A Histochemical Study of Epithelial Mucin in the Chick Chorioallantois [1]

JAMES L. CONKLIN
Department of Anatomy, The University of Michigan, Ann Arbor, Michigan

ABSTRACT Histochemical methods demonstrate the accumulation of two types of mucous inclusion in the allantoic epithelial cells of the chick embryo.

One type of inclusion contains a sialomucin that is PAS-reactive, unstained by Alcian blue and other anionophilic dyes and is susceptible to digestion by *Vibrio cholerae* neuraminidase.

The second inclusion contains an apparent sulfomucin which is Alcianophilic at pH 1.0, unstained by anionophilic dyes after a methylation-demethylation sequence and resistant to testicular hyaluronidase. This sulfomucin exhibits PAS reactivity after desulfation procedures.

Both types of mucus occur in individual cells and are normal components of the chorioallantois. Accumulation of the inclusions is enhanced by lowering the pH of the allantoic fluid or as a result of organ transplantation.

Microscopic examination of chick chorioallantoic membrane (CAM) revealed the presence of abundant mucus-like material within and on the surface of the allantoic epithelial cells. It was also observed that transplantation of certain organs to the CAM apparently enhanced the accumulation of this mucus. An interest in the morphology and function of the intact and transplant-recipient CAM prompted a more extensive histochemical investigation of the allantoic mucus. The objective was to obtain additional information about the normal CAM as well as to be able to interpret the results of subsequent studies of organ transplantation.

MATERIALS AND METHODS

The material employed in this study consisted of samples of CAM from white leghorn chick embryos ranging in age from 7–18 days (Hamburger and Hamilton, '51), although 17–18 day CAM was most extensively used.

Samples of CAM were fixed in either 10% neutral-buffered formalin (pH 7.0), Bouin's fluid or 10% formalin containing 0.5% cetylpyridinium chloride. Following fixation, the samples were washed, dehydrated in graded ethanol and embedded in paraffin.

Sectioned samples were stained in the following:

(1) periodic acid Schiff (PAS) and colloidal iron-PAS (Mowry, '58); (2) aldehyde fuchsin (Gomori, '50) (prepared with basic fuchsin, C.I. 42500), with and without oxidation in Lugol's solution; (3) aldehyde fuchsin and Masson trichrome (Masson, '29) (acid fuchsin, C.I. 4285, Ponceau R, C.I. 16150, light green SF, C.I. 42095); (4) 0.01% aqueous azure A, cert. no. NAz 17, adjusted to pH 2.0 and 4.2 with hydrochloric acid; (5) 0.1% Alcian blue 8 GX (C.I. 74240) in 3% acetic acid (pH 2.5) or 0.5% Alcian blue in 0.1 N hydrochloric acid (pH 1.0).

Tissues stained with azure A were either rinsed in water and examined both before and after mounting in glycerogel or dehydrated in tertiary butyl alcohol and mounted in HSR. Routinely, all other stained tissues were dehydrated in graded ethanol, cleared in xylene and mounted in HSR. The only exception to the preceding routine was to blot rather than dehydrate some sections after staining in Alcian blue (Lev and Spicer, '64).

Prior to the use of certain methods, tissue sections were treated by incubation in acidified methanol (0.3 cm^3 concentrated HCl in 40 cm^3 methanol) for four hours at 37°C (mild methylation) or 60°C (strong methylation). After methylation, some sections were subjected to a saponification step involving immersion in 1%

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KOH in 80% ethanol at 25°C for 20 minutes (Conklin, '62).

To confirm the absence of reactive lipids, sections were additionally extracted with chloroform-methanol (1:1) at 60°C for 24 hours.

Before staining, for enzymatic digestion, sections were incubated in solutions of either 0.5% α-amylase (Nutritional Biochemicals) in 0.004 M acetate buffer (pH 5.5) at 37°C for three hours, 0.5% testicular hyaluronidase in 0.1 M phosphate buffer (pH 5.5) at 37°C for four hours, or 100 units of *Vibrio cholearae* neuraminidase (Behringe Chemical Works) in 0.05 M acetate buffer (pH 5.5) at 37°C for four hours. As a control procedure, other sections were incubated in "buffer only" solutions for a similar length of time.

Since digestion with hyaluronidase did not alter the staining reactions, sections of umbilical cord were incubated in the hyaluronidase solutions (controlled as above) to confirm the activity of this enzyme. Such sections exhibited a marked reduction in staining after enzymatic digestion. As the control procedure confirmed the specificity of the enzymatic digestions, the results to be described include only the effects of digestion upon the staining reaction (table 1).

