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CHARACTERIZATION OF MUCUS GLYCOCONJUGATES IN NORMAL HUMAN CONJUNCTIVA BY LECTINS IN LIGHT MICROSCOPY, TRANSMISSION AND SCANNING ELECTRON MICROSCOPY

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Abstract

Maintenance of tear film in normal conditions is dependent on 1) mucus layer integrity and 2) the presence and distribution of conjunctival epithelial cell microvilli.

In the present work a new methodology has been developed to gain correlative information about microprojection assessment and mucus composition, from the same specimen, by Light Microscopy (LM), Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM).

We have characterized the glycosidic residues secreted by goblet cells in normal human conjunctiva, by means of four lectins (WGA, ConA, PNA and SBA), conjugated with FITC for LM and with colloidal gold for TEM and SEM.

The cytochemical reactions were performed on histological sections of paraffin-embedded material and on semithin and ultrathin sections of both Epon embedded material directly processed for TEM and of blocks recovered from SEM and reprocessed for TEM.

WGA, ConA, PNA and SBA receptors were found to be constituents of the mucus produced by goblet cells in human conjunctiva. The granules of the so-called "second mucus system" (SMS) cells were labelled mainly by WGA. A difference in the quality of glycoconjugates between goblet cells and SMS cells has been also demonstrated.

Our results provide an improved method to evaluate alterations of tear film that occur in many conjunctival diseases.

KEY WORDS: Human conjunctiva, glycoconjugates, goblet cells, second mucus system, lectins, colloidal gold, light microscopy, scanning and transmission electron microscopy.

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Introduction

In normal conditions the conjunctiva is moistened by a tear film that consists of 3 layers. The innermost layer is a mucus layer, produced principally by goblet and non-goblet epithelial cells of the conjunctiva (Takakusaki, 1969; Srinivasan et al., 1977; Greiner et al., 1979, 1980; Greiner and Allansmith, 1981). The non-goblet epithelial cells comprise the "second mucus system" (SMS).

The mucus layer plays an important role in the stability of tear film by decreasing its surface tension (Lemp et al., 1970). Microprojections of the superficial conjunctival (and also corneal) cells contribute to the maintenance of the film adherence, as they represent an anchoring point for the mucus layer. Thus, when analyzing problems related to the tear film alterations, one must study both morphological status of microprojections and mucus composition.

For microvilli, SEM is the most suitable method as it permits the investigation at the ultrastructural level of large areas of tissue, providing an immediate assessment of microprojection distribution (Versura et al., 1985). With regard to mucus, conjunctival goblet cell glycoproteins have been studied utilizing routine histochemical staining on paraffin sections (Spicer and Mayer, 1960; Matsumoto and Mimura, 1974; Srinivasan et al., 1977). They were classified as non sulphated mucopolysaccharides containing sialic acid. Greiner et al. (1985), using the Alcian Blue-PAS-pH technique found in non-goblet epithelial cell granules sialomucins and neutral mucins. Kawano et al. (1984) have utilized paraffin embedded and frozen biopsies labelled with FITC-lectins observed at LM level. Lectins are proteins which very specifically bind glycosidic residues (Goldstein and Hayes, 1978) and therefore they have been used to detect the carbohydrate content of

mucus cells in various cases (Essner et al., 1978; Freeman et al., 1980; Gorelick et al.,1982).

In our study, we have made an attempt to get as much complementary information as possible from the same specimen, recognizing that the conjunctival biopsy consists of a very small quantity of tissue.We have developed a method which permits correlative study of conjunctival tissue first by SEM, in order to analyze the microprojection distribution, and, secondly by LM, TEM, backscattered electron mode (BEI) in SEM of the previously scanned specimen, embedded in resin, in order to study the internal structural and cytochemical details.

