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Distribution of Mucosubstances in Adenoid Cystic Carcinoma

A Light and Electron Microscopic Study

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Summary. The distribution of mucosubstances in adenoid cystic carcinoma was investigated, and an attempt was made to characterize histochemically the various mucosubstances present. For these purposes the high iron diamine technique (HID), as well as the Astra blue, aldehyde fuchsin and Alcian blue staining methods were employed. Alcian blue was further combined with the periodic acid-Schiff (PAS) technique, the Alcian blue being applied at pH levels between 0.5 and 2.5. In addition the effects of neuraminidase and hyaluronidase treatment as well as methylation and acid hydrolysis procedures on the staining qualities were studied.

Acidic mucosubstances with varying histochemical properties were present in different structures of the neoplasm. The characteristic pseudocyst, a major structural component of the neoplasm, stained strongly with HID, Astra blue, aldehyde fuchsin and Alcian blue at low pH. These staining reactions were markedly suppressed by hyaluronidase treatment, and are apparently attributable to the presence of chondroitin 4- and/or 6-sulfate. Employing the Alcian blue-critical electrolyte concentration technique, the basophilia of the pseudocysts was suppressed at a concentration of 0.5–0.6 M $MgCl_2$, which might indicate polysaccharides of relatively low degree of sulfation. An additional, non-sulfated acid mucin could also be demonstrated in these structures.

In certain duct and gland like structures of the tumours, a change in staining pattern from blue or blue-red to red could be observed after exposure of the sections to neuraminidase and subsequent staining with the Alcian blue (pH 2.5)-PAS sequence. Similar observations were also made when the pH of the Alcian blue was lowered to 1.5–1.0, as well as after acid hydrolysis. These findings afford evidence for the presence of a neuraminidase

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susceptive sialomucin in certain epithelial secretions of the tumor.

At the ultrastructural level the replicated basement lamina of the pseudocysts displayed a strong positive reaction with the PA-CrA-silver staining technique. Furthermore, amorphous material within the lumina of small duct like structures also displayed a positive reaction. The amorphous material of the cystic compartments was less reactive.

Key words: Adenoid cystic carcinoma — Cylindroma — Salivary glands — Mucosubstances — Cytochemistry — Electron microscopy.

Introduction

Adenoid cystic carcinomata (ACC) of salivary gland origin have been subjected to numerous light microscopic histopathological investigations. Furthermore, ultrastructural, immunofluorescent and enzyme histochemical studies of this neoplasm have been presented (Markert, 1965; Bruce and Wertheimer, 1967; Eneroth et al., 1968; Fukushima, 1968; Hübner et al., 1969; Kleinsasser et al., 1969; Hoshino and Yamamoto, 1970; Tandler, 1971; Anthony and James, 1975). Comparatively little attention has been paid to a closer characterization of the various mucosubstances produced by the tumour.

Benign mixed salivary gland tumours have been found to contain both typical epithelial and connective tissue mucins. The latter contain highly sulfated glycosaminoglycans, whereas the epithelial mucins are characterized by their high content of neutral glycoprotein (Quintarelli and Robinson, 1967). In ACC tumours the mucins have also been separated into two types, epithelial mucins and, in the pseudocysts, hyaluronidase susceptible alcianophilic mucins (Azzopardi and Smith, 1959). Although histochemical studies of adenoid cystic carcinoma have previously been presented, there have been considerable developments in histochemical methods for mucosubstances during recent years. It was therefore considered to be of interest to investigate ACC applying these modern histochemical techniques. In addition ultrastructural cytochemical techniques were employed to locate the sites of periodic acid reactive carbohydrates in the tumours at the electron microscopic level.

Material and Methods

Specimens from seven patients with adenoid cystic carcinoma (cylindroma) of salivary gland origin or from the lacrimal gland (one patient) were studied. For light microscopical purposes tumour specimens were fixed in 10% neutral formalin, in Carnoy's fixative or in an absolute ethanol-formalin mixture.

Fixed specimens were embedded in paraffin, and serial sections were cut at 6μ. The sections were deparaffinized and dehydrated according to conventional laboratory methods.

Light microscopic histochemical methods:

Sulfated Mucosubstances. For the visualization of highly anionic mucosubstances sections were stained: a) 30 min in 1% Astra blue (AsB) pH 0.2 (Bloom and Kelly, 1960); b) 30 min in 1% Alcian blue 8 GX (AB) adjusted to pH 1.0 with 0.1 N HCl, and blotted dry prior to dehydration as recommended by Lev and Spicer (1964); c) 30 min in aldehyde fuchsin (AF) (Spicer et al., 1967); and d) 18 h in the high iron diamine reagent (HID) of Spicer (1965).

Weakly Sulfated and Carboxyl Rich Mucosubstances. Sections were stained with 0.1 per cent Alcian blue (AB) dissolved in 3% acetic acid at pH 2.5 (Mowry, 1963).

Sequential Staining. Sections were exposed to dye sequences of a) HID and AB, pH 2.5, and AF-AB, pH 2.5 to demonstrate carboxylated and sulfated mucosubstances within the same section and b) periodic acid Schiff (PAS) and AB at pH 2.5, 2.0, 1.5, 1.0 and 0.5 in both untreated and diastase digested sections to simultaneously demonstrate vicinal hydroxyls and polyanionic sites.

