from different tissues of the same subject.

Reaction of Antisera with Sera and Urines from Patients with Amyloid, and Reaction of Sera from These Patients with Amyloid.—Twelve sera and two urines were available from patients with amyloidosis. Four of these had multiple myeloma and the remainder had either primary amyloidosis or secondary amyloidosis associated with chronic infection. It seemed possible that their sera might contain products antigenically related to amyloid. However, none of the sera reacted with any of the antisera by double diffusion in agar. Conversely, the possibility existed that some of the sera might contain antibodies to amyloid. Here, too, no precipitin reactions were noted when the sera were allowed to diffuse against each of the DAM preparations, including the one prepared from the patient whose serum was tested. Negative reactions were also obtained with three of the sera which contained myeloma proteins when they were tested with the patients' own DAM preparations.

DISCUSSION

One of the major conclusions from this study appears to be that amyloid fibrils contain one or more antigenic substances which are not present in normal human serum or tissue. Because of the nature of the antigen and difficulties in obtaining clearcut results by double diffusion studies, it is not possible to determine the precise number of antigenic constituents involved. An equally important result of this study is the failure to demonstrate any serum protein constituents in these preparations of purified amyloid fibrils. When we compare these results, which at first glance appear contradictory to those previously reported in the literature (1–5), three points of difference must be considered. Firstly, the purified amyloid fibrils used in this study were thoroughly extracted with physiological saline to remove all soluble proteins. Thus, it seems possible that γ-globulins, α-globulins, and lipoproteins may all be intimately associated with the fibrils without being integral constituents of the major fibrillar protein component of amyloid. Secondly, since the amyloid fibrils appear to be weak antigens (1–5), it seems possible that they may not induce the formation of antibodies regularly when injected with other proteins. Since it seems likely that none of the previous investigators had pure amyloid fibrils, it is possible that antibody formation was induced by the associated proteins rather than the amyloid fibrils, and that antibody formation to the fibrils was inhibited by anti-

genic competition. Thirdly, the degradation of the amyloid fibrils by sodium hydroxide, and possibly by other agents, appears to have significantly increased the immunogenicity of the protein.

The results of this study, taken together with the preceding chemical analyses, permit certain tentative conclusions concerning the relations of amyloid to DAM and of different DAM preparations to each other. Thus, different preparations of amyloid appear to share certain common characteristics, yet to have others which appear to distinguish one preparation from the other. While one of the antisera appeared to be very limited in its reactivity, most of the antisera reacted with most or all of the preparations of amyloid tested. The relatively high degree of purity of these preparations and the nature of the quantitative precipitin curves which cannot be explained readily by the presence of varying amounts of contaminants suggest that these differences are indeed real and not due to contaminating substances. It seems rather surprising that the relatively great similarity in physical and chemical properties between different preparations of amyloid could give rise to the fairly marked immunological differences. However, the possibility exists that greater differences will become apparent once their primary structure is known. We can cite a precedent for this type of phenomenon in myeloma proteins which, in spite of their physical and chemical similarities, can have major differences by antigenic analysis. In myeloma proteins the magnitude of the differences in primary structure often does not become clearly apparent until amino acid sequence studies are performed, or until sufficiently good antisera are available to allow the demonstration of classes and subclasses. The number of preparations of amyloid and antisera available at this time does not permit any conclusions regarding the existence of classes and subclasses of amyloid; in fact, the chemical and physical data in the previous article suggest this existence more strongly than do the immunologic studies.

A few comments should be made on the immunogenicity of amyloid and the antigenic relationship of amyloid and DAM. The results of others, taken with the few experiments cited in this article, would suggest that amyloid, like other fibrillar proteins, is a poor antigen. This feature may help to explain many of the results previously published. Once the fibril is degraded, however, it appears to become more immunogenic. Whether this is related to its greater susceptibility to proteolysis in vivo or other as yet unknown factors remains obscure. The experiments, comparing the antigenic properties of amyloid and DAM and using the antisera to DAM, suggest that some of the determinants present in amyloid are either completely or partially hidden. Thus, they are not accessible to the antiserum. In one instance, where amyloid was able to remove all the antibody reactive with DAM, five times the amount of antigen was required to reach equivalence, while in three others only a fraction of the potential determinants was actually available for reaction with the antibody.

The search for soluble precursors of amyloid in serum and urine as well as attempts to find antibodies to amyloid in the sera of patients were negative. However, additional studies with more sensitive methods appear warranted to rule out these possibilities with absolute certainty. Many other questions remain to be answered, and many new approaches are potentially feasible as a result of the availability of antisera, such as these. Thus, if enough different antisera and different preparations of amyloid are available, a clear-cut idea of the relation of amyloids from different patients and from different tissues from the same patient would permit a more clear-cut classification of amyloid. Of even greater importance, however, is the possibility to localize amyloid fibrils or their precursors within cells presumably involved in their synthesis. Attempts to obtain information in these areas with light and electron microscopes are in progress (12).

SUMMARY

Eight preparations of soluble amyloid and degraded amyloid (DAM) were compared immunologically. Unlike amyloid fibrils, six of eight preparations of DAM proved to be relatively strong immunogens. Antisera to DAM reacted weakly or not at all with normal human serum or extracts of normal tissues, but were specifically reactive with amyloid fibrils or DAM.

Comparative studies of DAM's from eight different subjects showed some degree of cross-reactivity among them, yet demonstrated that they were not identical. Similar conclusions were obtained by quantitative precipitin and complement fixation analyses.

Comparison of the amyloid fibrils with the homologous DAM by complement fixation and absorption studies demonstrated the existence in DAM of antigenic determinants that were lacking or inaccessible in the native fibrils.

A search for amyloid precursors and antibodies to amyloid in the sera of 12 patients proved unsuccessful.

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