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Characterization of the glycoconjugate sugar residues in developing chick esophageal epithelium

G. Gheri[1], S. Gheri Bryk[1], E. Sgambati[1] and M. Gulisano[2]

[1]Department of Human Anatomy and Histology, University of Florence, Florence and

[2]Institute of Normal Human Anatomy, University of Sassari, Sassari, Italy

Summary. The development of the esophagus in the chick embryo is characterized by remarkable morphological changes especially at the level of the epithelium. Using horseradish peroxidase-conjugated lectins (DBA, PNA, SBA, WGA, ConA, LTA, UEAI) we have studied, at the level of the esophagus of chick embryos from the 8th to the 21st day of incubation and of 1- and 2-day-old chicken, the evolution of the saccharidic moieties of glycoconjugates, which precedes and/or is concomitant with the epithelial morphological transformations. We have found differences in content and cellular distribution of oligosaccharides during the histogenetic processes which characterize the lining and glandular epithelium. Before the appearance of cilia and mucus secretion at the bathyprismatic epithelial cells, the sugar residues D-galactose-(ß1→3)-N-acetyl-D-galactosamine, ß-N-acetyl-D-galactosamine and α-L-fucose were detected only at the luminal cell surface. These oligosaccharides were probably involved in giving rise to the polarization of the esophageal epithelial cells. The esophageal gland mucus was first characterized by the presence of α-L-fucose and afterwards also by the presence of D-galactose-(ß1→3)-N-acetyl-D-galactosamine, D-glucosamine and sialic acid.

Key words: Sugar residues, Esophagus, Chick embryo, Lectins

Introduction

During embryonic development of the esophagus marked changes occur, as reported by many morphological and histochemical investigations on mammalian (Johns, 1952; Botha, 1959; Parakkal 1967; Mottet, 1970; Kober and Herbst, 1975; Calvert et al., 1991) and birds (Schumacher, 1926; Ivey and Edgar, 1952; Van Halten and Fennel, 1957; Romanoff, 1960; Allenspach and Hamilton, 1962; Allenspach, 1964; Hinsch, 1967; Mottet, 1970; Lim and Low, 1977). In the chick embryo the esophageal epithelium, in the course of its development, is in a first stage characterized by the presence of a single layer of non-ciliated bathyprismatic cells and, afterwards, by bathyprismatic ciliated and mucous secreting cells; successively, a stratified squamous epithelium replaces the ciliated mucous one. In the last days of incubation, and sometimes after hatching, at the surface of the squamous epithelium, islets of ciliated cells are still present.

Following an ongoing program aimed at understanding the changes and the role of the oligosaccharidic moieties of glycoconjugates during epithelial development and differentiation (Gheri et al., 1990, 1991, 1992; Ghery Bryk et al., 1991, 1992), we have employed, for the present research, a battery of seven horseradish peroxidase-conjugated lectins to study the sugar residues in the chick embryo esophagus which, for its peculiar developmental features, seems to be a good model.

Materials and methods

Tissue collection and preparation

The esophageal anlage of White Leghorn chick embryos was studied each day from the 6th to the 21st of incubation (stages 29-46 of Hambuger and Hamilton, 1951) and in 1- and 2-day old chicks. All the specimens (a number of five for each day of incubation) were taken from the mid-portion, at the level of the tracheal bifurcation. The samples were fixed either in Carnoy's fluid or in 6% mercuric chloride in 1% sodium acetate containing 0.1% glutaraldehyde. The samples were routinely processed and 5 µm-thick paraffin sections were obtained. Sections from tissue fixed in buffered glutaraldehyde-mercuric chloride were treated with Lugol's solution prior to staining.

Offprint requests to: Prof. Gherardo Gheri, Dipartimento di Anatomia umana ed Istologia, Policlinico di Careggi, 50134 Firenze, Italy

Table 1. Lectin characterisitics.