Critical Electrolyte Concentration Series (CEC). An attempt to characterize mucosubstances was also made by adding to 0.1% solutions of AB, pH 5.7, graded concentrations (0.1–1.0 M) of $MgCl_2$ (Scott and Dorling, 1965). In addition the PAS reaction for the detection of neutral carbohydrates was employed in combination with the Alcian blue-CEC technique.

Staining Controls for Sulfated Mucosubstances. Prior to staining with AB at pH 1.0 or with AF, sections were methylated at 60° C for 4 h in acidified methanol which serves to hydrolyze SO_4-groups, resulting in loss of SO_4-engendered alcianophila which cannot be restored by subsequent saponification (see Spicer et al., 1967). Furthermore, sections were digested for 2–6 h in 0.05% testicular hyaluronidase in 0.1 M phosphate buffer, pH 7.6, to demonstrate chondroitin 4- or 6-sulfates.

Staining Controls for Carboxylated Mucosubstances. Prior to staining with AB at pH 2.5, sections were: a) treated for 4 h at 37° C with acidified methanol which esterifies carboxyl groups and suppresses carboxyl engendered alcianophilia at pH 2.5 (Spicer and Lillie, 1959). Subsequent saponification for 5 min in 1% KOH dissolved in 70% ethanol restores carboxyl-derived alcianophilia, b) digested for 18–24 h in *Vibrio cholerae* neuraminidase, 100–500 units/ml, pH 5.3 at 40° C to demonstrate sialomucins, c) digested for 2 h in RNase (1 mg/ml) in aqueous medium adjusted to pH 6.8 to suppress any alcianophilia caused by phosphoric esters. Furthermore, sections were treated with 0.1 N sulphuric acid for 2 h at 60° C to demonstrate non-labile sialomucins (Jones and Reid, 1973 a).

As control tissues, trachea, sublingual gland and pancreas of the mouse and rat, were included in all staining tests to ensure the validity of the dye reactions and enzyme digestions. For the study of tumour morphology every other serial section was also counterstained with hematoxylin.

Electron Microscopy. Tissue specimens from two tumours were processed according to the following procedures: a) some specimens were fixed for 2 h at 4° C in 4% phosphate buffered glutaraldehyde (pH 7.4), rinsed in buffer and postfixed in 1% osmium tetroxide for 2 h. Others, b) were fixed primarily in 1% phosphate buffered osmium tetroxide (pH 7.4) (Millonig, 1961) for 2 h at 4° C. Following OsO_4-fixation or postfixation, the specimens were rinsed in phosphate buffer for 2 h, dehydrated in graded ethanol solutions followed by propylene oxide, and were embedded in Epon 812. Semithin and thin sections were cut on an LKB Ultratome. The thin sections were collected on naked copper grids and stained with lead citrate or with uranyl acetate followed by lead citrate. For demonstration of periodate reactive substances at the ultrastructural level, tumour specimens were c) fixed for 2 h in 2.5% glutaraldehyde (0.1 M phosphate buffer, pH 7.4). Following an overnight buffer rinse, the tissues were dehydrated in ethanol and styrene and embedded in Vestopal W. Thin sections were collected on gold grids, and treated with the periodic acid-chromic acid-silver (PA-CrA-silver) technique of Rambourg et al. (1969). All thin sections were examined in a Philips EM 300 electron microscope.

Results

Light Microscopy

The seven tumours studied were all classical adenoid cystic carcinomas, and the specimens displayed several basic histopathological similarities. The tumour nodules and cords were composed of small, rather uniform cells with darkly

stained nuclei and with a sparse cytoplasm. Numerous cystic formations (pseudocysts) of varying size were observed within all tumours. No apparent differences were detected in staining behavior between specimens fixed in neutral formalin, Carnoy's fixative or in the absolute ethanol-formalin mixture.

In general the contents of the cysts displayed only a moderate reactivity towards PAS (Fig. 1). With this technique the material at the periphery of the cyst appeared threadlike in nature and these concentrically arranged fibrillar structures were more intensely stained with PAS than the central cystic portions (Fig. 2). On the other hand, the material of the cystic compartments showed a strong staining reaction towards Astra blue (pH 0.2), Alcian blue (pH 2.5–0.5), aldehyde fuchsin or high iron diamine staining techniques (Figs. 3, 4 and 5). Minor differences were noted with respect to staining intensity between the cysts. Lowering the pH of Alcian blue from 2.5 to 0.5 gave only a slight decrease in staining intensity. The different cysts were fairly uniformly stained with Alcian blue (pH 5.7) at 0.1, 0.2 and 0.4 M $MgCl_2$. The highest concentration of $MgCl_2$, at which a majority of the pseudocysts was stained, was 0.5 M $MgCl_2$. However, a positive staining could also be detected in a few cysts at 0.6 M $MgCl_2$.

After staining with Alcian blue (pH 2.5–0.5) followed by PAS, positive PAS-staining sites were present within the cysts together with strongly alcianophilic material. A mixture of blue, blue-red, red or purple material also occurred in certain sites after this sequential staining procedure. Furthermore, after staining with the HID-AB (pH 2.5) sequence, purple-black iron diamine precipitates occurred together with alcianophilic material. A similar staining pattern was also noted after AF-AB staining.