For the cytochemistry we utilized four lectins: Wheat Germ Agglutinin (WGA) specific for N-acetyl-glucosamine and N-acetyl-neuraminic acid, Arachis Hipogea (Peanut lectin, PNA) specific for galactosil β-(1-3)-N-acetyl-galactosamine and non reducing terminal galactose, Soybean Agglutinin (SBA) specific for α-N-acetyl-D galactosamine and Concanavalin A (ConA) specific for trimannosidic cores substituted by two N-acetyl-glucosaminyl residues (Roth, 1978; Kornfeld and Kornfeld, 1978; Lis and Sharon, 1981; Debray et al., 1981). FITC-conjugated lectins have been employed for light microscopy and colloidal gold-conjugated lectins for TEM and SEM.

Materials and Methods

Normal conjunctival biopsies were obtained from eighteen patients (11 females and 7 males; medium age 55), undergoing surgery for diseases not involving the anterior segment. Normalcy of conjunctival tissue was determined by slit-lamp examination of the patient 24 h prior to surgery. After local anaesthesia (ossybuprocain 0.5%), biopsies were taken from the inner lower tarsus, by surgical forceps and scissors. Specimens were divided into three groups and processed for microscopy as follows:

GROUP A - 10 biopsies mounted on ashless filters to prevent tissue from folding were briefly washed in saline and then immediately immersed in 2.5% glutaraldehyde - 1.6% p-formaldehyde in 0.1 M cacodylate buffer for 3 h at 4°C. We omitted post-fixation in OsO_4 following the suggestion of Thomopoulos et al. (1983) who recommends to be careful in the choice of fixatives as they influence the sensitivity of detection of the glycoconjugates. After an overnight buffer wash (0.1 M cacodylate buffer plus 2.7% (sucrose) biopsies were dehydrated through a graded series of ethanol, critical point dried, mounted on aluminium stubs and sputter coated with gold (20 nm). They were observed with a Philips SEM 505, operating in a range of 10-30 kV. Following SEM observation, the specimens were removed from the stubs and processed for TEM. Specimens were embedded in Epon. Semithin and ultrathin sections were obtained with the Reichert UM3 ultratome.

GROUP B - 4 biopsies were immediately fixed in 2.5% glutaraldehyde - 1.6% p-formaldehyde in 0.1 M cacodylate buffer for 3 h at 4°C, washed overnight, dehydrated through a graded series of ethanol and embedded in Epon. Semithin and ultrathin sections were obtained with the Reichert UM3 ultratome.

GROUP C - 4 biopsies were immediately fixed in 10% p-formaldehyde for a minimum of 24 h at room temperature, dehydrated through a graded series of ethanol, embedded in Paraplast, as previous works have indicated a good preservation of lectin receptors with respect to other histological procedures (Bonvicini et al., 1983, 1984). Sections (4-5 μm thick) were obtained with the Leitz microtome.

Cytochemical reactions

Table 1 summarizes the cytochemical reactions applied to the different types of sections.

The reactions utilizing lectins were performed as follows:

FITC-lectins on thick and semithin sections. FITC-WGA, ConA, PNA, SBA and their respective inhibitor sugars (N-acetyl-glucosamine, α-methyl-D-mannoside, D(+)galactose, N-acetyl-D-galactosamine) are commercially available from Sigma Co., St. Louis, Mo.USA.

Semithin sections from groups A and B were:
- etched in 3% Na-OH in absolute ethanol for 3 minutes at room temperature
- briefly (5 minutes) washed in PBS
- incubated in FITC lectins for 1 h in a moist chamber at room temperature. The optimum concentration was 1 mg/ml of PBS pH 7.4 for FITC-WGA, PNA and SBA; ConA was used 1 mg/ml of distilled water pH 7.4.
- washed (two changes, 5 minutes each at 4°C) in PBS
- mounted on a glass coverslip with a drop of glycerol.

Thick sections of group C were deparaffinized and then labelled following the same schedule as above, excluding etching.

As control, sections from each group were incubated with a solution containing the same concentrations of FITC lectins but with the addition of 0.5 M specific inhibitor sugar.