LECTIN (COMMON NAME), ACRONIM	CARBOHYDRATE BINDING SPECIFICITY	INHIBITORY SUGAR (1)	REFERENCES
Arachis hypogaea (peanut), PNA	D-GAl(ß1→3)-D-GalNAc	ß-D-Gal	Lotan et al., 1975
Canavalia ensiformis (Jack bean), ConA	α-D-Man>α-D-Glc	Methyl-α-Man	Goldstein and Hayes, 1978
Dolichos biflorus (Horse gram) DBA	α-D-GalNAc	---	Hammarström et al., 1977; Leathem and Atkins, 1983
Glycine max (Soybean), SBA	α/ß-D-GalNAc>D-Gal	D-GalNAc	Lis et al., 1970; Debray et al ., 1981
Lotus tetragonolobus (Asparagus pea), LTA	α-L-Fuc	α-L-Fuc	Pereira and Kabat, 1974; Sugii et al., 1982
Triticum vulgare (Wheat germ), WGA	$(ß\text{-D-GlcNAc})_n$, sialic acid	D-GlcNAC	Goldstein et al., 1975; Bhavanandan and Katlic, 1979
Ulex europaeus (Gorse seed), UEA-I	α-L-Fuc	---	Matsumoto and Osawa, 1969; Sugii et al., 1982

Fuc: fucose; Gal: galactose; GalNAc: N-acetylglalactosamine; Glc: glucose; GlcNAc: N-acetylglucosamine; Man: manose; Methyl-α-Man: methyl-α-mannopyranoside; (1): sugar not listed failed to inhibit.

Lectin histochemistry (Table 1)

After hydration, sections were treated with 0.3% hydrogen peroxide for 10 min (to inhibit the endogenous peroxidase), rinsed in distilled water and washed with 1% bovine serum albumin (BSA) (Murata et al., 1983) in 0.1M phosphate-buffered saline (PBS), pH 7.2. The sections were then incubated for 30 min at room temperature in horseradish peroxidase-conjugated lectins (HRP-lectin conjugate) dissolved in phosphate-buffered saline (0.1M PBS, pH 7.2, 0.1M each of NaCl, 0.1 mM $CaCl_2$, $MgCl_2$ and $MnCl_2$) and then rinsed three times in PBS. The optimal concentration for each lectin (Sigma Chemical Co., St. Louis, MO) which allowed maximum staining with minimum background was as follows: DBA *(Dolicus biflorus)* 25 µg/ml; PNA *(Arachis hypogea)* 25 µg/ml; SBA *(Glycine max)* 20 µg/ml; WGA *(Triticum vulgare)* 20 µg/ml; ConA *(Canavalia ensiformis)* 20 µg/ml; LTA *(Lotus tetragonolobus)* 25 µg/ml; and UEAI *(Ulex europaeus)* 25 µg/ml. Staining of the sites containing bound lectin-HRP was obtained by incubating the slides with PBS (pH 7.0), containing 3,3'diaminobenzidine (DAB) (25 mg/100 ml) and 0.003% hydrogen peroxide, for 10 min at room temperature. Specimens were rinsed in distilled water, dehydrated using graded ethanol solutions, cleared in xylene and mounted in Permount. Controls for lectin staining included: 1) substitution of unconjugated lectins for lectin-HRP conjugates; 2) exposure to HRP and substrate medium without lectin; 3) oxidation with 1% periodic acid for 10 min prior to lectin staining; 4) exposure of sections to 20-25 µg/ml of each lectin-HRP conjugate containing 0.2M D-galactose, D-glucose, D-mannose, L-fucose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine and methyl-α-mannopyranoside.

Sialidase digestion

In some experiments, prior to PNA and WGA staining, sialic acid was removed by pretreating the sections for 18 hr at 37 °C in a solution of sodium acetate buffer 0.25M, pH 5,5 containing 0.1 unit/ml sialidase (neuraminidase Type X from *Clostridium perfrigens* (Sigma Chemical Co., St. Louis, MO)), 5.0 mM $CaCl_2$ and 154 mM NaCl, prior to staining with lectin-HRP conjugates. Controls containing the sialidase buffer without the enzyme were also prepared.