A marked decrease in staining ability of the cysts with HID or AF was noted after hyaluronidase digestion. This was also the case with respect to Astra blue or Alcian blue stainability (Fig. 6). However, after such digestion staining with AB at pH 2.5 caused a weak positive staining of a few cysts. Employing the CEC method after hyaluronidase digestion it was found that the latter cysts stained with 0.1 M $MgCl_2$ (Alcian blue pH 5.7) but not at higher molarity.

Enzyme digestion with neuraminidase, RNase or diastase did not markedly alter dye binding as compared to that in buffer controls. On the other hand, acidified methylation at 60° C almost completely abolished the pH 1.0 alcianophilia of the cysts, and subsequent attempts to restore the staining by saponification failed. Acid hydrolysis was without effect on cyst staining.

In the cytoplasm of the tumour cells clusters of PAS-positive granules were frequently observed. This staining was completely abolished by diastase digestion.

The staining reaction of the secretory material in ducts differed markedly from that of the cysts. Ducts or duct-like structures were found in all tumours but were rather inconspicuous. The ducts were lined with epithelial cells in one or two layers.

The secretory material of the duct lumina stained strongly with PAS (Fig. 7) and sometimes with Alcian blue at pH 2.5. However, it did not stain with Alcian blue at lower pH, nor did it stain at all with HID, AF or AsB. Employing

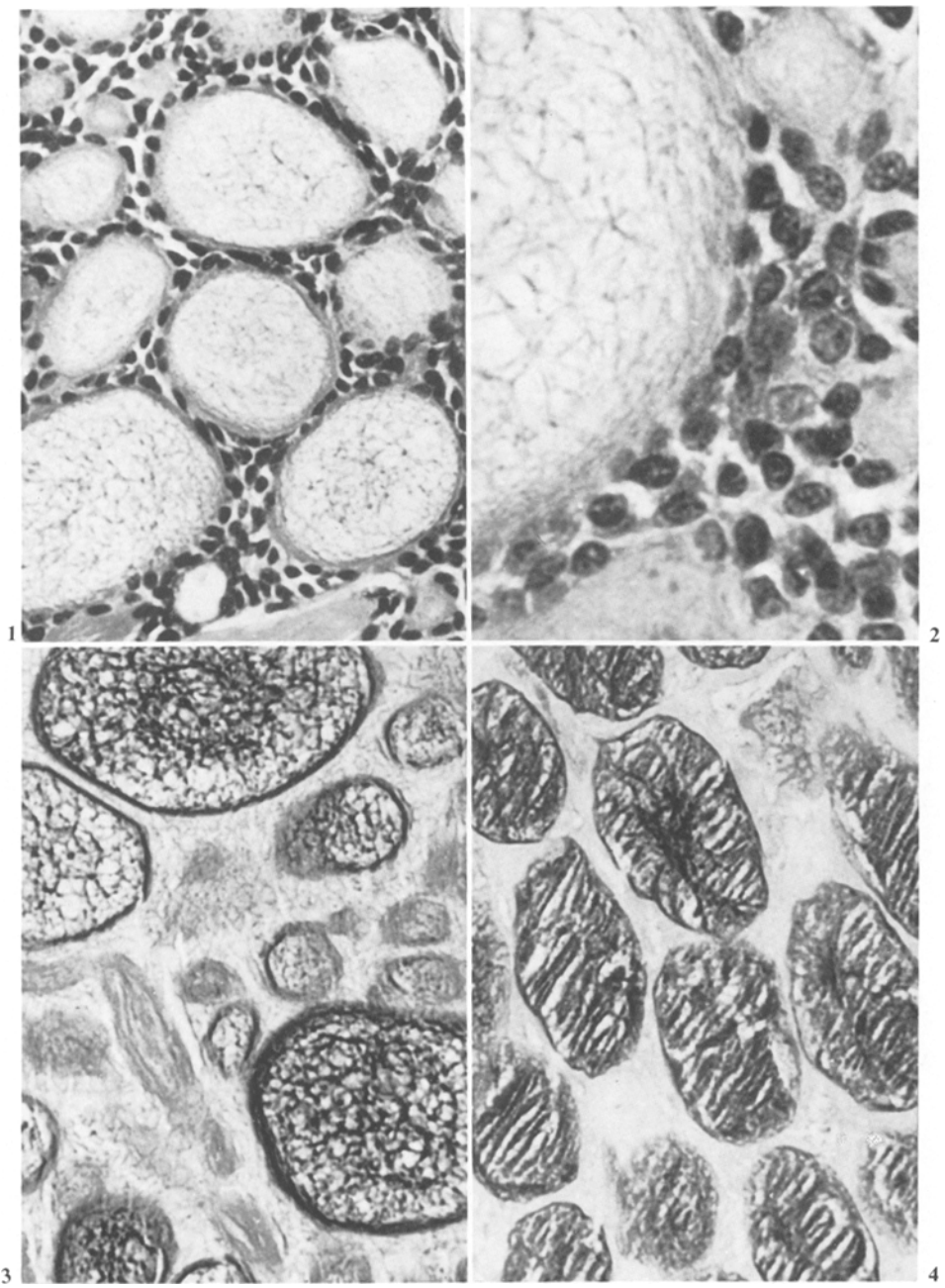

Fig. 1. Cribriform pattern of adenoid cystic carcinoma. Specimen stained with PAS-hematoxylin. A strong PAS-staining reaction is observed at the periphery of the pseudocysts. Light micrograph. ×210

Fig. 2. Light micrograph of ACC section stained with PAS-hematoxylin. Note the fibrillar configuration at the periphery of a large pseudocyst. ×530

