A STUDY OF THE HISTOCHEMICAL AND STAINING CHARACTERISTICS OF AMYLOID*

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During a study of senile cardiac amyloidosis,[1] it was observed that the manifestation of metachromasia with acidified crystal violet[2] constituted the most reliable indication that suspicious hyaline deposits were indeed amyloid. However, when the staining characteristics of other varieties of amyloid were investigated, it was noted that orthochromatic reactions were frequently encountered in amyloidosis associated with multiple myeloma. This observation prompted a general survey of the staining and histochemical reactions of several varieties of amyloid, the results of which are herein reported.

MATERIAL AND METHODS

Tissues were secured at necropsy from patients dying at the Cincinnati General Hospital and affiliated institutions. Processing by a variety of techniques was unavoidable since a portion of the material had been obtained many years previously. Paraffin blocks from 3 cases of amyloidosis associated with multiple myeloma were furnished us through the kindness of Dr. D. C. Dahlin, Mayo Clinic, Rochester, Minnesota. The manner of preparation and number of cases of each type are indicated at the head of each column in Table I. The techniques applied are indicated in the extreme left-hand column of the chart. Unless otherwise indicated below, the techniques utilized were those in standard use. Sections from cases of senile cardiac, generalized primary, secondary, multiple myeloma and isolated cutaneous amyloidosis were examined. Because of the paucity of material, not all reactions were performed upon all varieties.

Crystal violet staining was performed at concentrations of 0.45 per cent both in dilute hydrochloric acid solution[2] and in buffers at graded pH from 2.0 to 10.0. Hyaluronidases of testicular (Nutritional Biochemicals Corporation, Cleveland, Ohio) and streptococcal (furnished through the courtesy of Wyeth and Company, Philadelphia, Pennsylvania) origin were utilized in digestion studies. The methylation[3] and

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TABLE I

Staining and Histochemical Properties of Amyloid

Reaction	Forms of amyloidosis, manner of preparation, and number of cases				
	Senile cardiac, 5 cases (F/P, F/F)*	Generalized primary, 1 case (Z/P)* Heart, tongue	Secondary, 3 cases (F/P, F/F)* Kidney, spleen	Multiple myeloma, 5 cases: 1 (F/P, F/F), 3 (F/P), 1 (Z/P)* Bone, liver	Isolated cutaneous, 1 case (F/P)*
Crystal violet (0.45%)	Metachromatic pH 2.0 – 9.0	Same	Same	4 of 5 orthochromatic	Metachromatic pH 2.0 – 9.0
Methylation 20 – 25° C.	No change in 72 hr.	Same	Same	No block (1 case)	Same
Methylation 60° C.	Block in 12 hr.	Same	Same	Block (1 case)	Same
Methylation-saponification	Restores metachromasia	Restores metachromasia	Restores metachromasia	Restores metachromasia (1 case)	Restores metachromasia
Acetylation	No block	No block	No block	No block (1 case)	No block
Diastase digestion	No block	No block	No block	No block (1 case)	No block
Pepsin digestion	No block	No block	No block	No block (1 case)	No block
Trypsin digestion	Amyloid dissolved	Same	Same	Same	Same
Hyaluronidase digestion	No effect	Same	Same	Same	Same
Ribonuclease digestion	No effect	Same	Same	Same	Same
Toluidine blue metachromasia					
(a) 3%, 70° C.	+	+	+	4 of 5 orthochromatic	Weak +
After methylation	o	o	o	o (1 case)	o
Methylation-saponification	+	+	+	+ (1 case)	+
(b) 0.25%, room temperature	Weak to absent	Weak to absent	Weak to absent	4 of 5: o 1 of 5: weak to absent	o
After sulfation	Strong	–	Same	+	+
After pepsin digestion	+	–	+	1 of 4 probably +	?
After trypsin digestion	o	o	o	o	–
After pepsin-methylation	o	o	o	o	–
After pepsin-methylation-saponification	**Probably +**	**–**	**Probably +**	**?**	**–**
Hyaluronidase	**No effect**	**Same**	**Same**	**Same**	**Same**

