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STUDIES ON THE PATHOLOGY OF DICROCOELIASIS AND FASCIOLIASIS IN THE GOAT

II. THE HISTOCHEMISTRY OF BILE-DUCT MUCOSUBSTANCES*

By
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RAHKO, TIMO: *Studies on the pathology of dicrocoeliasis and fascioliasis in the goat. II. The histochemistry of bile-duct mucosubstances.* Acta vet. scand. 1972, *13*, 563—574. — Histochemical studies were made on the characteristics of carbohydrate-rich compounds in the bile-duct mucosa of goats infected either with Dicrocoelium dendriticum or with Fasciola hepatica. In a previous study the author showed that a chronic infection with these parasites produces glandular hyperplasia in the walls of bile ducts. This study revealed a definitely increased secretion of qualitatively normal mucosubstances by the hyperplastic epithelial cells.

Epithelial mucosubstances in the bile-duct walls were characterized by the presence of periodate-reactive hydroxy groups and both carboxyl and sulphate radicals. The intensity of the PAS reaction was moderate or strong and not definitely inhibited by the acidic or acetyl radicals. Specific enzymatic digestions did not reveal periodate-reactivity produced by glycogen or basophilia due to hyaluronic acid or chondroitin-4 and -6-sulphates in the epithelial cells. On the other hand, sialidase digestion showed that the main part of the carboxymucins was sialidase-labile sialomucins. Their susceptibility towards sialidase was not definitely increased after deacetylation. The single and sequential histochemical methods showed a more intense staining reaction for sulphate radicals in the mucosubstances of the deep glandular and goblet cells than in those of the superficial glands, which were characterized by high concentration of carboxyl groups.

Dicrocoelium dendriticum; Fasciola hepatica; liver; bile ducts; mucosubstances.

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In parasitic cholangitis normal regular bile-duct epithelium is transformed into more or less thick glandular mucosa (*Rahko* 1971). However, the capacity of different parasites to induce the proliferation of bile-duct epithelial cells is variable, and some produce a primarily fibrous reaction but practically no epithelial hyperplasia (*Lumsden & Karin* 1970). Fasciola and Dicrocoelium also seem to differ in this respect, the glandular hyperplasia produced by fascioliasis being more pronounced than that in dicrocoeliasis (*Rahko* 1972).

Many workers consider that glandular hyperplasia in the walls of bile ducts is accompanied by increased mucin secretion (reviewed by *Rahko* 1971). On the other hand, only a few publications are known to the author describing the histochemical changes produced by parasites in the secretion of bile-duct mucosubstances. Such investigations have been carried out by *Dhar & Singh* (1963) on hill-bulls with dicrocoeliasis, by *Chou & Gibson* (1970) on man and some experimental animals with clonorchiasis and by *Rahko* (1971) on cattle and mice with fascioliasis. This comparative study describes the main characteristics of carbohydrate-rich compounds in the bile-duct walls of goats infected either with Dicrocoelium dendriticum or with Fasciola hepatica.

MATERIAL AND METHODS

The histochemical staining methods for the characterization of carbohydrate-rich compounds were employed on the formalin-fixed material described in a previous paper (*Rahko* 1972). The staining methods were carried out according to the instructions given by *Lillie* (1954), *McManus & Mowry* (1960), *Pearse* (1961) and the *Manual* of Histologic Staining Methods of the Armed Forces Institute of Pathology (1968). The terminology proposed by *Spicer et al.* (1965) for carbohydrate-rich compounds is employed in the present work.

The histochemical staining methods were selected to characterize certain radicals (1,2 hydroxy, carboxyl and sulphate groups) or to demonstrate particular compounds and end groups by specific enzymatic digestion.

