Hyalinosis Cutis et Mucosae: Histochemical Study of Hyaline Material

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The histochemical property of the hyaline material was studied in a case of hyalinosis cutis et mucosae. Alcian blue, azure A and periodic acid Schiff were used for staining mucopolysaccharides. Bromphenol blue was employed for detection of basic proteins. In a further attempt to identify various polyanions histochemically, staining was carried out with alcian blue containing different concentrations of electrolytes. Methylation, saponification, mild acid hydrolysis and digestions by hyaluronidase and sialidase were also employed. The results obtained suggest that the hyaline material found in the case of hyalinosis cutis et mucosae contains a sialic acid-containing mucoprotein.

Hyalinosis cutis et mucosae (lipoidosis cutis et mucosae; lipoid proteinosis) was first defined clinically and histologically by Urbach and Wieithé in 1929. It is a rare, peculiar, usually benign disease which is characterized by widespread papules, nodules, indurated plaques and ulcerated lesions involving skin and mucous membranes. Histological examination discloses that the essential abnormality, namely, a focal deposit of hyaline material, is located in the dermis or subepithelial connective tissue.

In regard to the nature of hyaline material, Urbach and Wieithé, and Ramos e Silva, using the Smith-Dietrich stain, suggested the presence of phospholipids. Using Baker’s acid hematin test, however, which is a reliable stain for phospholipids, a number of other investigators have reported negative results. Weyhbrecht and Korting thought that the material contained a large amount of saturated triglycerides. McCusker and Caplan considered it to consist of a glycoprotein with associated free or loosely bound lipid in the form of cholesterol, a small amount of neutral fat and only a trace of phospholipid. Ungar and Katzenellenbogen, in a re-investigation of tissue from one of Urbach’s earliest cases, concluded that the material contained carbohydrate and probably little or no protein. Heyl and de Kock reported their chromatographic analysis of the lipids in the skin specimens from eight patients. They could identify cholesterol and its esters as the principal lipid components of this material, but found no qualitative difference in them between the patients and normal subjects.

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The identification of the carbohydrate components also has been undertaken by many investigators. Weynbrecht and Korting\textsuperscript{7} reported that the material gave a positive periodic acid Schiff (PAS) reaction which became negative in sections pre-treated with hyaluronidase. Ungar and Katzenellenbogen\textsuperscript{5} observed that the PAS reaction did not diminish by prior treatment of the sections with saliva, but became negative by prior acetylation. Recently, Fleischmajer et al.,\textsuperscript{10} in a re-investigation of Ramos e Silva's case, observed that most of the PAS-positive material disappeared by prior treatment with pepsin, and suggested the presence of glycoprotein in the material. On the other hand, several investigators\textsuperscript{5,11,12} noted an occurrence of metachromasia in some areas, utilizing basic metachromatic dyes. Furthermore, Grosfeld et al.\textsuperscript{13} found a positive reaction for colloidal iron in metachromatic areas, and suggested the presence of acid mucopolysaccharide. However, no report has been presented on the precise nature of the carbohydrate component. This study was designed to analyze histochemically the carbohydrate component of the hyaline material by applying the concept of the critical salt concentration to the differentiation of polyanions.

**Materials and Methods**

Biopsy skin specimens were obtained from a 28-year-old male with hyalinosis cutis et mucosae. Each specimen was cut into two portions, one of which was fixed in 10\% neutral buffered formalin, and the other in 10\% formalin containing 0.5\% cetylpyridinium chloride. After being dehydrated and paraffin-embedded in usual fashion, they were cut at 6\,\mu in thickness. In addition to routine staining methods (hematoxylin and eosin, picrofuchsin, trichrome, phosphotungstic acid hematoxylin, and resorcin fuchsin), the following techniques were employed.

1) Alcian blue. Alcian blue was dissolved to make a 0.5\% solution in 0.05 M acetate buffer of pH 5.8. The solution was distributed in several dye baths and to these magnesium chloride was added sequentially so as to give the molarities from 0.025 to 1.5. Sections were stained for 12 hours in respective dye baths, then rinsed three times for 5 minutes in baths containing the same buffer and same concentrations of magnesium chloride as used for staining. After rinsing, the sections were washed three times with distilled water in 3-minute baths and dehydrated, cleared and mounted.

2) Azure A. Sections were stained for 30 minutes with 0.05\% azure A in a 0.1 M phosphate-HCl or phosphate-citrate buffer of a selected pH at room temperature. Sections were blotted dry, not dehydrated in alcohol, and cleared in acetone-xylol. Some sections were stained with 3\% azure A heated at 70\(^\circ\)C.

3) PAS. Sections were stained with the technic of Lillie.\textsuperscript{14} Some sections were stained after immersion in a 1:1 chloroform-methanol mixture for 12 hours at 70\(^\circ\)C.