Fig. 3. Tumour specimen stained at low pH (0.2) with Astra blue. A positive staining is observed in small as well as large pseudocysts. Light micrograph. ×210

Fig. 4. Tumour specimen stained with high iron diamine. A strong positive staining is detected in the cystic compartments. Light micrograph. ×210

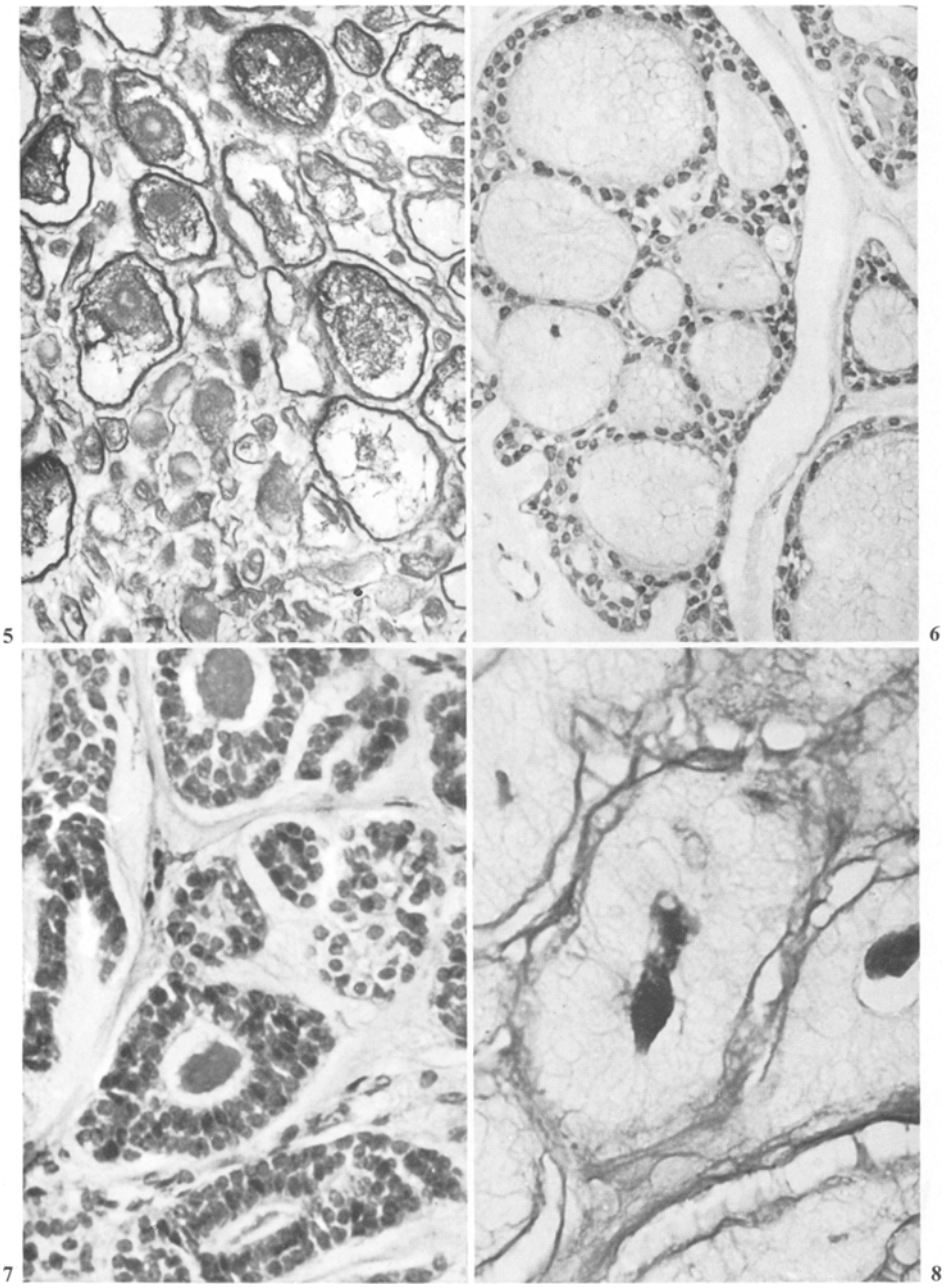

Fig. 5. ACC tumour section stained with Alcian blue at pH 1.0. Numerous pseudocysts of varying size are heavily stained. Light micrograph. ×210

Fig. 6. Tumour specimen treated with hyaluronidase for 4 h followed by staining with Alcian blue, pH 1.0 and counterstained with hematoxylin. The basophilia of the pseudocysts is abolished. Light micrograph. ×210

Fig. 7. Duct structures in ACC. Specimen stained with PAS-hematoxylin. Note the strong PAS staining reaction over duct lumina. Light micrograph. ×210

Fig. 8. Section of ACC stained with Alcian blue at pH 2.5 followed by the PAS technique. The luminal contents of several ducts are heavily stained with Alcian blue. Light micrograph. ×530