1,2 hydroxy groups (vicinal glycols) were revealed by periodic acid-Schiff (PAS). PAS-reactivity of the mucosubstances was also investigated after acetylation (acetylation-PAS) and

deacetylation (deacetylation-PAS) of the tissue sections. In the acetylation procedure the sections were treated at room temperature for 24 hrs. in acetic anhydride-pyridine solution. Deacetylation was carried out at 37°C in ammonia ethanol solution for a period of 24 hrs. Sections were treated with 1 % KOH in 70 % ethanol for 1 hr.

Acid radicals (carboxyl and sulphate groups) were revealed with Mayer's mucicarmine (MC), alcian blue staining at pH 2.5 (AB 2.5) or at pH 1.0 (AB 1.0) and with aldehyde fuchsin at pH 1.7 (AF). Alcian blue staining was also employed at pH 2.5 or pH 1.0 on sections treated with strong HCL-methylation (methylation-AB 2.5 or -AB 1.0) followed by saponification (methylation-saponification-AB 2.5 or 1.0). Further, alcian blue staining at pH 2.5 was carried out on sections treated for 1 hr. in 0.1 N sulphuric acid at 80°C (*Gibbons* 1963).

The following sequential stainings revealing vicinal glycols and carboxyl or sulphate radicals were used to differentiate neutral from acidic mucins or carboxymucins from sulphomucins in the same sections: alcian blue staining at pH 2.5 or 1.0 followed by PAS (AB 2.5/PAS, AB 1.0/PAS) and aldehyde fuchsin at pH 1.7 followed by alcian blue at pH 2.5 (AF/AB).

The following enzymatic digestions were employed to demonstrate particular compounds or end group: Diastase digestion followed by PAS (diastase-PAS) was performed as previously described (*Rahko* 1972). Hyaluronidase digestion (hyaluronidase 350 u./ml, Type I Sigma) was performed in 0.1 M phosphate buffer pH 7.1 at 37°C for 1 hr. Control sections were treated in buffer without enzyme. After washing, the sections were stained with alcian blue at pH 2.5 (hyaluronidase-AB). Sialidase digestion (Neuraminidase 0.05—0.20 u./mg, Type V Sigma) was carried out in 0.1 M acetate buffer pH 5.3, containing 0.04 M calcium chloride, at 37°C for 42 hrs. or at pH 8.9 for 3 hrs. Control sections were digested in corresponding solutions without enzyme. After washing, sections were stained with alcian blue at pH 2.5 (sialidase-AB). Sialidase digestion was also performed in a corresponding manner at pH 5.3 after deacetylation of the tissue sections (deacetylation-sialidase-AB).

Haematoxylin and nuclear fast red were used as counterstains if necessary.

RESULTS

The identification of neutral mucins, carboxymucins and sulphomucins on the basis of reactions to different histochemical staining methods was carried out according to Table 1.

Table 1. The identification of neutral, carboxy- and sulphomucins.

Staining method	Neutral mucins	Carboxy-mucins	Sulpho-mucins
PAS	red	—*	—
d-PAS	red	—	—
acetylation-PAS	—	—	—
deacetylation-PAS	red	—	—
methylation-PAS	red	—	—
methylation-saponification-PAS	red	—	—
MC	—	red	red
AB 2.5	—	blue	blue
AF	—	—	purple
AB 1.0	—	—	blue
methylation-AB 2.5	—	—	—
methylation-AB 1.0	—	—	—
methylation-saponification-AB 2.5	—	blue	—
methylation-saponification-AB 1.0	—	—	—

* no staining reaction.

No qualitative differences were noted in the main characteristics of epithelial mucosubstances between normal and infected bile ducts. On the other hand, dicrocoeliasis and fascioliasis produced a pronouncedly increased mucin secretion, as judged by the intenser staining and the larger masses of stainable substances in the cytoplasm of bile-duct epithelial cells (Figs. 1 and 2). In normal bile ducts the staining reactions for different mucosubstances occurred in a narrow apical zone of the cytoplasm of the cells in the surface epithelium and the superficial glands but in the whole cytoplasm of only the deep glandular cells and the infrequent goblet cells. On the other hand, in the infected bile ducts, which showed more or less pronouncedly increased frequency of superficial glands and goblet cells, larger masses of intensely stainable mucin were observed in the different epithelial cells (Table 2).