4) Periodic acid phenylhydrazine Schiff. A 1/2 to 2 hour blockade in 5\% aqueous phenylhydrazine hydrochloride was interposed between oxidation with periodic acid and treatment with the Schiff reagent in the PAS procedure.

5) Mercuric bromphenol blue. Sections were stained with the technic of Mazia, Brewer and Alfert.\textsuperscript{15}

6) Methylation. Sections were placed in 0.1 N HCl in absolute methanol for 4 hours at 60\(^\circ\)C.
7) Saponification. Sections were immersed in a 1% potassium hydroxide solution in 70% ethanol for 30 minutes at 25°C.

8) Acetylation. Sections were placed in a 2:3 mixture of acetic anhydride : pyridine for 6 hours at 22°C.

9) Mild acid hydrolysis. Sections were placed in 0.1 M acetate buffer at pH 2.5 for 1 to 4 hours at 37°, 55° or 70°C.

10) Diastase digestion. Malt-diastase was dissolved in 0.02 M phosphate buffer at pH 6.0, in a concentration of 1 mg per ml. Sections were incubated for 1 hour at 37°C.

11) Hyaluronidase digestion. Testicular hyaluronidase was dissolved in a 0.1 M phosphate buffer at pH 6.5, in a concentration of 0.5 mg per ml. Sections were incubated for 1 hour at 37°C.

12) Sialidase digestion. Sialidase digestion was carried out as described by Luna. Malt-diastase and testicular hyaluronidase were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Sialidase was obtained from General Biochemical Co., Chagrin Falls, Ohio, U.S.A.

Observations

The epidermis was moderately atrophic, and occasionally there was a loss of epidermal rete ridges. In the upper and middle dermis, there was hyaline material arranged in thick homogeneous bundles, staining diffusely and intensely with eosin (Fig. 1). The tissue in this area included numerous distended or slit-like capillary spaces, which were lined with flat endothelial cells and did not show distinct walls. Elastic fibers were diminished in the hyaline areas, between which agglomerations of coarse and clumped elastotic fibers were often seen (Fig. 2).

The hyaline material stained yellow with picrofuchsin, greenish grey with trichrome, brownish grey with phosphotungstic acid hematoxylin, and showed a positive reaction for PAS (Fig. 3). When the phenylhydrazine treatment for 30 minutes was interposed between periodic acid oxidation and the Schiff stain, the reaction markedly diminished, and was not completely reduced even in a 4-hour exposure. Also, the hyaline material showed a strong affinity for alcian blue at pH 5.8 (Fig. 4). When the sections were immersed in alcian blue solutions containing different concentrations of magnesium chloride from 0.01 to 0.15 M, no variation was found in the affinity for alcian blue. The affinity was markedly reduced in the presence of 0.3 M or higher concentrations of magnesium chloride (Fig. 5), and completely disappeared at 0.45 M (Fig. 6). On the other hand, the material showed an azurophilia at pH 4.0 or a higher pH range. However, when the sections were stained with 3% azure A heated at 70°C, it showed a strong metachromasia at pH 2.5 or a higher pH range.

Methylation for 4 hours at 60°C slightly diminished the PAS reaction of the hyaline material. The 30-minute saponification following methylation failed to change the reaction intensity. The affinity for alcian blue completely disappeared after methylation. When saponification was carried out after methylation, the affinity was partly recovered in the presence of magnesium chloride at 0.1 M or less.
Mild acid hydrolysis for 4 hours at 37°C failed to change the staining behavior with any of the methods. However, when the sections were incubated in an acid solution for 4 hours at 55°C, the PAS reaction slightly diminished, while the affinity for alcian blue was unaffected. The latter showed a decrease in intensity after incubation for 4 hours at 70°C.

Treatment with malt-diastase or testicular hyaluronidase failed to change the staining with any of the methods used. Sialidase digestion for 6 hours weakened the intensity of staining, although the degree differed from one staining to others.

On the other hand, the hyaline material showed a weak reaction with mercuric bromphenol blue, and after methylation for 4 hours at 60°C, it was enhanced prominently.
Fig. 2. Hyalinosis cutis et mucosae. Resorcin fuchsin and picrofuchsin stain. Elastic fibers are diminished in the hyaline areas, between which agglomerations of coarse and clumped elastotic fibers are seen. ×120.

Fig. 3. Hyalinosis cutis et mucosae. Periodic acid Schiff stain. The hyaline material is strongly positive. ×120.
Fig. 4. Hyalinosis cutis et mucosae. Alcian blue stain at pH 5.8. In the absence of MgCl₂, the hyaline material shows a strong affinity for alcian blue. ×120.