Fluorescence microscopy - All sections were observed under a ZEISS III photomicroscope equipped with an epi-fluorescence condenser III RS, using 10x, 16x and 63x objectives.

Fluorescence was evaluated semiquantitatively

Table 1

The cytochemical reactions applied to the different types of sections are summarized

	GROUP A		GROUP B		GROUP C
	semithin sections	ultrathin sections	semithin sections	ultrathin sections	thick sections
FITC-lectins (LM)	●		●		●
Au_5/Au_{17}-lectins (LM; SEM)	●		●		●
Au_5/Au_{17}-lectins (TEM)		●		●	

and graded from - to +++ jointly by trained observers.

Colloidal gold-lectins on thick and semithin sections (gold-silver staining). Colloidal gold was prepared as follows:
- Au_5 was obtained by reduction of 240 ml of 0.01% $HAuCl_4$ with 2 ml of white phosphorus ether according to Horisberger (1981).
- Au_{17} was obtained by reduction of 100 cc of 0.01% $HAuCl_4$ with 4.2 ml of 1% Na-citrate according to Frens (1973).

WGA, PNA, ConA, SBA, purchased from Sigma Co., St. Louis, Mo., USA, were conjugated with colloidal gold according to Horisberger (1981).

Semithin sections from groups A and B were:
- etched in 3% Na-OH in absolute ethanol for 3 minutes at room temperature
- incubated with undiluted Au_5/Au_{17}-WGA, ConA, SBA, PNA for 1 h at room temperature in a moist chamber
- washed in 0.01 M phosphate buffer plus 0.5 M NaCl pH 7.4 (3 changes, 5 minutes each)
- developed in silver nitrate according to Danscher and Nörgaard (1983) for 1 h in a dark room
- washed in distilled water (10 minutes)
- mounted on glass coverslip with a drop of glycerol

Deparaffinized thick sections of group C were labelled in the same way, excluding etching.

As a control, sections of each group were incubated in a solution containing the same concentration of colloidal gold-lectins but with the addition of 0.5 M specific inhibitor sugar.

Light microscopy - The product of the reaction gold-silver was detected first at LM level with the ZEISS III.

Scanning Electron Microscopy - We have then reduced the size of the glass slide, mounted it on an aluminium stub, covered it with a thin film of carbon (20 nm) and observed it with the Philips SEM 505. The product of the reaction gold-silver was detected in backscattered electron (BSE) and secondary electron (SE) modes, following the method of Pasquinelli et al.(1985). To facilitate the correlation of LM and SEM, SEM observations were recorded at reverse polarity mode.

Colloidal gold-lectins on ultrathin sections. Lectins and colloidal gold were conjugated as described above. On serial sections from groups A and B we performed the reactions as follows; the sections were:
- collected on nickel grids covered with a thin film of formvar
- etched in 3% NaOH in absolute ethanol for 35 seconds
- washed in Tris Buffer Saline (TBS, pH 7.0 for WGA, PNA, SBA and pH 7.2 for ConA)
- incubated in Au_5/Au_{17}-WGA, ConA, PNA, SBA for 1 h at room temperature. The optimum dilution found was for WGA, PNA, and SBA 1:10 in TBS 0.02 M pH 7.0; ConA was used undiluted
- washed in distilled water with stirring (7 minutes)
- counterstained with uranyl acetate and lead

Table 2

The intensity of fluorescence detected in conjunctival goblet cells is shown

	GROUP A semithin sections	GROUP B semithin sections	GROUP C thick sections
WGA	++	++	+++
Con A	½ +/+	½ +/+	-
PNA	+/++	+/++	++/+++
SBA	+/++	+/++	-/+

- = absence; + = traces; from ++ to +++ = presence

citrate.

The controls were obtained by incubating sections in a solution containing the same concentration of colloidal gold-lectin as used before but with the addition of 0.5 M specific inhibitor sugar, except for D(+) galactose which was used 1.0 M.

Transmission Electron Microscopy - Sections were observed in a Zeiss EM 9 and a JEOL 100 B TEM.