PAS-reactivity observed at different epithelial sites varied from moderate to strong and was not increased after deacetyla-

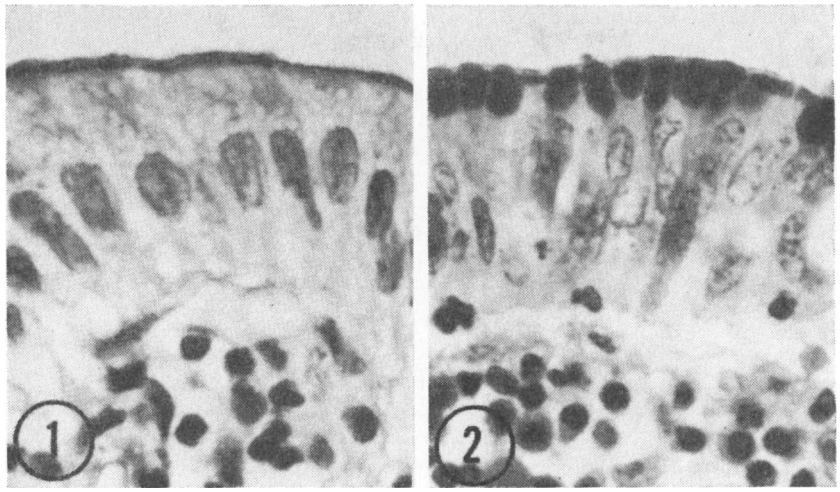


Figure 1. Normal location of periodate-reactive mucosubstances (dark apical zone) in the surface epithelium of a main bile duct. PAS, haematoxylin counterstain × 630.

Figure 2. Fascioliasis. Large masses of periodate-reactive mucosubstances are seen in superficial epithelial cells of a main bile duct. PAS, haematoxylin counterstain × 630.

tion. The periodate-reactive substances were completely resistant to diastase digestion. The sections stained with methylation-PAS and methylation-saponification-PAS had an overall "washed-out" appearance, representing weakening of the PAS-reactivity.

Acidic mucins were present at all the epithelial sites containing periodate-reactive mucosubstances (Figs. 3a and 4a). The MC-method produced uneven carminophilia and usually no staining in the surface epithelium. AB 1.0 and AF stainings for sulphate groups showed similar distribution of sulphomucins while somewhat larger masses of alcianophilic substances were observed in sections stained with AB 2.5 after acid hydrolysis with sulphuric acid. Methylation-AB 2.5 and 1.0 stainings and the methylation-saponification-AB 1.0 method revealed no blue-stainable substances and an overall "washed-out" appearance in the corresponding control sections. This was the reason for the less intense alcianophilia of the sections stained with methylation-saponification-AB 2.5 compared with that observed after AF/AB sequential staining. Deacetylation did not affect the alcianophilia of acidic mucins. The single and sequential stainings for differentiation of acid radicals revealed higher con-

centrations of sulphate groups in the mucosubstances of the deep glandular and goblet cells than in those of the superficial glands (Table 2).

Table 2. The chief location and some histochemical characteristics of epithelial mucosubstances in the infected bile ducts.