Schiff reaction	o	o	o	o	o
After HIO_4	+	+	Strong +	Usually strong + (4 of 5 cases)	+
Diastase PAS	No effect	Same	Same	Same	Same
Pepsin PAS	o	o	o	o	o
Bisulfite PAS	Block 2 hr	Same	Same	2 of 5 require 24 hr.	Block 2 hr.
Acetylation PAS	Block 2 hr.	Same	Same	2 of 5 require 24 hr.	Block 2 hr.
Methylation PAS	Block 72 hr. 60° C.	Same	Same	Same	Same
Proteins	+	+	+	+	+
Naphthol Y SX	+	−	+	−	−
Millon	+	+	+	+	+
Ninhydrin-Schiff†	Weak +	Same	Same	Same	Same
Coupled tetrazonium	+	+	+	+	+
Post PTA-aniline blue (Masson, Mallory-Heidenhain)	Blue	Blue	Blue	2 blue; 2 red; 1 mixture	Blue
Phosphotungstic acid-hematoxylin	Pink	Pink	Pink	3 of 5 blue	Pink
Acid fuchsin (van Gieson)	o	o	o	o	o
Feulgen	o	o	o	o	o
Lipids					
Oil red O	o	o	o	o (1 case)	o
Luxol fast blue	o	o	o	o	o
Sudan black B	o	o	o	o	o
Baker's acid hematein	o (1 case)	−	−	o (1 case)	−
Miscellaneous					
Alcian blue	Usually +	+	Usually o	o	Weak +
Alcian green	Usually +	+	Usually o	o	Weak +
Alcian blue-PAS	Mixed	Mixed	Usually red	Usually red	Mixed

* F/P = Formalin fixation, paraffin embedded.
F/F = Formalin fixation, frozen sections.
Z/P = Zenker fixation, paraffin embedded.
† Only formalin-fixed tissue available.

o = Negative reaction.
+ = Positive reaction.
? = Reaction indeterminate because of poor preservation.
— = Reaction not performed.

saponification[4] techniques were those described by Lillie. When these rigorous techniques followed peptic digestion, survival of the tissue posed a considerable problem, but the use of the concentrated toluidine blue technique described below provided confirmation of the findings to be described. Sulfation was accomplished by means of the technique described by Moore and Schoenberg.[5] Toluidine blue was used both in buffered dilute (0.25 per cent) solution at room temperature and in concentrated (3 per cent) solution, buffered to pH 2.9 at 70° C.[6]

Results

The results are indicated in Table I. The salient features are summarized as follows:

1. In all varieties of amyloid, protein content was prominent.
2. Amyloid in multiple myeloma, in contrast to other varieties tested, usually was orthochromatic with crystal violet.
3. Other varieties of amyloid manifested strong metachromasia with crystal violet or with 3 per cent toluidine blue at 70° C., but weak to absent metachromasia with dilute (0.25 per cent) toluidine blue at room temperature.
4. Sulfation or pepsin digestion resulted in the production of metachromasia with toluidine blue; methylation blocked metachromasia with crystal violet. Methylation also blocked toluidine blue metachromasia produced by peptic digestion or the use of 3 per cent toluidine blue concentration at 70° C.
5. When methylation was followed by saponification with potassium hydroxide, metachromasia was restored.
6. The periodic acid-Schiff (PAS) reaction was positive in all cases; frequently it was stronger in secondary amyloid and amyloid with myeloma.
7. Two-hour bisulfite treatment or acetylation, or peptic digestion all blocked the PAS reaction. There was, however, greater resistance to the blocking by bisulfite and acetylation in some cases of amyloid with myeloma.
8. Phosphomolybdic acid-phosphotungstic acid-aniline blue and phosphotungstic acid-hematoxylin (PTAH) methods produced reactions resembling those of collagen in all varieties of amyloid except in that associated with multiple myeloma; all reacted with acid fuchsin (van Gieson) stain in a fashion unlike collagen.
9. Lipid was absent.

Discussion

Many definite and certain speculative interpretations of these results are warranted. It seems reasonable to conclude that most amyloids contain two carbohydrate components manifesting independent histochemical characteristics. One of these is a PAS-positive component (probably glycoprotein[7]), blocked by acetylation and digested by pepsin. The other is an acid mucopolysaccharide which yields metachromasia with crystal violet but not with dilute toluidine blue. This metachromasia is neither blocked by acetylation nor digested by pepsin. The observation that metachromasia with toluidine blue is revealed after peptic digestion has been interpreted by Windrum and Kramer[8] to indicate that anionic dye binding sites are blocked in the amyloid moiety by combination with glycoprotein.