Results

Morphological remarks

From the 6th to the 7th day of incubation the chick embryo esophageal epithelium was represented by a single layer of non-ciliated columnar cells. Afterwards, until the 9th day, a progressive pseudostratification of the nuclei was observable. At the 10th day of incubation the epithelium showed two layers of low columnar cells; the superficial one was characterized by the presence of some mucus-secreting cells and very few ciliated ones. From the 10th to the 13th incubation day the esophageal epithelium remained two-layered showing a progressive increase in the number of mucous and ciliated cells. At the 13th day small knots of epithelial cells began to project downwards into the underlying mesenchyme, representing the first step of gland bud formation. At the 14th day an intermediate layer appeared, which gave rise to the first developmental feature of a pluristratified squamous epithelium. On the same day the gland anlage began to show an irregular lumen. On the 16th day of incubation a typical multilayered squamous epithelium was observable, showing, at its luminal surface, islets of ciliated cells. Islets of ciliated cells were still present at hatching and, more rarely, in 1- and 2-day-old chicks. From the 16th day till hatching an increase in number of the epithelial layers and in mucus secretion by the glands appeared.

Lectin histochemistry at the lining epithelium (Tables 2, 3)

At the 6th and 7th day of incubation the monolayered epithelium was characterized by cytoplasmic granules which reacted with PNA (Fig. 1), WGA, SBA and ConA (Fig. 10).

The apical surface of the epithelial cells showed reactivity with PNA, WGA, SBA and LTA in different periods of incubation and not continuously. In fact, from