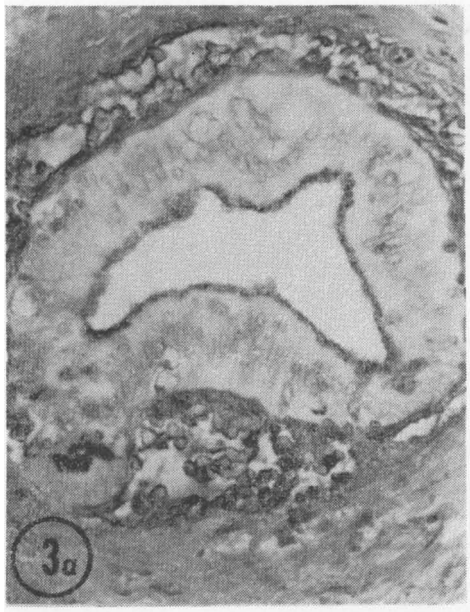
Staining method	Surface epithelium	Superficial glands	Deep glands	Goblet cells
d-PAS	red 1-2*	red 2	red 3	red 3
AB 2.5	blue 1-2	blue 2	blue 3	blue 3
AB 1.0	blue 1	blue 1	blue 2-3	blue 3
AB 2.5/PAS	purple 1-2	blue or purple 2	blue or purple 3	blue or purple 3
AB 1.0/PAS	purple 1, red 1-2	red 2	purple 2-3	purple or red 3
AF/AB	purple 1, blue 1-2	blue 2	purple 3	purple 3

* intensely stainable mucin located in narrow (1) or broad (2) apical zone of the cytoplasm or in the whole cytoplasm (3) of epithelial cells. The symbols 1-2 and 2-3 indicate uneven distribution of the intense reaction.

In hyaluronidase-AB stained sections, hyaluronidase treatment did not affect the blue-staining of epithelial mucosubstances, only producing a decrease in the alcianophilia of connective tissues. In contrast, sialidase digestion produced a significant decrease of alcianophilic epithelial mucosubstances. The short digestion with sialidase at pH 8.9 (Fig. 3) appeared almost as effective as the digestion carried out at pH 5.3 for 42 hrs. (Fig.

Figure 3. Fascioliasis. a: shows alcianophilic substances (dark-stained areas), indicating the presence of acid radicals, in the apical zone of the cytoplasm of the surface epithelium and in wide cytoplasmic areas of the gland (to the left). Compare a with b and note that 3 hrs. treatment with sialidase has eliminated alcianophilia of the surface epithelium. 3a: AB 2.5. 3b: sialidase-AB, counterstained with nuclear fast red, × 318.

Figure 4. Fascioliasis. a: presents AB 2.5-stained section through the wall of a main bile duct and shows the presence of carboxymucins and sulphomucins (dark-stained areas) in superficial, glandular and goblet cells. b: shows the contiguous section stained by the same method after 42 hrs.' digestion with sialidase, which removed alcianophilia due to sialidase-labile sialomucins. 4a: AB 2.5; 4b: sialidase-AB, × 318.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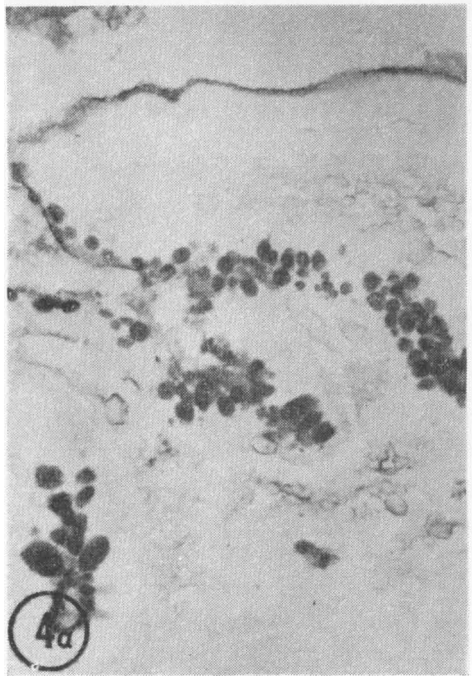
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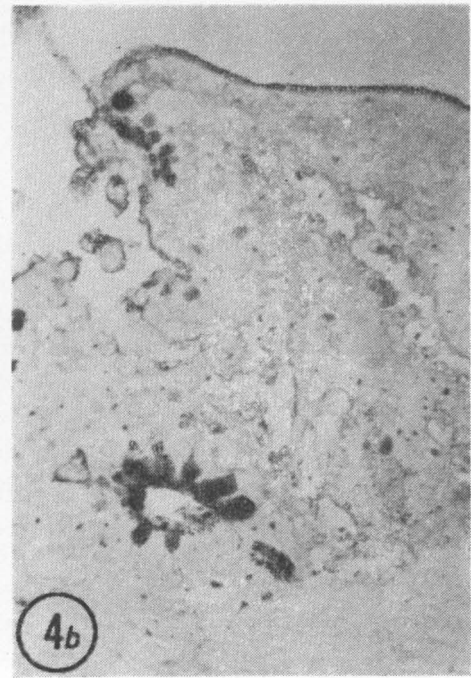
4b