TABLE 1

Staining response of allantoic epithelial mucin

Procedure	Response
PAS	+
Colloidal iron	+
Alcian blue	+
Aldehyde fuchsin	+
Azure A, pH 4.2	+
Azure A, pH 2.0	+
Colloidal iron, PAS	+, + [1]
Methylation, PAS	+
Methylation, colloidal iron	−
Methylation, azure A, pH 4.2	−
Methylation, azure A, pH 2.0	−
Methylation, aldehyde fuchsin	−
Methylation, demethylation, PAS	+ [2]
Methylation, demethylation, colloidal iron	−
Methylation, demethylation, aldehyde fuchsin	−
Methylation, demethylation, azure A, pH 4.2	−
Methylation, demethylation, azure A, pH 2.0	−
Amylase, PAS	+
Neuraminidase, PAS	−
Neuraminidase, colloidal iron	+
Neuraminidase, alcian blue	+
Hyaluronidase, PAS	+
Hyaluronidase, colloidal iron	+
Hyaluronidase, alcian blue	+

− indicates that no staining occurred; + indicates an affinity for the stain.
[1] After this sequence some inclusions are PAS-positive, while some are stained by colloidal iron.
[2] After this sequence, inclusions that were formerly PAS-negative are now PAS-reactive.

RESULTS

The allantoic epithelium is a single layer of approximately cuboidal cells which line the inner surface of the chorioallantoic membrane (CAM).

The nuclei of the cells are located basally while the cytoplasm is most abundant at the apical end bordering on the allantoic lumen. The apical surface of the cells exhibits short microvilli (Leeson and Leeson, '63) which are indistinct when viewed with the light microscope.

On about the ninth day of development, inclusions appear in the supranuclear cytoplasm of the allantoic epithelial cells. These inclusions increase until, by the eighteenth day, they appear to fill most of the apical cytoplasm (fig. 1–3). Concomitantly, similar material accumulates on the surface of the cells within the allantoic lumen.

The inclusions, because of their affinity for certain stains, are subsequently termed *mucous inclusions*. It seems probable that these mucous inclusions correspond to the weakly osmiophilic vacuoles observed by Leeson and Leeson ('63) in an electron microscopic study of the chorioallantois.

As indicated in table 1, the mucous inclusions of the allantoic epithelium are stained by many of the methods commonly employed in carbohydrate histochemistry. They are colored a deep red by the PAS reaction and also stained by colloidal iron, Alcian blue, aldehyde fuchsin and azure A. However, when the inclusions are stained with a colloidal iron-PAS sequence, it is apparent that, while most inclusions are stained by colloidal iron, some inclusions are unstained by colloidal iron yet are PAS-reactive. This duality in the staining of the inclusions is most evident in 10–12 day CAM when fewer inclusions are present. The same duality in staining is exhibited by the mucus covering the surface of the epithelium.

Both types of mucous inclusions are present in most epithelial cells. The presence of both PAS-reactive and colloidal iron-positive inclusions in individual cells as well as the occurrence of mixed reactive material in the lumen of the allantois suggests that the allantoic cells contain at least two kinds of mucus and that this same material is secreted into the allantoic lumen.

To obtain additional information about the nature of the reactive groups in the mucous inclusions, control procedures were employed prior to the application of certain staining reactions.

While the use of alcohol-dehydrated, paraffin-embedded sections indicated that the PAS-reactive material was not lipid in character, some sections were further treated with hot chloroform-methanol before staining with PAS. Chloroform-methanol extraction did not alter the PAS staining of the inclusions. Also, predigestion with amylase did not alter the the PAS staining of inclusions. In contrast, incubation of the sections in neuraminidase abolished all PAS staining of the inclusions. As anticipated, methylation of the tissue sections did not alter PAS staining. However, after a methylation-demethylation sequence, PAS staining of inclusions was greater than in untreated sections.

After either methylation or a methylation-demethylation sequence, the inclusions were not stained by colloidal iron, Alcian blue, aldehyde fuchsin or azure A (fig. 4). Colloidal iron or Alcian blue staining of the inclusions was unaffected by either hyaluronidase or neuraminidase digestion (fig. 5).

The preceding results were nearly the same following fixation in either of the three fixatives. In general, staining was less intense following fixation in neutral-buffered formalin. Results after fixation in CPF or Bouin's fixative were comparable.

DISCUSSION

The methods employed in this study fall into two categories. The first, the PAS reaction, is generally considered specific for reactive vicinal hydroxyl groups, although certain lipids are also capable of reacting (Lillie, '65). The remaining stains are those which exhibit an affinity for tissue anions such as sulfate, phosphate or carboxyl groups (Pearse, '60). The specificity of these *anionophilic* stains is dependent, to some extent, on the pH at which they are employed.