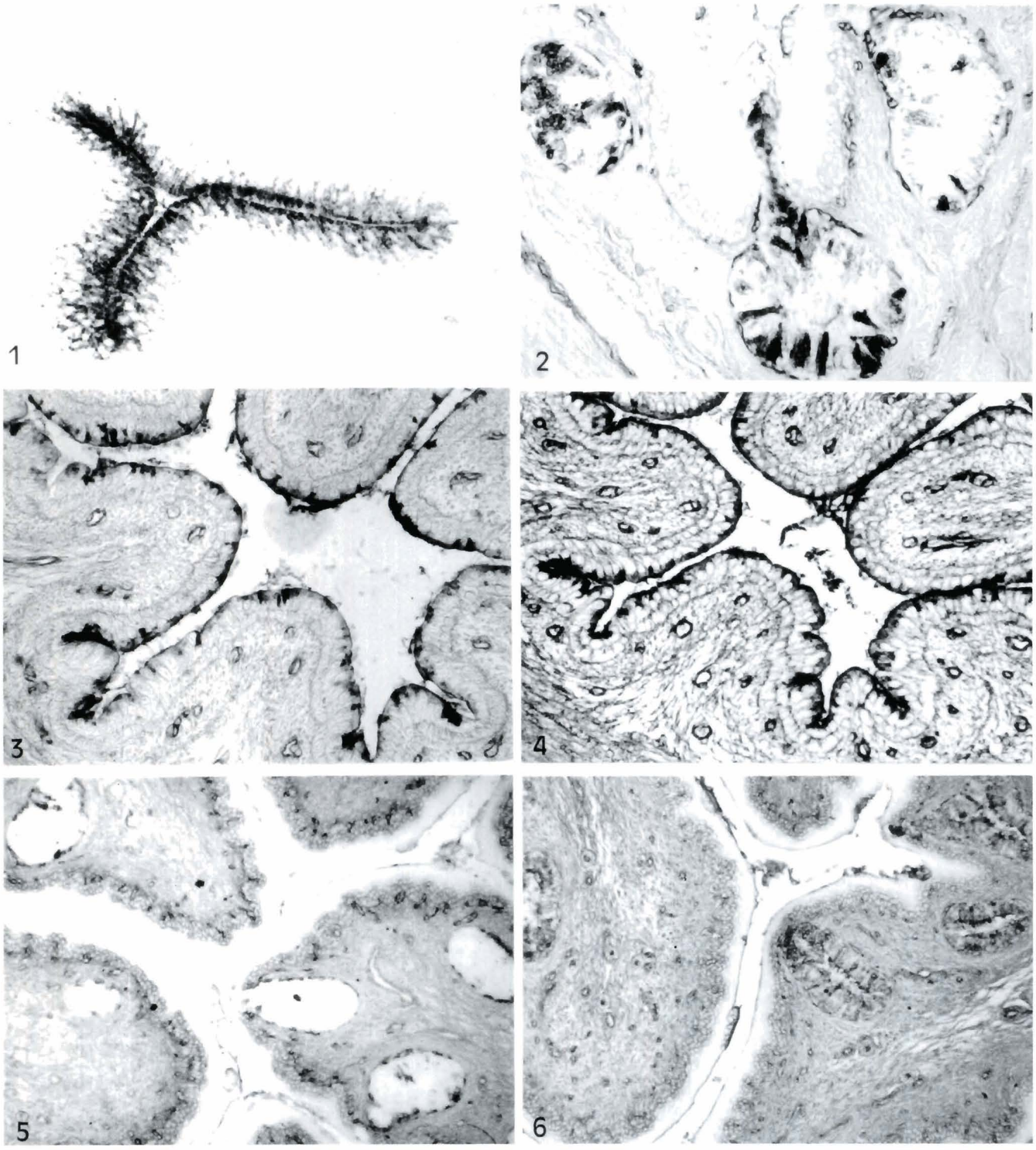

Fig. 1. PNA. 7th day of incubation. Esophageal epithelium. At this day of incubation reactive supranuclear cytoplasmic granules are observable. x 270

Fig. 2. PNA. 1-day-old chicken. Esophageal glands. Some strongly reactive secreting cells are obervable within the esophageal gland. x 270

Fig. 3. PNA. 11th day of incubation. Esophageal epithelium. Positive secreting cells along the lining epithelium. x 270

Fig. 4. Neuraminidase-PNA. 11th day of incubation. Esophageal epithelium. Following neuraminidase digestion, no appreciable increase in stainability at the secreting cells is detectable. An increase in PNA reactivity is observable at the surface and at the apical portion of the epithelial cells. An increase in mesenchymal cell stainability is evident. x 270

Fig. 5. PNA. 21st day of incubation. Esophageal glands and epithelium. Reactivity is observable at the luminal surface and at the surface of the cells located in the inner epithelial layers. Most cells of the esophageal glands are unreactive. x 135

Fig. 6. Neuraminidase-PNA. 21st day of incubation. Esophageal glands and epithelium. Following neuraminidase digestion, all cells of the glands appear reactive. x 135

Table 2, Lectin reactivity in the cells of the esophageal epithelium from the 6th to the 13th day of incubation.

	DAYS							
	6	7	8	9	10	11	12	13
PNA								
LS	0	0	3	3	0	0	0	0
AS	0	0	0	0	0	0	0	0
CG	3	3	0	0	0	0	0	0
SM	-	-	-	-	3	3	3	3
GR	0	0	0	0	0	0	0	0
DS	0	0	0	0	0	0	0	0
WGA								
LS	0	0	1	2	2	2	2	0
AS	1	2	2	2	2	2	2	2
CG	3	3	0	0	0	0	0	0
SM	-	-	-	-	0	3	3	0
GR	0	0	0	0	0	0	0	0
DS	0	0	0	0	0	0	0	2
SBA								
LS	0	0	1	2	2	0	0	0
AS	1	1	0	0	0	0	0	0
CG	2	1	0	0	0	0	0	0
SM	-	-	-	-	0	0	0	0
GR	0	0	0	0	0	2	1	1
DS	0	0	0	0	0	1	1	1
ConA								
LS	0	0	0	0	0	0	0	0
AS	2	1	2	2	2	1	1	1
CG	2	2	0	0	0	0	0	0
SM	-	-	-	-	0	0	0	0
GR	0	0	0	0	0	0	0	0
DS	0	0	0	0	0	0	2	2
LTA								
LS	0	0	0	1	1	2	1	0
AS	0	0	0	0	0	0	0	0
CG	0	0	0	0	0	0	0	0
SM	-	-	-	-	0	1	2	3
GR	0	0	0	0	0	0	0	0
DS	0	0	0	0	0	0	0	0

LS: luminal surface; AS: abluminal cell surface; CG: cytoplasmic granules; SM: secretory material within the goblet cells; GR: Golgi region of the ciliated cells; DS: supranuclear diffuse cytoplasmic positivity at the ciliated cells. The number indicates the intensity of the reaction: 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction. DBA and UEAI lectins gave negative results at any site.