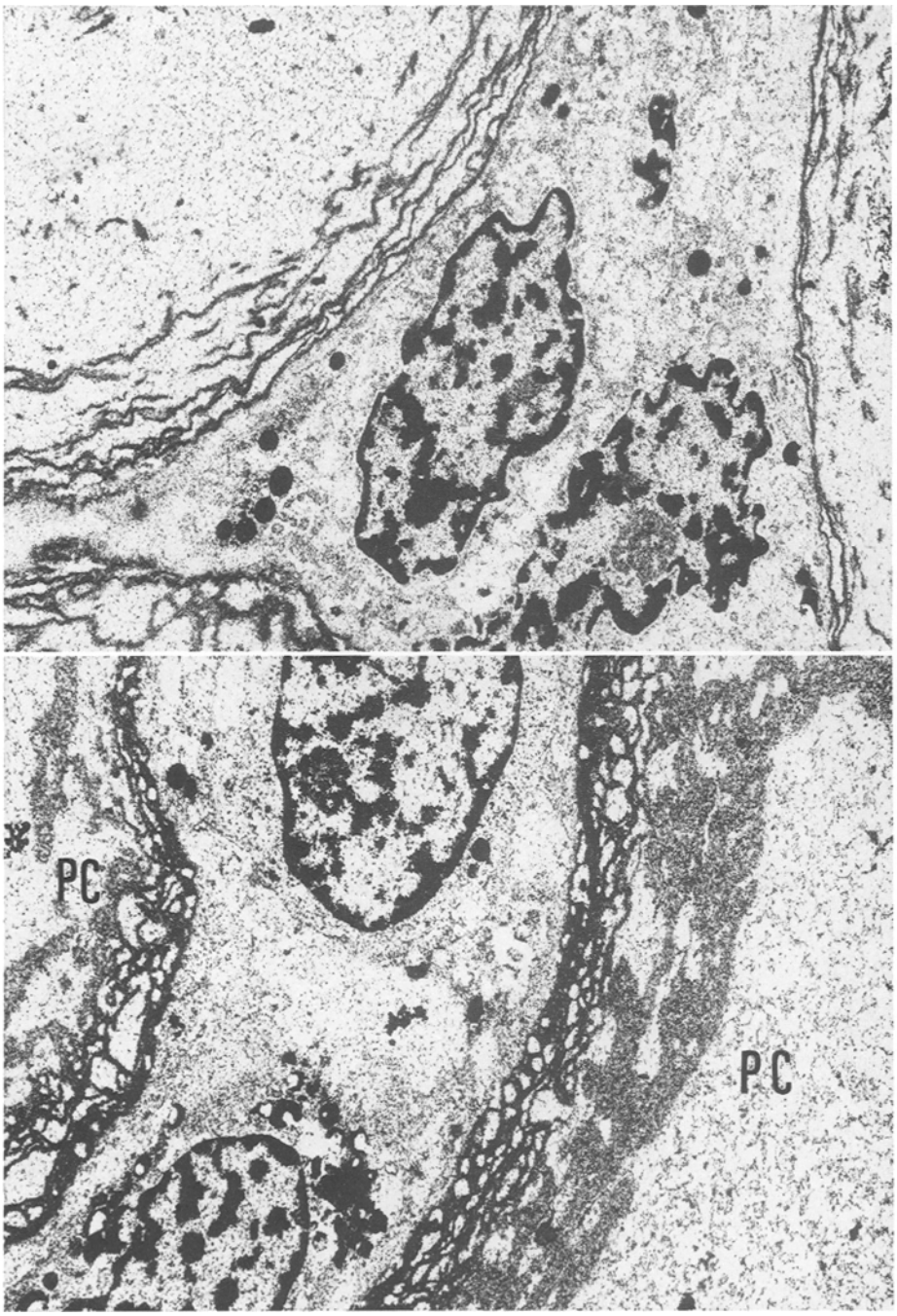

Fig. 9. Electron micrograph of a Vestopal embedded specimen stained with the PA-CrA-silver technique. The replicated basal lamina of three cystic spaces are strongly reactive as are several cytoplasmic dense bodies of the lining neoplastic cells. ×8300

Fig. 10. Electron micrograph of ACC showing two pseudocysts (*PC*). Specimen treated as in Figure 9. The complex replicated membrane material at the extreme periphery of the cysts is more reactive than adjacent material. ×6600

