Histochemical Study on the Pathogenesis of 
Epidermolysis Bullosa Acquisita

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Tissue changes in epidermolysis bullosa acquisita were investigated by histological and histochemical methods. In an attempt to identify acid mucopolysaccharides, staining was carried out with alcian blue solutions containing different concentrations of electrolytes. Methylation, saponification and digestion by hyaluronidase, sialidase and diastase were also applied. The alteration of collagen was examined by the luxol fast blue, trichrome and sulfation toluidine blue methods. The most striking abnormalities of the skin, whether clinically involved or not, were an increase of dermatan sulfate and a decrease of luxol fast blue-positive collagen and oxytalan fibers in the upper dermis. The pathogenesis of epidermolysis bullosa acquisita was discussed on the basis of these results.

Epidermolysis bullosa is a rare hereditary disease characterized by an extraordinary tendency to blister formation after slight trauma. Occasionally this symptom appears in adult with no pertinent familiar background. Siemens\(^1\) and Bloom\(^2\) found that some of the so-called acquired forms of epidermolysis bullosa (epidermolysis bullosa acquisita) are the expression of severe drug reactions. Turner and Obermayer\(^3\) and Brunsting and Mason\(^4\) recognized the similarity of epidermolysis bullosa acquisita to bullous forms of porphyria cutanea tarda. However, there are cases of idiopathic epidermolysis bullosa acquisita with no recognizable background.

On the differentiation between idiopathic epidermolysis bullosa acquisita and porphyria cutanea tarda, Epstein et al.\(^5\) noted that the most striking histological feature in the former was the disruption of the periodic acid Schiff-positive basement membrane in the clinically normal as well as abnormal tissue. Sams and Smith\(^6\) and Pass and Dobson\(^7\) found the abnormalities of dermal connective tissue in patients with idiopathic epidermolysis bullosa acquisita and indicated its close relationship to the dystrophic form of epidermolysis bullosa hereditaria. Our previous histochemical study\(^8\) suggested that the dermo-epidermal separation in epidermolysis bullosa dystrophica was due to the disturbance of mucopolysaccharide metabolism in the dermis. Thus, it seemed interesting to investigate tissue change in idiopathic epidermolysis bullosa acquisita using histochemical methods.

**Materials and Methods**

Biospy skin specimens were obtained from the anterior aspect of the legs of two patients with idiopathic epidermolysis bullosa acquisita before or after rubbing the normally

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looking skin with a glass rod. Each specimen was fixed in 10% neutral formalin containing 0.5% cetylpyridinium chloride for 48 hours prior to routine paraffin embedding and cut of 6 μ in thickness. In addition to the stainings with hematoxylin and eosin, picric fuchsin, trichrome, luxol fast blue MBS and silver impregnation, the following technics were employed.

1) Alcian blue. Alcian blue was dissolved to make a 0.05% 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 up to 1.5. Sections were stained for 12 hours in respective dye baths, then rinsed three times in 5-minute 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) Toluidine blue. Sections were stained according to the method of Kramer and Windrum.9

3) Periodic acid-Schiff. Sections were stained by the technic of Lillie.10

4) Periodic acid phenylhydrazine-Schiff. In the periodic acid-Schiff procedure, sections were placed in 5% aqueous phenylhydrazine hydrochloride for 1/2 to 2 hours after the oxidation with periodic acid, then treated with the Schiff reagent.

5) Aldehyde fuchsia, and peracetic acid aldehyde fuchsia. Sections were stained by the technic of Fullmer and Lillie.11

6) Methylation. Sections were placed in 0.1 N HCl in absolute methanol for 4 hours at 60°C.

7) Saponification. Sections were immersed in 1% potassium hydroxide solution in 70% ethanol for 30 minutes.

8) Sulfation. Sections were immersed in a 1:1 acetic and sulfuric acid mixture for 15 minutes.

9) Hyaluronidase digestion. Testicular hyaluronidase was dissolved in 0.1 M phosphate buffer of pH 6.5 to give a concentration of 0.5 mg per ml. Sections were incubated for 5 hours at 37°C.

10) Sialidase digestion. Sections were incubated for 24 hours in a solution of influenza virus vaccine diluted with 4 volumes of 0.005 M phosphate buffer of pH 6.0 containing 0.85% sodium chloride.

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

Observations

Histological and histochemical features of the specimens obtained either before or after rubbing were essentially similar, except that the dermo-epidermal separation was seen in the latter.

The epidermis was moderately thin and flat. The basal cell layer was normal, though somewhat flattened on the blister roof. Separation was found at the dermo-epidermal junctional zone (Fig. 1). The periodic acid Schiff-positive and silver-impregnated basement membrane was thin and poorly defined even in unseparated skin, and at the blister edge, it was divided into two sheets (Fig. 2).
Fig. 1. *Epidermolysis bullosa acquisita*. Hematoxylin and eosin stain. Separation is found at the dermo-epidermal junctional zone. The dermis shows no inflammatory reaction. $\times 120$.

Fig. 2. *Epidermolysis bullosa acquisita*. Periodic acid-Schiff stain. The periodic acid Schiff-positive basement membrane is thin, and at the blister edge, it is divided between roof and floor. $\times 480$. 
In the papillary and subpapillary layers of the dermis, there were observed a striking decrease of luxol fast blue-positive collagen (Fig. 3), absence of elastic fibers (Fig. 4), and a decrease of oxytalan fibers. With picrofuchsin and trichrome, no abnormality was found. The superficial blood vessels were surrounded by round cell infiltrate.