Table 3, Lectin reactivity in the cells of the esophageal epithelium from the 14th to the 21st day of incubation and in 1- and 2-day-old chicks.

	DAYS							
	14-15	16	17-18	19	20	21	1	2
PNA								
LS	0	3[a]	3[a]	2[a]	2[a]	2[a]	2	0
AS	0	0	0	2[b]	1[b]	1[b]	1[b]	0
CG	0	0	0	0	0	0	0	0
SM	3	0	0	0	0	0	0	0
GR	0	2[c]	2[c]	0	0	0	0	0
WGA								
LS	0	4	4	4	3	3	3	2
AS	2	3	3	3	3	3	3	3
CG	0	0	0	0	0	0	0	0
SM	0	0	0	0	0	0	0	0
GR	0	0	0	0	0	0	0	0
SBA								
LS	0	0	1	2[a]	2[a]	1	0	0
AS	0	0	0	1	1	0	0	2
CG	0	0	0	0	0	0	0	0
SM	0	0	0	0	0	0	0	0
GR	1[c]	1[c]	1[c]	2[c]	2[c]	0	0	0
ConA								
LS	0	2	2	1	1	1	1	1
AS	1	2	2	1	1	1	1[d]	1[d]
CG	0	0	0	0	0	0	0	0
SM	0	0	0	0	0	0	0	0
GR	0	0	0	0	0	0	0	0
LTA								
LS	0	2[a]	2[a]	2[a]	0	0	0	0
AS	3	1	1[a]	1[a]	2	2	2	2[b]
CG	0	0	0	0	0	0	0	0
SM	3	2	0	0	0	0	0	0
GR	0	0	0	0	0	0	0	0

LS: luminal cell surface; AS: abluminal cell surface; CG: cytoplasmic granules; SM: secretory material within the goblet cells; GR: Golgi region. The number indicates the intensity of the reaction: 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction; 4, very strong reaction. [a]: also the surface of the cells of the islets was reactive; [b]: only cells in the deepest layers; [c]: only islets of ciliated cells; [d]: only cells in the outer layers. DBA and UEAI lectins gave negative results at any site.

the 13th to the 15th day the luminal surface of the esophageal cells was characterized by the loss of positivity to all the above mentioned lectins. Reactivity with ConA at this site was notable only from the 16th day onwards. In 1- and 2-day-old chicks the luminal epithelial surface showed affinity for PNA, WGA (Figs. 11, 12) and ConA.

Binding of WGA and ConA to the abluminal surface of the epithelial cells was seen during the whole considered period of incubation. After hatching, positivity to WGA at this site was observable at all epithelial layers, while ConA reactivity was restricted to the outer ones. LTA bound to the surface of all epithelial cells from the 14th day till hatching and in 1-day-old chicks, while in 2-day-old chicks positivity was observed only at the inner layers. For limited and different time periods the cell surface reacted with PNA (Fig. 5) and SBA. The supranuclear cytoplasm of the mucous secreting cells showed affinity for PNA (Fig. 3) and LTA (Fig. 7) from the 10-11th until the 15-16th day of incubation. WGA positivity was observable only on the 11-12th day.

Following neuraminidase treatment an increase in PNA reactivity (Fig. 4) and a decrease in WGA reactivity was observed at the undifferentiated mesenchyme and at the surface of all the epithelial cells.

DBA and UEAI did not bind at all to the chick esophageal epithelium.