Larsen[6] demonstrated metachromasia of amyloid with toluidine blue when acidified concentrated solutions (3 per cent) and high temperatures (70° C.) were used. He also showed that the presence of free proteins interfered with the demonstration of metachromasia of acid mucopolysaccharides when dilute solutions of toluidine blue were utilized. He postulated that there was a competition between proteins and toluidine blue molecules for the anionic dye binding sites of acid mucopolysaccharides. At high temperatures (70°) and low pH (2.9), highly concentrated dye cations could exchange with the blocking proteins and metachromasia thus become manifest. Confirmatory evidence indicating the presence of acid mucopolysaccharides was provided by the usually positive reactions with both Alcian blue and Alcian green.

The data herein described would further tend to indicate that the acid polysaccharide was carboxylated rather than sulfated or phosphorylated. As pointed out by Kantor and Schubert,[9] methylation of acid mucopolysaccharides eliminates their metachromasia, but by different mechanisms in the case of sulfated, as opposed to phosphorylated or carboxylated compounds. In sulfated polysaccharides, the sulfate group is removed by means of methylation, resulting in the substitution of a hydroxyl group and the formation of free methyl sulfate esters. With carboxyl or phosphoryl groups, the acidic groups remain attached and become esterified. In these latter cases, saponification restores the original structure[4,10,11] and, therefore, the property of metachromasia, whereas with sulfated mucopolysaccharides, restoration of the property of metachromasia by saponification is im-

possible, since the sulfate groups are no longer attached to the polysaccharide molecules.

For sulfated acid mucopolysaccharides:

$$R-OSO_3K + CH_3OH \xrightarrow{HCl-60^\circ} ROH + CH_3OSO_3K$$

For carboxylated acid mucopolysaccharides:

$$R-COOH + CH_3OH \xrightarrow{HCl-60^\circ} RCOOCH_3$$

$$R-COOCH_3 + KOH \xrightarrow{70\%\ \text{alcholic}} R-COOR + CH_3OH$$

This sequential procedure causes the restitution of crystal violet metachromasia and the metachromasia with toluidine blue following peptic digestion or the use of 3 per cent toluidine blue at 70°. These findings appear to preclude the possibility that a sulfated compound may account for the metachromasia of amyloid. The possibility that the metachromasia is due to phosphorylated nucleic acid appears to be eliminated by the lack of effect of ribonuclease digestion on metachromasia and a negative Feulgen reaction.

The data concerning the character of the protein component of amyloid yielded by this survey are not specific. Although tests for various amino-acid components are positive, these results offer no definite indication of the nature of the protein. In particular, they do not demonstrate the presence of serum globulins.

The usually positive reaction of amyloid with aniline blue following treatment with phosphotungstate or phosphomolybdate in the Mallory-Heidenhain or the trichrome staining techniques is noteworthy. According to Puchtler and Isler,[12] this quality indicates a high content of basic groups, the phosphomolybdic acid (PMA) forming a bridge between the basic substrate and the alkaline component of the amphoteric dye. The more basic proteins (collagen, reticulin, and presumably amyloid) are thus stained selectively by the aniline blue or other similar dye. The smaller number of such groups contained in less basic proteins (cytoplasm, sarcoplasm, etc.) bind only a relatively small amount of the PMA, resulting in their staining minimally with such dyes. These latter components may be stained with an acid dye before treatment with the phosphotungstic acid (PTA) or PMA.

Since amyloid is usually indistinguishable from collagen by the PTA-PMA-aniline blue or trichrome techniques, it is necessary to make this distinction with the van Gieson method. The failure of amyloid to stain with acid fuchsin by this means might be interpreted to indicate a less basic protein than that of collagen.

The apparently unique behavior of the amyloid seen in 4 of 5 cases

of multiple myeloma is noteworthy and would appear to indicate a difference in composition. Most cases revealed no metachromasia with crystal violet, nor was there metachromasia with toluidine blue following peptic digestion or with 3 per cent concentration at 70° C. The PAS reaction usually was stronger in amyloid with multiple myeloma, and, in some cases, was more resistant to blocking by bisulfite treatment and acetylation. One might speculate that a greater proportion of glycoprotein and protein causes a more profound inhibition of the metachromasia of any acid mucopolysaccharide present, or, as seems more likely, that the acid mucopolysaccharide component is frequently lacking in the "amyloid" seen in multiple myeloma. The variability of other tinctorial reactions of amyloid in myeloma contrasted with other forms further supports this latter hypothesis and suggests variation in chemical composition from case to case. Indeed, amyloid in multiple myeloma is unique histologically in that it may be encountered intracellularly within neoplastic plasma cells,[13] here resembling the hyaline material of Russell bodies.[14] The single case of 5, which manifested metachromasia with crystal violet, also was metachromatic with 3 per cent toluidine blue at 70° C. The acid polysaccharide in this case appeared histochemically similar to that of other amyloids.