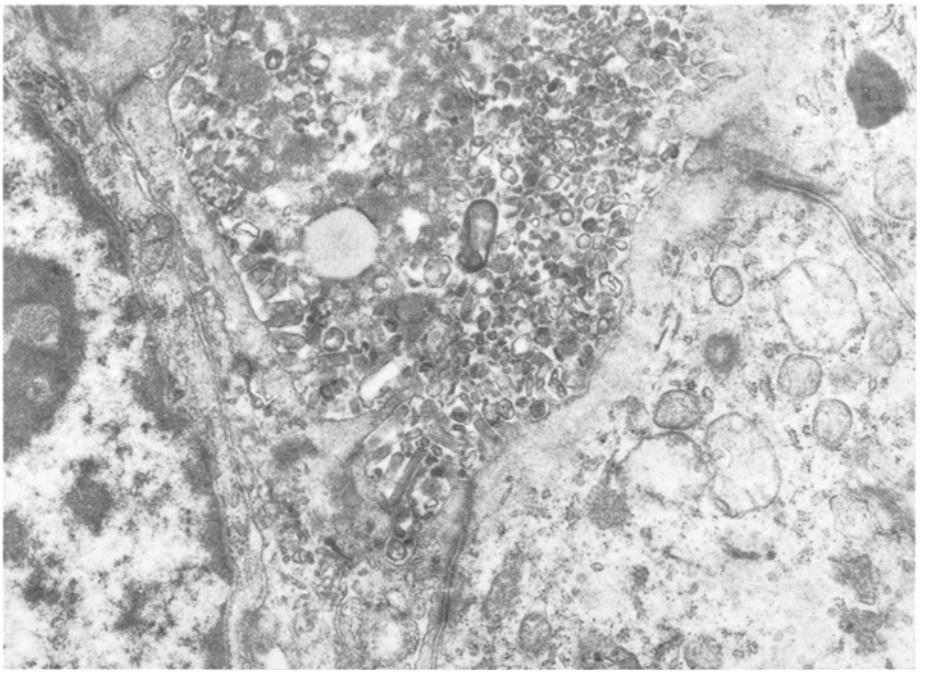

Fig. 11. Duct lumen in ACC specimen fixed in glutaraldehyde followed by osmium tetroxide. Note the numerous microvilli which protrude from the duct cells. The luminal contents consist of minute vesicles and granules as well as an electron dense, amorphous material. Desmosomes are present between the epithelial cells. Section stained with uranyl acetate and lead citrate. Electron micrograph. ×15500

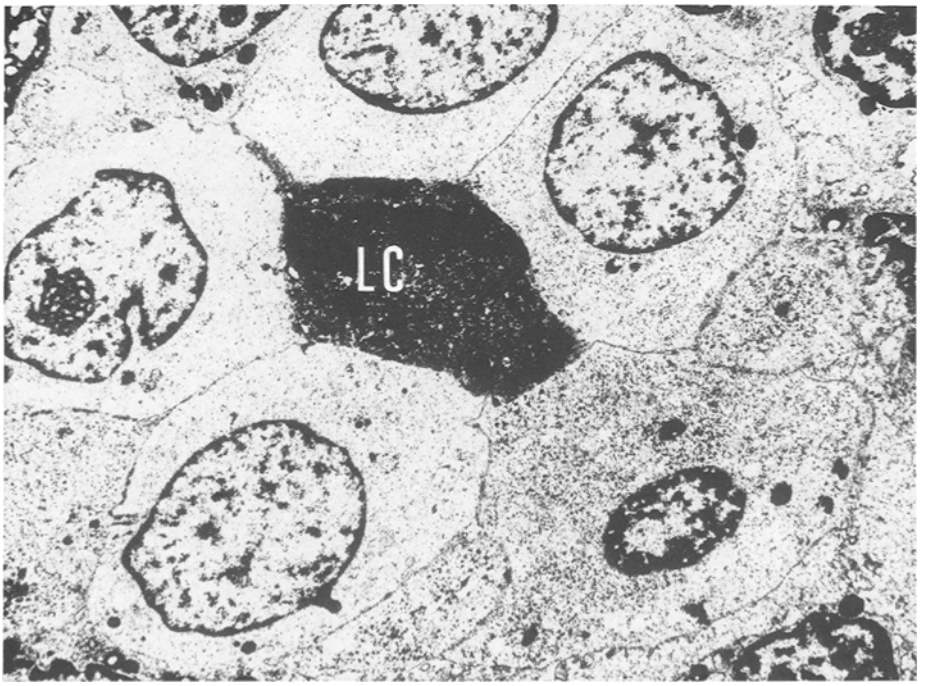

Fig. 12. Duct in an adenoid cystic carcinoma displaying dense silver deposits over luminal contents (*LC*). Specimen treated as in Figure 9. Electron micrograph. ×5700

the Alcian blue (pH 2.5)-PAS sequential staining most of the ductal material stained red. However, in certain locations ductal lumina displayed a blue (Fig. 8) or blue-red colour. By comparing serial sections stained with Alcian blue (pH 2.5–1.0–0.5) followed by PAS, a change in colour from blue to red was observed in these ducts, as the pH of Alcian blue was lowered. A similar change in staining pattern with AB pH 2.5–PAS was also observed after neuraminidase treatment or after acid hydrolysis.

Electron Microscopy

In the electron microscope the fibrillar material at the periphery of the cysts was observed to be composed of concentric, multiple basement lamina. In the two neoplasms examined the laminae were found to form various patterns within different pseudocysts. The thickness of the basement membrane structures also varied. The lamina densa of the membranes was split up and replicated into several layers of concentric membranes (Fig. 9). In other cysts the material formed complex networks (Fig. 10). The central portions of the cysts contained an amorphous or occasionally a finely fibrillar material (Figs. 9 and 10). On applying the PA-CrA-silver technique to ultrathin Vestopal embedded specimens, silver deposits were observed over the basement membranes at the periphery of the cysts (Figs. 9 and 10). The most peripheral membranes showed a stronger reaction than those centrally located (Fig. 10). The bulk of the amorphous cystic material displayed, at most, a faint staining reaction (Figs. 9 and 10). The contents of the lumina of the small duct-like structures observed in the tumours were comprised of minute vesicles and dense granules dispersed in an amorphous material (Fig. 11). The luminal surfaces of the cells lining these ducts exhibited numerous microvilli, and along lateral cell borders, tight junctions and desmosomal attachments were observed (Fig. 11). The contents of these duct lumina displayed a strong positive staining reaction with PA-CrA-silver (Fig. 12).