Lectin histochemistry of the esophageal glands (Table 4)

The surface of the cells of the esophageal glands strongly reacted with WGA from the 14th day of

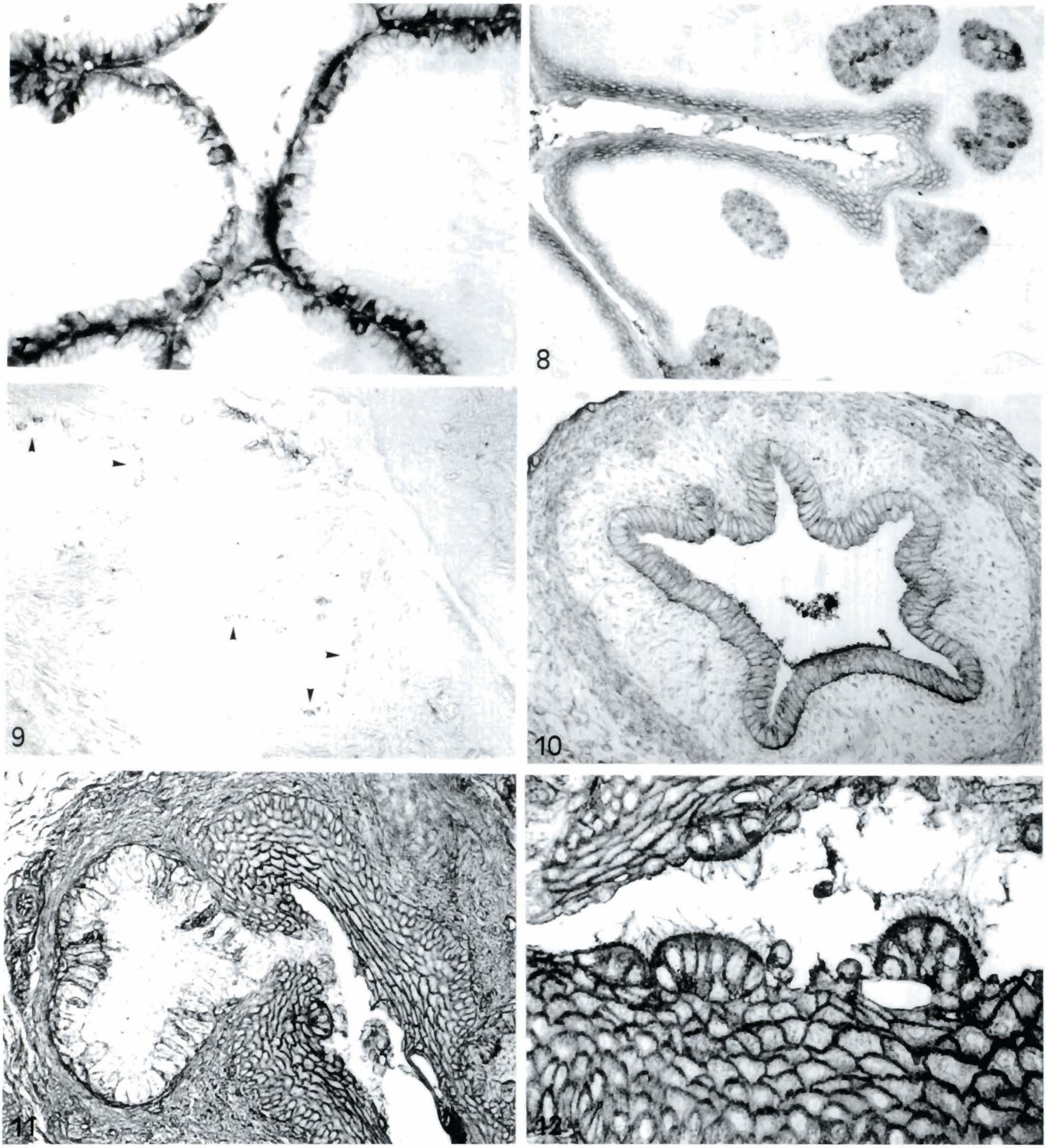

Fig. 7. LTA. 13th day of incubation. Esophageal epithelium. Reactive secreting cells are observable. x 270

Fig. 8. LTA. 1-day-old chicken. Esophageal glands and epithelium. The surface of the epithelial cells reacts with LTA. At the level of the esophageal glands, «foamy» reactive secretory material is detectable. x 270

Fig. 9. SBA. 21st day of incubation. Esophageal glands and epithelium. Weak reactivity is observable at the Golgi region of the glandular cells (arrows). x 270