Furthermore, the characteristics of the protein component of amyloid in myeloma are essentially different from those noted in other varieties. The reaction with the PTAH and aniline blue techniques, as in Mallory-Heidenhain's or Masson's stains is frequently (3 of 5 cases) unlike that of connective tissue. On the other hand, other varieties showed tinctorial qualities resembling those of collagen with these stains. This behavior would suggest that the protein in these cases differs in nature from that seen in other forms of amyloid, probably being of a less basic character. It would tend to confirm the presence of variation in chemical composition. One cannot avoid speculating that this type of "amyloid" may represent a simple precipitate of abnormal proteins (globulins ?) or glycoproteins.

Although there were slight differences observed in the reactions of secondary amyloid from those of the primary form in our material, these were relative rather than absolute. While the PAS reaction generally appeared somewhat stronger in the secondary variety, it was blocked as readily as that in the primary type. While there was usually a negative reaction with Alcian blue and no metachromasia with 0.25 per cent toluidine blue, metachromasia of secondary amyloid after pepsin digestion or when stained with 3 per cent toluidine blue at 70° C. did not differ from that of primary amyloid. The limitation of material precluded the possibility of a complete survey of isolated cutaneous

amyloidosis, but the several reactions described were similar to those of the primary cardiac variety.

A brief comparison with other reports of this nature is in order. From the standpoint of metachromasia with toluidine blue, our data generally agree with those of Windrum and Kramer,[8] and Larsen[6] rather than those of Wagner,[15,16] who described amyloid as metachromatic when stained with dilute toluidine blue solutions. Our observations on the effects of peptic digestion and the use of concentrated solutions of toluidine blue at high temperatures also confirm those of Windrum and Kramer[8] and Larsen[6] and extend them to the primary variety.

The suggestion that the acid polysaccharide component is carboxylated rather than sulfated is at variance with the conclusions reached by Hass,[17] who believed that it was chondroitin sulfate. Ehrström[18] observed that a mixture of serum and chondroitin sulfate manifested similar tinctorial properties to those of amyloid. Meyer,[19] on the other hand, suggested that the acid polysaccharide present was monosulfated and related to heparin. More recently, Giles and Calkins[20] reported that chondroitin sulfate was not a prominent component of secondary amyloid. They found, on chemical analysis, that it contained protein and no more than 4 per cent carbohydrate, with both glycoprotein and polysaccharide contributing to the latter component. Wagner[15] found mucopolysaccharide and globulin in amyloid on chemical analysis. Larsen[7] pointed out the prominence of the glycoprotein component. Diametrically opposed to all these observations is the study of Eppinger[21] which revealed no polysaccharide component.

Histochemical study offers the advantage of certain knowledge of the tissue component that is yielding the reaction utilized. It is possible that the variability in chemical analytic data is the result of the existence in tissue extracts of other carbohydrate tissue components (glycoprotein, chondroitin sulfate) universally present in all stromal tissues.[22] The present study appears to offer strong evidence that a non-sulfated acid mucopolysaccharide and a glycoprotein are present in amyloid.

The data of Vazquez and Dixon[23,24] and of Wagner,[25,26] using fluorescent antibody techniques and chemical analysis respectively, tend to indicate that globulin is an important component of amyloid. These investigators have suggested that this substance represents an antigen-antibody precipitate. Calkins, Cohen, and Gitlin[27] have secured data using immunochemical methods tending to deny this hypothesis. The ordinary histochemical techniques may not be expected

to resolve this problem, but the data secured in this study appear to constitute evidence against the hypothesis that amyloid represents a simple antigen-antibody precipitate.

Summary and Conclusions

1. A study was made of the staining and histochemical reactions of 5 different varieties of amyloid.

2. The results tended to indicate that metachromasia with crystal violet, and with toluidine blue after peptic digestion, or in 3 per cent concentration at 70° C. is due to the presence of non-sulfated acid mucopolysaccharide, probably of carboxylated nature.

3. Three components are identifiable—protein, carbohydrate (or glycoprotein), and acid mucopolysaccharide.

4. "Amyloid" associated with myeloma was variable in tinctorial reactions, usually not metachromatic, and its protein and carbohydrate components frequently differed, histochemically, from those of other varieties of amyloid. It is suggested that this variety of "amyloid" may frequently possess little or no acid mucopolysaccharide, and perhaps less basic protein than other varieties.

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