4). On the other hand, the deacetylation procedure did not produce any definite increase in the susceptibility of the acidic mucins towards sialidase. The enzymatic digestions showed the presence of sialomucins particularly in the cells of the superficial glands. In addition a decrease in alcianophilia was observed in the mucosubstances of the surface epithelium as also in occasional deep glandular cells. The mucosubstances of the goblet cells, on the other hand, seemed to be fairly resistant to sialidase digestion.

DISCUSSION

The histochemical methods at present available provide rather little information on the exact chemical structure of carbohydrate-rich tissue components compared with that which might be obtained by biochemical analysis. However, difficulties are encountered in such an analysis of mucins, because the secretion of a certain type of cell readily becomes contaminated by other secretions. This also applies to the secretion in bile ducts, where several types of cells are capable of producing mucin (*Rahko* 1971). On the other hand, with histochemical methods it is possible to analyse the composition of mucosubstances in different epithelial cells, either by demonstrating different groups and radicals or by studying their location within the macromolecule (*Spicer et al.* 1965).

Previous studies on the histochemical composition of mucosubstances have shown that epithelial acidic mucins are usually periodate-reactive whereas those in the connective tissues mostly fail to react with PAS (e.g. *Lev & Spicer* 1965). No periodate-nonreactive mucins were demonstrated in the bile-duct mucins of the goat. This indicates that the sulphate esters visualized, e.g. by AB 1.0 staining, are evidently not located on vicinal glycols. Such sulphomucins are periodate-nonreactive and have been reported to be present in sublingual glands of certain animals (*Spicer et al.* 1965).

The PAS-reaction of the mucosubstances in the epithelial cells of the bile ducts was moderate or strong and was completely abolished by the acetylation procedure. This indicates, according to *Pearse* (1961), that vicinal glycol groups were responsible for the periodate-reactivity. Moreover, the absence of diastase labile material eliminates the possibility that the PAS-reaction originated from glycogen.

The deacetylation treatment failed to increase the intensity of the PAS-reaction. This procedure removes acetyl radicals and makes more hydroxyl groups available for oxidation with periodic acid (*Pearse*). Since the originally strong reaction with PAS may readily mask any increase in periodate-reacting groups, the result of the deacetylation-PAS method does not necessarily indicate the absence of acetyl groups from the PAS-reactive mucosubstances. The epithelial sites containing compounds with periodate-reactive hydroxy groups showed a staining reaction for sulphate groups, too. Hence it seems possible that sulphate radicals may be located in the PAS-reactive mucosubstances and possibly inhibit the periodate oxidation. However, such an inhibitory effect could not be shown after desulphation with the methylation procedure. On the other hand, in the colonic mucosa the originally weak PAS reaction was greatly increased by both the deacetylation and methylation methods (*Mäkelä et al.* 1971).

The strong HCL-methylation blocks the basophilia of acidic mucins, producing hydrolysis of sulphate groups but esterifying the carboxyls (*Spicer & Lillie* 1959). Therefore the methylation-blocked basophilia of carboxyl groups alone is restored after demethylation with KOH. The absence of alcianophilic substances in sections stained with methylation-AB at pH 2.5 and 1.0 indicates the effectiveness of the methylation blockade in bile-duct mucins. The following saponification with KOH, however, failed to restore the alcianophilia to the extent which might be expected from the results of AF/AB sequential staining. The latter method stains carboxymucins blue with alcian blue and sulphomucins purple with aldehyde fuchsin (e.g. *Spicer et al.* 1967). The disagreement between the results with methylation-saponification-AB and AF/AB staining possibly indicates that the saponification procedure did not demethylate all the carboxyl groups. *Spicer* (1960) also obtained somewhat variable results with these techniques.