Fig. 10. ConA. 7th day of incubation. Esophageal epithelium. The surface of the cells of the cuboidal epithelium shows reactivity with the lectin. x 270

Fig. 11. WGA. 1-day-old chicken. Esophageal gland and epithelium. Strong reactivity is observable at the surface of the cells of the pluristratified epithelium. Some cells of an esophageal gland are characterized by a reactive secretory material. x 270

Fig. 12. WGA. 2-day-old chicken. Esophageal epithelium. The surface of the epithelial cells is strongly reactive. Some WGA reactive islets of ciliated cells are still observable; one of them is detaching from the luminal surface. x 675

Table 4, Lectin reactivity in the cells of the esophageal glands from the 14th to the 21st day of incubation and in 1- and 2-day old chicks..

	DAYS							
	14-15	16	17-18	19	20	21	1	2
PNA								
CS	0	0	0	0	0	0	0	0
SM	0	0	0	0	3	3	4	4
GR	0	0	0	0	0	0	0	0
WGA								
CS	2	3	3	3	3	3	3	3
SM	0	0	0	0	4	4	3	3
GR	0	0	0	0	0	0	0	0
SBA								
CS	0	0	0	0	0	0	0	0
SM	0	0	0	0	0	0	0	0
GR	0	0	0	0	0	1	2	1
ConA								
CS	1	1	1	1	0	0	0	0
SM	0	0	0	0	0	0	0	0
GR	0	0	0	0	0	0	0	0
LTA								
CS	0	0	0	1	1	2	2	2
SM	0	2	2	3	3	3	3	3
GR	0	0	0	0	0	0	0	0

CS: cellular surface; SM: secretory material; GR: Golgi region. The number indicates the intensity of the reaction: 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction; 4, very strong reaction. DBA and UEAI lectins gave negative results at any site.

incubation till hatching and in 1- (Fig. 11) and 2-day-old chicks. At this site ConA affinity was observable from the 14th to the 19th day of incubation. LTA positivity was detected from the 19th day till hatching and in 1- (Fig. 8) and 2-day-old chicks. Mucus secreting cells reacted with LTA from the 16th day and with PNA (Fig. 2) and WGA (Fig. 11) from the 20th day. Lectin reactivity at secreting cells remained observable till hatching and in 1- and 2-day-old chicks. SBA bound to the Golgi region of the glandular epithelial cells from the 21st day onwards (Fig. 9). After enzymatic cleavage of sialic acid, an increase in number of PNA-reactive cells (Fig. 6) and a decrease in WGA reactivity were observed at the epithelial cells surface and at the supranuclear cytoplasm of the mucus secreting cells.

DBA and UEAI did not bind at all in the chick esophageal glands.

Discussion

SBA and DBA nominally show the same sugar specificity to N-acetylgalactosaminyl residues (Schulte et al., 1985). The lack of reactivity with DBA at any site of the esophageal epithelium suggests the presence of ß-N-acetyl-D-galactosamine anomer only.

UEAI did not react at all at any site of the esophageal epithelium, while LTA reactivity was observable. This fact has been previously observed in other embryonic (Gheri et al., 1990, 1991; Gheri Bryk et al., 1991, 1992) and adult (Madrid et al., 1989) organs in the fowl. According to other authors (Debray et al., 1981; Schulte and Spicer, 1983; Foster et al., 1991) reactivity with LTA and the lack of binding to UEAI suggest the presence of reactive sites containing terminal L-fucosyl residues which bind via α(1➔6) linkages to penultimate glucosaminyl residues and/or difucosylated oligosaccharides.