The papillary and subpapillary layers stained pale red diffusely after the periodic acid-Schiff procedure. The staining was resistant to diastase, hyaluronidase and sialidase. However, when sections were exposed to phenylhydrazine for 30 minutes or more after the periodic acid oxidation, no staining could be obtained by subsequent treatment with the Schiff reagent. Also, these layers showed an affinity for alcian blue (Fig. 5). When the alcian blue solution was buffered at pH 5.8, the affinity was slightly reduced in the presence of 0.1 M magnesium chloride and suppressed at 0.7 M (Fig. 6). Methylation for 4 hours at 60°C completely abolished the affinity for alcian blue. When saponification was carried out for 30 minutes after methylation, the affinity was partly restored in the presence of 0.1 M or lower magnesium chloride. Treatment with hyaluronidase for 5 hours strikingly diminished, though not completely, the affinity in the
Fig. 4. *Epidermolysis bullosa acquisita*. Resorcin fuchsine and trichrome stain. Note an absence of elastic fibers in the papillary and subpapillary layers. ×280.

...presence of 0.05 M or lower magnesium chloride. The affinity was not affected by 24 hours' digestion by sialidase.

On the other hand, the papillary and subpapillary layers showed metachromasia on staining with toluidine blue, even in the sections which were sulfated after immersion in 1.0 M magnesium chloride for 24 hours.

**DISCUSSION**

On the pathogenesis of *epidermolysis bullosa*, many hypotheses have been presented, but none is entirely convincing. Engman and Mook12 and Leon13 thought that an inadequate development of elastic fibers in the upper dermis played an important role. However, some investigators14,15 suggested that the absence of elastic fibers in the upper dermis was not the primary but secondary feature resulting from their destruction during the process of the disease. Langhof16 and others17,18 attributed blister formation to the disturbance of mucopolysaccharide metabolism in the dermis. By electron microscopic examination, Pearson19,20 found that there were distinct pathologic feature in each form of *epidermolysis bullosa*, and stated that *epidermolysis bullosa simplex* blisters resulted primarily from the disintegration of the cytoplasm of the basal cells, whereas
blisters in the dystrophic form occurred as a result of the disintegration of collagen of the upper dermis. Similar opinions have been advanced by others.\textsuperscript{21-25}

The histological and histochemical abnormalities found in the present study consist in a decrease of luxol fast blue-positive collagen, elastic and oxytalan fibers and an increase in affinity for alcian blue in the upper dermis. Since these abnormalities are observed irrespective of rubbing with a glass rod, it is unlikely that they are the consequences of trauma.

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 provided that the salt concentration is maintained above a certain critical concentration. Scott, Dorling and Quintarelli,\textsuperscript{26} using spots of acid mucopolysaccharide solution on filter paper, found that alcian blue behaved similarly to cetylpyridinium, and applied this principle to tissue section to identify individual acid mucopolysaccharides. 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,
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Fig. 6. *Epidermolysis bullosa acquisita.* Alcian blue stain at pH 5.8. Alcianophilia is completely suppressed in the presence of MgCl₂ at 0.7 M. ×120.

behave in similar fashion as the carboxyl group-containing polyanions, showing the requirement of about the same level of low critical salt concentration. On the other hand, 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. In the present study, the upper dermis showed an affinity for alcian blue in the presence of 0.6 M or lower magnesium chloride. The affinity was abolished by methylation, and partially restored by saponification. However, treatment with testicular hyaluronidase failed to change the affinity for alcian blue. Accordingly, it seems that the affinity for alcian blue in the upper dermis is related to chondroitin sulfates, presumably dermatan sulfate.

For the demonstration of collagen in tissue sections, many modifications of the standard methods have been reported. The standard methods for staining collagen fall into four: 1) picric acid method, 2) phosphotungstic acid/or phosphomolybdic acid method, 3) hydrochloric acid method, 4) luxol fast blue method. Hale et al.²⁷ and Constantine and Mowry²⁸ compared these methods with one another, and found that luxol fast blue did not stain immature fine collagen fiber. Chemically, collagen is a protein characterized by a glycine residue content of approximately 1/3 of the total, a combined proline-hydroxyproline residue content
of approximately 1/3, and the absence of tyrosine. Pass and Dobson\textsuperscript{7} showed the decrease of hydroxyproline content in the dermis of clinically uninvolved skin in \textit{epidermolysis bullosa acquisita}. Because the properties of hydroxyproline are by no means distinct from those of other hydroxyls, no specific histochemical method for hydroxyproline has appeared up to the present. On the other hand, sulfate esters of hydroxyls introduced by the sulfation procedure impart characteristic sulfate type basophilia to tissue sections, which stains metachromatic with thiazin dyes. Mowry\textsuperscript{29} found strong orthochromasia in collagen with sulfation toluidine blue sequence, and speculated that the orthochromatic reaction of collagen was due to its hydroxyproline content. In the present study, the papillary and subpapillary layers stained metachromatic with sulfation toluidine blue sequence, and their metachromatic reaction persisted even when the sequence was carried out after immersion in 1.0 M magnesium chloride for 24 hours. This fact, together with the decrease of luxol fast blue-positive fibers, seems to suggest a decrease in mature collagen in the upper dermis.

It is generally acknowledged that acid mucopolysaccharides play an important role in collagen formation. Keech,\textsuperscript{30} who investigated the effects of acid mucopolysaccharides on collagen formation, could produce collagen susceptible to degradation by mild mechanical stress. In the normal skin, however, dermanatan sulfate binds itself so tightly to the collagen fibers, that it is scarcely visualized by staining methods without collagen-degradation treatments. Thus, it seems likely that the dermal fragility in \textit{epidermolysis bullosa acquisita} is due to abnormal synthesis of collagen.

References

5) Epstein, J.H., Epstein, N.N. & Greenlee, M. Epidermolysis bullosa acquisita (tardive) and porphyria cutanea tarda. \textit{Arch. Derm.}, 1959, \textbf{80}, 713–724.