The histochemical methods employed for acidic mucins revealed the presence of both carboxyl and sulphate radicals in the epithelial mucins. The absence of hyaluronidase-labile material indicates that the basophilia in epithelial cells was not produced by hyaluronic acid or chondroitin-4 and -6-sulphates, which, according to *Pearse*, are readily digested with this enzyme. On the other hand, one or more of these substances were present in the connective tissues of the bile-duct walls.

The enzymatic digestion by sialidase showed that most or all of the carboxymucins produced by bile-duct epithelial cells were sialomucins. In the goat sialomucins were highly labile towards sialidase, being largely digested within as little as 3 hrs., as were also those in the bile ducts of the mouse (*Rahko* 1971). In contrast, bile-duct carboxymucins of cattle were highly resistant to sialidase treatment (*Rahko* 1971). Of the different sialic acids, N-acetyl-O-diacetyl-like forms are known to be resistant to sialidase, becoming labile with the deacetylation procedure (*Gibbons* 1963, *Ravetto* 1968). However, the deacetylation-sialidase-AB procedure did not reveal the presence of these forms of sialic acid in bile-duct mucins of the goat.

The different types of epithelial cells in the bile ducts of the goat do not seem to be strongly differentiated with respect to mucin production. Thus both neutral mucins and one or both of the acidic mucins could be demonstrated in the same type of cell. However, the staining characteristics seem to indicate that the superficial glands produce mainly sialomucins and the deep glandular and goblet cells primarily sulphomucins. Since it is precisely superficial glands and goblet cells which proliferate most extensively in dicrocoeliasis and fascioliasis, it is possible that the proportion of sialomucins and sulphomucins increases in parasitic cholangitis. However, this supposition cannot be confirmed by histochemical methods.

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SAMMANFATTNING

Undersökningar med avseende på dicrocoeliasis- och fascioliasis-infektionens patologi hos get. II. Gallgångarnas mukosubstansers histokemi.

Histokemiska undersökningar med avseende på bestämmandet av karaktären hos kolhydrat-rika substanser i gallgångarnas slemhinna hos get infekterad med Dicrocoelium dendriticum eller med Fasciola hepatica presenteras. Författaren har enligt en tidigare undersökning visat att en kronisk infektion med dessa parasiter framkallar en körtelartad hyperplasi i gallgångsväggarna. Denna undersökning visade även en definitivt tilltagande sekretion av kvalitativt normala mukosubstanser från hyperplastiska epitelceller.

Epiteliala mukosubstanser i gallgångsväggarna var karakteriserade genom perjodat-reaktiva hydroxy grupper och genom carboxyl och sulfat radikaler. Intensiteten av PAS-reaktionen var moderat eller stark och inte definitivt inhiberad av de sura och acetyl-radikalerna. Specifika enzymatiska digestioner visade i epitelceller inte någon perjodat-reaktivitet producerad av glykogen och ingen basofili fram-

kallad av hyaluronsyra eller chondroitin-4 och -6-sulfater. Däremot visade sialidase-digestionen att största delen av karboxymucinerna representerade sialidase-labila sialomuciner. Dessas labilitet mot sialidase blev inte definitivt ökad efter deacetyleringen. De enkla och sekventiella histokemiska metoderna visade en intensivare reaktion för sulfat radikaler i mukosubstanser hos profunda kjörtlar och bägarceller än hos mukosubstanser i de superficiella körtlarna, som karakteriserades genom höga koncentrationer med carboxyl grupper.

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