Discussion

Previous histochemical studies have established that adenoid cystic carcinomas of salivary gland origin are rich in various mucosubstances. Based on light microscopic histochemical and polarization microscopic investigations, Friborsky (1966) proposed that the pseudocysts contained large amounts of sulfated mucopolysaccharides, chiefly chondroitin 4- and/or 6-sulfate. These findings have been substantiated by e.g. Kleinsasser et al. (1969). Azzopardi and Smith (1959) have moreover pointed out the marked histochemical differences between the mucosubstances present in the tumour ducts and those of the pseudocysts.

The histochemical methods employed in the present study reveal additional information concerning the various mucosubstances present in ACC. The specificity of the histochemical techniques employed under carefully regulated condi-

tions of pH, dye concentration and ionic strengths, permit the conclusion that both carboxylated and sulfated forms of mucosubstances occur in the tumour. High iron diamine, Astra blue, aldehyde fuchsin and Alcian blue staining provide evidence for a sulfated mucosaccharide in the pseudocysts. These reagents are known to show a high affinity for sulfated mucosubstances in various tissues and cellular sites, e.g. cornea, cartilage, mast cells, and colonic goblet cells (Bloom and Kelly, 1960; Spicer, 1960, 1965; Spicer et al., 1967). The blocking of Alcian blue staining by methylation for 4 h at 60° C supports the supposition that sulfate groups are present in the pseudocysts. The alcianophilia was not restored by brief saponification with alcoholic KOH. The findings are further substantiated by the hyaluronidase digestion experiments. Testicular hyaluronidase hydrolyses chondroitin 4- and 6-sulfates as well as hyaluronic acid. Although chondroitin 4- or 6-sulfates are stained with Alcian blue at low pH and high molar concentrations of $MgCl_2$, as well as with HID, hyaluronic acid, as a carboxylated polysaccharide, is not stained. The blue staining of certain pseudocysts with the HID-Alcian blue sequence or with Alcian blue at pH 2.5 after testicular hyaluronidase treatment indicates that a non-sulfated acid mucin is also present at these sites. Hyaluronidase, which was very effective as far as the cyst material was concerned, did not abolish the basophilia of the epithelial secretions. Diastase did not affect the PAS positivity of either the pseudocysts or the epithelial secretions. The PAS reactivity of certain cytoplasmic granules, was however, abolished by diastase treatment, indicating that they were composed of glycogen. A persistent basophilia in spite of RNase digestion eliminates polynucleotide phosphates as a source of polyanions binding cationic dyes in the tumours.

Sialomucins are included among the acid epithelial glycoproteins. Certain histochemical techniques have been developed to identify these substances. The most reliable are a) neuraminidase digestion of sensitive sialomucins and b) acid hydrolysis (Jones and Reid, 1973a, b). The latter treatment removes all sialic residues in tissue sections. In the present investigation, acid hydrolysis did not markedly alter the staining pattern of the pseudocyst material. However in certain small duct-like structures, a change from blue or blue-red to red was observed after staining with the Alcian blue (pH 2.5)-PAS sequence after hydrolysis. A similar change in staining pattern was also noticed in these structures after pretreatment of the sections with neuraminidase. It would thus seem evident that sialomucins are present in certain duct secretory material.

In mixed salivary gland tumours there is great contrast between the histochemical properties of the chondroid and myxoid areas on one hand and the epithelial secretions on the other. The basophilia of chondroid and myxoid regions of such tumours is suppressed at a concentration of 0.6 M $MgCl_2$ employing the Alcian blue-CEC method (Quintarelli and Robinson, 1967; Spicer et al., 1967). These findings indicate the presence of a polymer (chondroitin-4 and/or chondroitin-6 sulfate protein complex) of either a low molecular weight or of low degree of sulfatation. As observed in the present study, the staining pattern of the pseudocysts of ACC is essentially the same as that of the chondroid and myxoid areas of the mixed tumours. Furthermore the staining of the epithelial secretions are similar in both tumour types, and sialomucins have been

demonstrated in epithelial structures of mixed tumours of both salivary and lacrimal gland origin (Quintarelli and Robinson, 1967; Jensen, 1970). Thus the histochemical resemblance in mucosubstance composition between the benign mixed tumours and adenoid cystic carcinomata is striking. Ultrastructural evidence has also been presented that cells of similar origin are responsible for the formation of both tumours (Koss et al., 1970).

The staining of the tumours studied in the present investigation is markedly different from that of the normal gland parenchyma adjacent to the neoplasms. The mucins produced in the sublingual, submandibular and minor salivary glands are neutral, carboxy, sialo and sulfomucins, often with a marked heterogeneity between individual cells (Eversole, 1972; Harrison, 1974). Also with regard to the mixed tumours of the lacrimal gland it has been shown that the mucosubstances of the tumour cells and their secretions differ from those of the normal epithelium (Jensen, 1970).

Previous ultrastructural studies have demonstrated that the cystic compartments of adenoid cystic carcinoma are delimited by a generally extremely thick basement membrane (see Tandler, 1970; Koss et al., 1970). In the present investigation, the basement membrane like material displayed a strong positive staining reaction towards the PA-CrA-silver technique of Rambourg et al. (1969). However the amorphous material present throughout the cyst was less reactive. There was a striking relation between the localisation of PAS positive material at the light microscopic level and the PA-CrA-silver deposits observed in the electron microscope.

It is apparent that certain mucosubstances abound at different sites in adenoid cystic carcinoma and that these substances must be produced by the various tumour cells. The mucins present in tumour ducts appear to be neutral glycoproteins and sialomucins, whereas those of the pseudocysts are mainly typical connective tissue mucopolysaccharides.