The mucous inclusions of the allantoic epithelial cells are also of two types. There are inclusions which are only PAS-reactive and inclusions which are colloidal iron-positive, PAS-negative, when stained by a sequential stain. The appearance of similar material in the allantoic lumen suggest that at least two types of mucus produced by the allantoic epithelial cells is subsequently secreted into the allantoic fluid, which does contain copious quantities of mucus (Needham, '50).

The identification of specific carbohydrates in tissue sections is difficult if not impossible with the methods employed. It is possible, however, to describe the nature of the reactive material in the mucous inclusions. The contents of one type of inclusion are PAS-reactive, unstained by anionophilic methods, resistant to hyaluronidase and amylase and susceptible to neuraminidase digestion. Avoiding the current dispute over the histochemical nomenclature of carbohydrates (Spicer et al., '65; Meyer, '66; Saunders and Rosan, '66), it seems reasonable to consider the contents of this inclusion as a neuraminic acid-containing sialomucin. While the significance of its occurrence remains obscure, its presence is not unexpected. Neuraminidase occurs in the CAM in large quantities (Ada and Lind, '61; Cook and Ada, '63); Bogach et al. ('62) have isolated a sialic acid-containing material, sialoresponsin, from allantoic fluid, following virus innoculation of the CAM. Although the localization of the CAM neuraminidase has not been demonstrated, it seems probable that it would occur in the allantoic epithelium where the sialomucin is found

The second, most numerous type of mucous inclusion is intensely stained by procedures which have an affinity for negative groups. While colloidal iron and Alcian blue stain both sulfate and carboxyl groups, they are more specific if the pH of the staining solution is maintained well below the pK of carboxyl groups, (Lev and

Spicer, '64) which indicates that staining is due to sulfate. (Phosphate groups are also weakly stained but can probably be discounted since they have not been reported in mucus). Staining with azure A, at pH 2.0, and with aldehyde fuchsin, while not specific, is also indicative of sulfate groups. That the anions in the mucous inclusions are sulfate rather than carboxyl groups is further indicated by the lack of staining which is observed after methylation-demethylation treatment of the tissue sections. It is assumed (Lillie, '65) that, after a methylation-demethylation sequence, only carboxyl groups are available for staining.

Neuraminidase digestion did not alter the staining with colloidal iron and this reaction was also unaffected by hyaluronidase digestion. Since hyaluronic acid, and chondroitin sulfates A and C are digested by testicular hyaluronidase (Pearse, '60; Lillie, '65), the sulfated mucus (sulfomucin) is probably other than one of these three carbohydrates. The enhancement of PAS reactivity of the mucous inclusions by methylation-demethylation suggests the presence of vicinal hydroxyl groups in the sulfomucin and the occurrence of sulfate in a position which prevents PAS reactivity (Lillie, '65). Desulfation removes the hindrance effect of the sulfate, and the molecule becomes PAS-reactive. For the present, the contents of the second type of mucous inclusion can only be identified as a sulfated mucin which also contains vicinal PAS-reactive groups and is lacking in demonstrable carboxyl groups. Protein is present in both types of epithelial mucus, as evidenced by bromphenol blue staining of the components following electrophoretic separation (unpublished observation).

Presently, the significance of the allantoic mucus remains obscure. It has been suggested that it is secreted in response to the accumulation of noxious materials in the allantoic fluid (Needham, '50). This view is supported by the observation that artificial lowering of the pH of the allantoic fluid enhances mucus accumulation. Other factors are also involved, however, since transplantation of certain tissues to the CAM also accelerates mucus accumulation. In any event, the allantoic epithelium should provide an interesting tissue for the study of mucus synthesis and secretion, since the cells are apparently capable of synthesizing two quite different types of mucous material.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

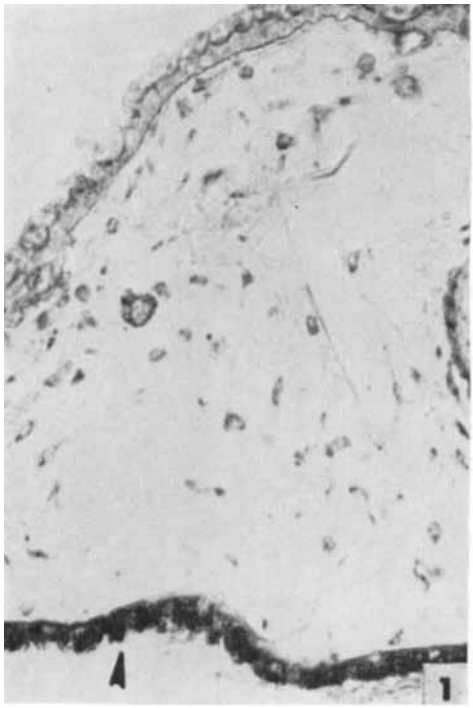
1 A section through the chorioallantoic membrane (CAM). From top to bottom are the ectodermal, mesodermal and entodermal components. The allantoic epithelial cells (arrow) contain copious amounts of mucus. Colloidal iron and PAS. × 600.