Results

FITC-lectins on thick and semithin sections.

The results are summarized in Table 2.

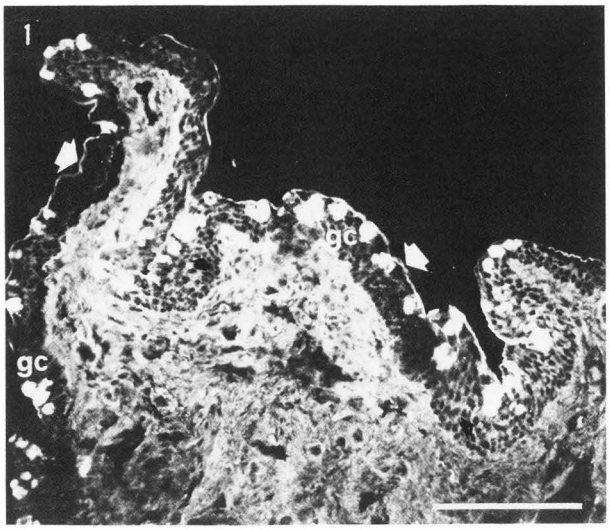
FITC-WGA. All the thick sections of group C showed goblet cells strongly labelled by FITC-WGA (Fig. 1). A band of fluorescence was also found at the epithelial surface and at the cellular borders of the conjunctival epithelial cells. The semithin sections of group B (Fig. 2) showed the same distribution of glycosidic receptors for WGA as described above. The fluorescence was very bright at the goblet cell level (++), but less than that detected in the thick sections of group C (+++). The semithin sections of group A (Fig.3) showed the same FITC-WGA labelling as described for the sections of groups B and C. The intensity of fluorescence (++) was estimated to be exactly the same as the semithin sections of group B.

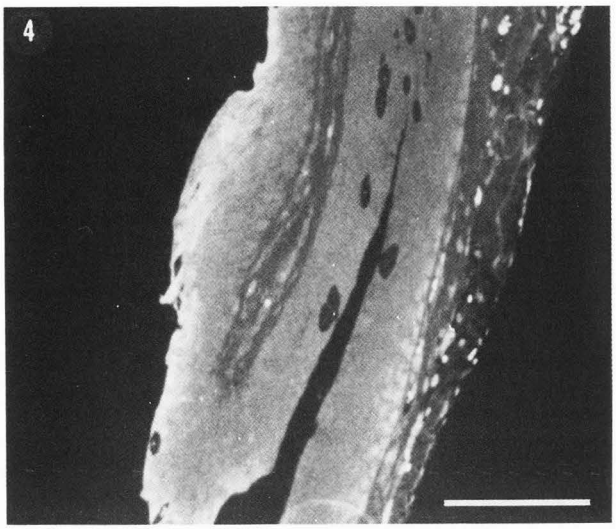
In all the three types of sections, the fluorescence was completely abolished by addition of N-acetyl-galactosamine to FITC-WGA during incubation (Fig. 4).

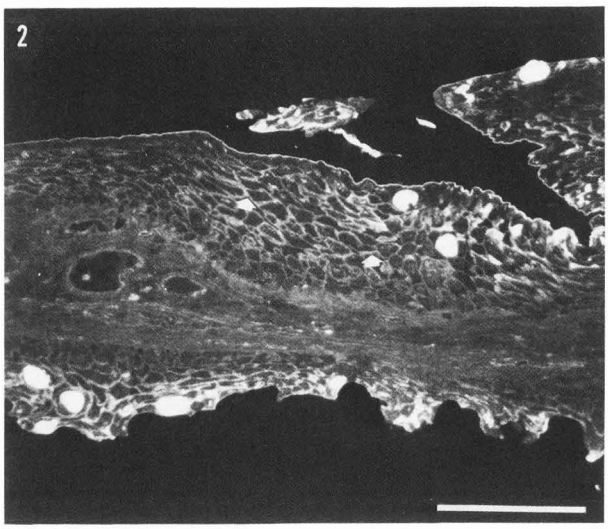
FITC-ConA. In the thick sections of group C, goblet cells were always found completely negative for FITC-ConA (Fig. 5). As to conjunctival epithelial cells, they were slightly labelled only in the external part of the cytoplasm. In the semithin sections of group B, both positive and negative goblet cells were found on the same section (½ +/+). In positive ones, small clusters of fluorescence were observed (Fig. 6). The fluorescence detected in the outer cytoplasm of conjunctival epithelial cells was less than for sections of group C. Also in the semithin sections of group A, the intensity of fluorescence in some goblet cells was the same detected in semithin sections of group B. By adding mannose in the FITC-ConA incubation medium, the fluorescence was reduced in all the three types of sections.