References

Anthony, P.P., James, P.D.: Adenoid cystic carcinoma of the breast: prevalence, diagnositc criteria, and histogenesis. J. clin. Path. **28**, 647–655 (1975)

Azzopardi, J.G., Smith, C.D.: Salivary gland tumours and their mucins. J. Path. Bact. **77**, 131–140 (1959)

Bloom, G.D., Kelly, J.W.: The Copper Phthalocyanin Dye "Astrablau" and its Staining Properties, especially the Staining of Mast Cells. Histochemie **2**, 48–57 (1960)

Bruce, R.A., Wertheimer, F.W.: Enzyme histochemistry of adenoid cystic carcinoma of minor salivary glands. J. Oral Surg. **25**, 30–37 (1967)

Eneroth, C.-M., Hjertman, L., Moberger, G., Wersäll, J.: Ultrastructural Characteristics of Adenoid Cystic Carcinoma of Salivary Glands. Arch. Ohr., Nas. u. Kehlk. **192**, 358–368 (1968)

Eversole, L.R.: The histochemistry of mucosubstances in human minor salivary glands. Arch. Oral. Biol. **17**, 1225–1239 (1972)

Friborsky, V.: Submicroscopical structure of adenoid cystic carcinoma of salivary glands. Acta. Morph. Ac. Scie. Hung. **14**, 105–116 (1966)

Fukushima, M.: An electron microscopic study of human salivary gland tumours-pleomorphic adenoma and adenoid cystic carcinoma—. Bull Tokyo Med. Dent. **15**, 387–408 (1968)

Harrison, J.D.: Minor Salivary Glands of Man: Enzyme and Mucosubstance histochemical studies. Histochem. J. **6**, 633–647 (1974)

Hoshino, M., Yamamoto, I.: Ultrastructure of adenoid cystic carcinoma. Cancer **25**, 186–198 (1970)

Hübner, G., Kleinsasser, O., Klein, H.J.: Zur Feinstruktur und Genese der Cylindrome der Speicheldrüsen. Virchows Arch. Path. Anat. **347**, 296–315 (1969)

Jensen, O.A.: Mucosubstances of mixed tumours of the human lacrimal gland. Acta path. microbiol. scand. **78**, 110–126 (1970)

Jones, R., Reid, L.: The effect of pH on Alcian Blue staining of epithelial acid glycoproteins. I. Sialomucins and sulphomucins (singly or in simple combinations). Histochem. J. **5**, 9–18 (1973a)

Jones, R., Reid, L.: The effect of pH on Alcian Blue staining of epithelial acid glycoproteins. II. Human bronchial submucosal gland. Histochem. J. **5**, 19–27 (1973b)

Kleinsasser, O., Hübner, G., Klein, H.J.: Zur Histogenese der Cylindrom. Arch. Ohr., Nas. u. Kehlk. **195**, 192–206 (1969)

Koss, L.G., Brannan, C.D., Ashikari, R.: Histologic and ultrastructural features of adenoid cystic carcinoma of the breast. Cancer **26**, 1271–1279 (1970)

Lev, R., Spicer, S.S.: Specific staining of sulphate groups with Alcian blue at low pH. J. Histochem. Cytochem. **12**, 309 (1964)

Markert, J.: Zur Ultrastruktur des Cylindrom. Arch. Ohr., Nas. u. Kehlk. **184**, 496–505 (1965)

Millonig, G.: Advantages of a phosphate buffer for OsO_4 solutions in fixation. J. Appl. Physics **32**, 1637 (1961)

Mowry, R.W.: The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins. With revised directions for the colloidal iron stain, the use of Alcian blue G8X and their combinations with periodic acid-Schiff reaction. Ann. N.Y. Acad. Sci. **106**, 402–423 (1963)

Quintarelli, G., Robinson, L.: The glycosaminoglycans of salivary gland tumours. Amer. J. of Pathology **51**, 1–32 (1967)

Rambourg, A., Hernandez, W., Leblond, C.P.: Detection of complex carbohydrates in the Golgi apparatus of rat cells. J. of Cell Biol. **40**, 395–414 (1969)

Scott, J.E., Dorling, J.: Differential staining of acid glycosaminoglycans (mucopolysaccharides) by Alcian blue in salt solutions. Histochemie **5**, 221–233 (1965)

Spicer, S.S.: A correlative study of the histochemical properties of rodent acid mucopolysaccharides. J. Histochem. Cytochem. **8**, 18–35 (1960)

Spicer, S.S.: Diamine methods for differentiating mucosubstance histochemically. J. Histochem. Cytochem. **13**, 211–234 (1965)

Spicer, S.S., Horn, R.G., Leppi, T.J.: Histochemistry of Connective Tissue Mucopolysaccharides. The Connective Tissue International Academy of Pathology Monograph 7. Edited by B.M. Wagner and D.E. Smith 251–303. Baltimore: The Williams and Wilkins Company, 1967

Spicer, S.S., Lillie, R.D.: Saponification as a means of selectively reversing the methylation blockade of tissue basophilia. J. Histochem. Cytochem. **7**, 123–125 (1959)

Tandler, B.: Ultrastructure of Adenoid Cystic Carcinoma of Salivary Gland Origin. Lab. Invest. **24**, 504–512 (1971)

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