At the 6th day of incubation the supranuclear cytoplasm of the cells of the esophageal non-ciliated columnar epithelium was characterized by minute granules containing D-galactose-(ß1➔3)-N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, ß-N-acetyl-D-galactosamine and α-D-mannose, as revealed by their reactivity with PNA, WGA, SBA and ConA. From the 7th to the 8th day of incubation such granules, except the ConA-positive ones, were observed even closer to the apical cytoplasm and, at the 9th day, they were found chiefly distributed at the cellular luminal surface. From the 8th-9th day of incubation, the sugar residues D-galactose-(ß1➔3)-N-acetyl-D-galactosamine, ß-N-acetyl-D-galactosamine and α-L-fucose were detectable only at the luminal cell surface. Such oligosaccharides were probably involved in giving rise to the polarization of the epithelial cells which preceded and/or went with differentiative cellular phenomena such as the appearance of cilia, and mucus secretion. According to Spicer et al. (1981), the existence of mechanisms which favour the transport of newly-synthesized glycoconjugates towards luminal more than abluminal plasma membrane ought to be considered. Until the 13th day, the sugar residue with characterized the abluminal cell surface was the α-D-mannose. N-acetyl-D-glucosamine was detected both at the luminal and abluminal cellular surfaces.

As the stratification of the epithelium into two layers took place, the outer layer showed two types of differentiated cells: ciliated and mucus secreting cells. The mucus secreting cells, which were first observable at the 10th day of incubation, strongly reacted with PNA. The onset of goblet cell mucous secretion occurred simultaneously with the acid production by the proventriculus (Toner, 1965; Mottet, 1970). One day later, the goblet cells also reacted with WGA and LTA. Interestingly, WGA reactivity was observed for a short period while PNA and LTA reactivity disappeared at the 15th-16th day of incubation.

An interesting finding was the loss of reactivity at the luminal epithelial surface, with all the used lectins, from the 13th to the 15th day of incubation. It is to be noted that, during this period, the esophageal epithelium underwent a process of «squamous development from a ciliated mucous to stratified squamous epithelium» (Mottet, 1970). From the 16th day of incubation, when the process of differentiation is completed, the reappearance of a lectin-reactive luminal surface was detected, characterized by a larger distribution of sugar residues in comparison to that detected in the previous period. Furthermore, from the 15th day onwards, a larger

number of sugar residues was detected at the abluminal plasma membrane of the epithelial cells. Conceivably the appearance, from the 14th day onwards, of a new sugar residue such as α-L-fucose at the plasma membrane was of some significance in the developing squamous epithelium. Occurrence of α-L-fucosyl residues in the developing squamous epithelium was also observed to characterize the dorsal and ventral lingual epithelium of the chick (Gheri et al., 1991).

Sialic acid was never detected in the epithelial goblet cells, while it was seen at the surface of all epithelial cells. It is noteworthy that the presence of sialic acid moieties at the cell surfaces coincided with the appearance of a bi- and successively multi-layered epithelium. As postulated by Suprasert et al. (1989) «multiantenneric glycoconjugates with sialic acid could form a bridge to positively charged groups». Such behaviour of the glycoconjugates rich in sialic acid would strengthen the membrane stability (Werner et al., 1992) and at the same time could contribute to the achievement of a certain degree of cell surface rigidity (Suprasert et al., 1989).

The cessation of mucous secretion by goblet cells was immediately followed by mucus production by the esophageal glands. The esophageal gland mucus was first characterized by the presence of α-L-fucose. Only on the last days of incubation two other sugar residues (D-galactose-(ß1⇒3)-N-acetyl-D-galactosamine and N-acetyl-D-glucosamine) were detected. The glycoconjugates released by esophageal glands are involved in mucosubstances covering the luminal surface of the alimentary tract (Fox, 1979; Suprasert et al., 1987). Such mucosubstances probably regulate the control of infections, heat transfer, prevention of dehydration and lubrication (Hafez, 1977; Fox, 1979; Werner et al., 1982; Suprasert et al., 1987).

In the last days of incubation, sialic acid was detectable in the mucous secreting cells of the esophageal glands; this finding disagrees with that of Mottet (1970) who stated the absence of sialic acid at the secreting cells of the esophageal glands in the chick embryo. Sialic acid residues, negatively charged at physiological pH, contribute to mucus viscosity and ion binding (Foster et al., 1991). The presence of sialic acid moieties in the last days of incubation seems to be necessary for the establishment of a mucous environment suitable for a radical change in the type and modality of feeding after hatching.

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