FITC-PNA. In the thick sections of group C, all goblet cells stained very bright (++/+++) (Fig. 7). Conjunctival epithelial cells were almost completely negative, only in some sections traces of fluorescence have been found. In the semithin sections of groups A and B fluorescence was of less intensity (+/++) with respect to group C (Fig. 8). Also, traces of fluorescence were detected in epithelial cells. In all the sections goblet cells did not stain when galactose and N-acetyl-galactosamine were added to FITC-PNA incubation solution.

FITC-SBA. In the thick sections of group C (Fig. 9) goblet cells and conjunctival epithe-







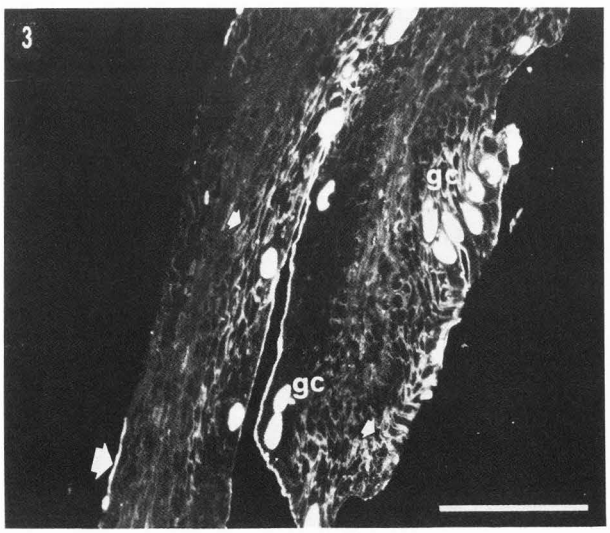
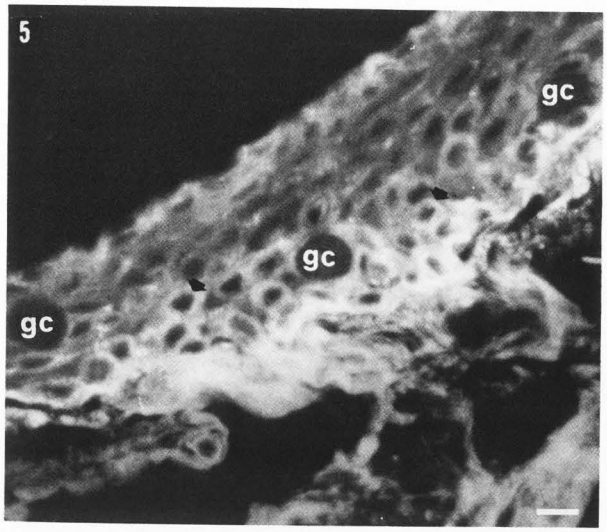