2 The mucus (arrow) of the allantoic epithelium as it appears after staining with aldehyde fuchsin. × 800.

3 The allantoic epithelium and mucous components. The distinction between the two types of mucous inclusions is not apparent. Note the presence of mucus in the allantoic lumen (arrow). × 1600.

4 A section of CAM after a methylation-demethylation sequence and staining with colloidal iron. Note the absence of stained material in the allantoic epithelium (arrow). × 400.

5 The CAM after treatment with neuraminidase. Only the mucous inclusions that are stained by colloidal iron (arrow) are visible (contrast with fig. 1). Colloidal iron and PAS. × 400.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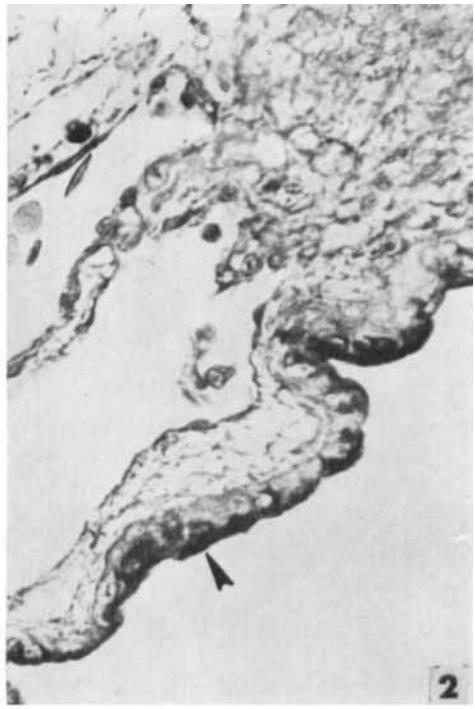
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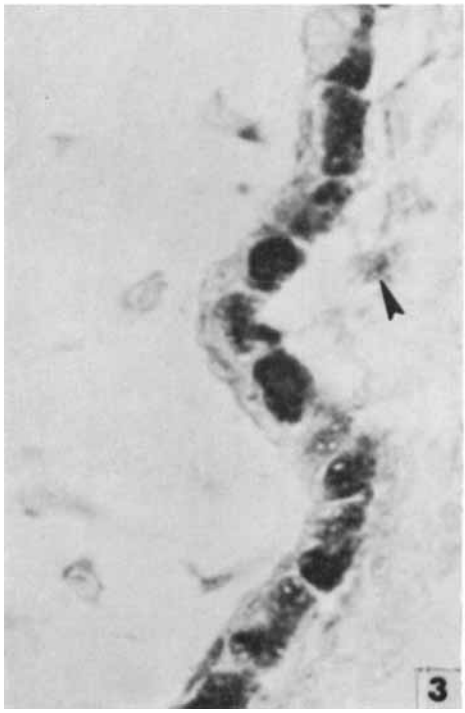
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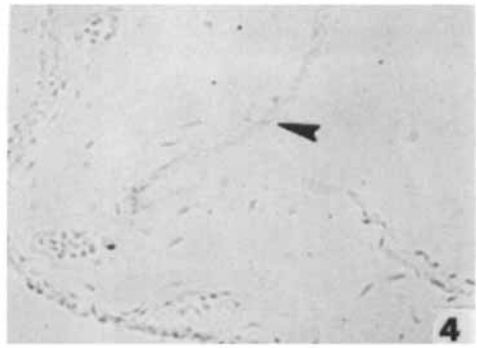
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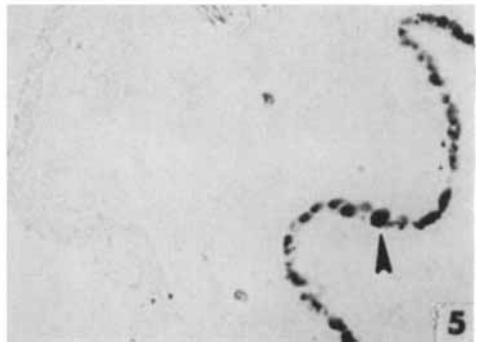
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