Fig. 5. Hyalinosis cutis et mucosae. Alcian blue stain at pH 5.8. The affinity of the hyaline material for alcian blue is markedly reduced in the presence of MgCl₂ at 0.3 M. ×120.
DISCUSSION

There is a general agreement that carbohydrates are mainly responsible for the PAS reaction. Theoretically, all periodate-oxidizable polysaccharides, regardless of acid or neutral, should be stained. And also, some lipids are capable of reacting to periodic acid, because they contain a primary acylated amino group adjacent to a hydroxyl group. In contrast to carbohydrate, however, the PAS reactivity of lipids cannot be prevented by prolonged acetylation, but disappears after lipid extraction. On the other hand, the Schiff reactivity of periodate-engendered aldehydes in acid mucopolysaccharides is less susceptible to phenylhydrazine blockade than that of aldehydes in neutral mucopolysaccharides, so that periodate-reactive acid mucopolysaccharides stain conspicuously with the periodic acid phenylhydrazine Schiff method. In the present study, treatment with phenylhydrazine, which interposed between the periodic acid oxidation and the immersion into the Schiff reagent, weakened the PAS reaction of the material, but did not reduce it to nothing even after exposed to phenylhydrazine for a long period of time. Acetylation for 6 hours prevented the PAS reaction of the material, while the treatment with chloroform-ethanol or diastase did not affect. These results indicate that the PAS reactivity of the hyaline material is related to the presence of neutral and acid mucopolysaccharides.

Acid mucopolysaccharides are identified histologically by a strong affinity for basic dyes. Dempsey et al.,17 taking into account the pK values of the carboxyl, phosphate and sulfate groups, suggested the possibility of recognizing the type of
acid group responsible for staining reaction by the use of basic dyes buffered at different pHs. At high pH values, however, the carboxyl, phosphate and sulfate groups are all ionized. When basic dyes are buffered at a low pH, the anionic sites of the substrate interact with the cations of protein, thus preventing a complete binding with the dye cations. Larsen\(^1\) showed that at a high temperature, highly concentrated dye cations can exchange with the blocking protein. Under these conditions, the material manifested a strong azurophilia at pH 2.5 or a higher pH range. However, when azure A stain was carried out at a low concentration and a low temperature, the material did not manifest an azurophilia up to pH 4.0. On the other hand, the mercuric bromphenol blue reaction is given by basic protein, particularly amino groups. This reaction was found to be weak in the hyaline material. When methylation was applied to the sections prior to the staining, the reaction became much stronger. This would imply that amino groups masked by the carbohydrate component are released by methyleation, freeing more protein amino groups. Therefore, these results suggest the co-existence of acid mucopolysaccharides, which are azurophilic at pH 2.5 or a higher pH range, and proteins.

In general, all polyanions are precipitated by quaternary ammonium salts such as cetylpyridinium chloride, and the water-insoluble polyanion cetylpyridinium complexes are soluble in salt solutions, concentration of which is maintained above the certain concentration. Scott and Dorling,\(^1\) using spots of acid mucopolysaccharide solution on the filter paper, found that alcian blue behaves similarly to cetylpyridinium, and applied this principle to the tissue section to identify the individual acid mucopolysaccharide. According to them, hyaluronic acid takes up stain at magnesium chloride concentrations <0.1 M, sialomucin <0.4 M, chondroitin sulfate <0.6 M, heparin <0.75 M and keratosulfate <1.0 M; and all phosphate group-containing polyanions, including polynucleotides, behave in the same fashion as the carboxyl group-containing polyanions, showing the requirement of about the same level of the low critical salt concentration. Methylation removes sulfate groups, resulting in the formation of free methyl esters, while it esterifies carboxyl and phosphate groups. And, subsequent saponification restores only the latter two groups. The hyaline material showed an affinity for alcian blue in the presence of 0.4 M or less magnesium chloride. The affinity was diminished by methylation, and partially restored by saponification. And treatment with hyaluronidase failed to change the affinity for alcian blue. These results suggest that the affinity of the hyaline material for alcian blue is based on the carboxyl groups of other acid mucopolysaccharides than hyaluronic acid.

Incubation of the sections with sialidase largely weakened the alcianophilia in the presence of 0.4 M magnesium chloride and the PAS reactivity. Similar results were obtained by the mild acid hydrolysis procedure. From the biochemical and histochemical experiments, Quintarelli \textit{et al.}\(^2\) pointed out that mild acid hydrolysis splits off sialic acid without further breaking the rest of the molecule. Therefore, it is likely that some reactive carboxyl groups of the hyaline material
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is related to those of sialic acid.

Thus, it may well be concluded that the hyaline material in hyalinosis cutis et mucosae consists of sialic acid-containing mucoprotein.

References