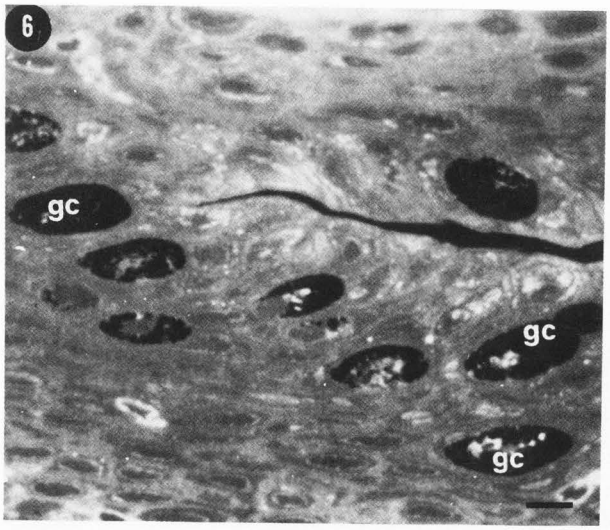
Fig. 1. Thick section of group C incubated with FITC-WGA. The lectin labels diffusely goblet cells (gc), the epithelial surface also shows a layer of fluorescence (arrows) as well as the intercellular borders of epithelial cells (small arrows). Corion is also labelled.
LM Bar = 100 µm

Fig. 2. Semithin section of group B incubated with FITC-WGA. The lectin binding pattern is similar to that shown in Fig. 1. Fluorescence is less intense, but better defined than in Fig. 1. The epithelial cell borders are well outlined (arrows). LM Bar = 100 µm

Fig. 3. Semithin section of group A incubated with FITC-WGA. No difference in fluorescence is detectable with respect to Fig. 2. The large arrow indicates an artefactual fluorescence due to the retention of the dye in a space between collagen fibers and the gold layer, sputtered on the specimen for the previous SEM observation. (gc = goblet cells; arrows = intercellular borders of epithelial cells).
LM Bar = 100 µm

Fig. 4. Semithin section of group A incubated with FITC-WGA plus N-acetyl-glucosamine. Note the complete disappearance of the fluorescence in the specific binding sites.
LM Bar = 100 µm


5
gc
gc
gc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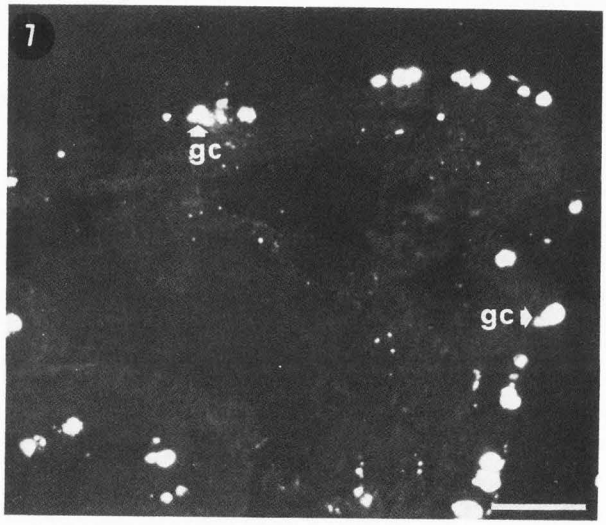
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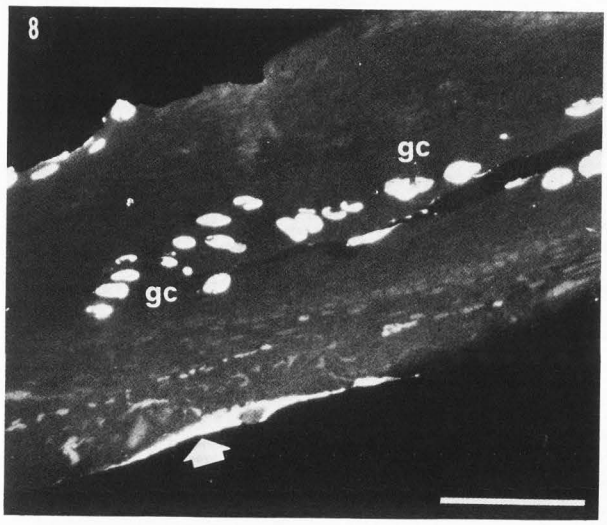
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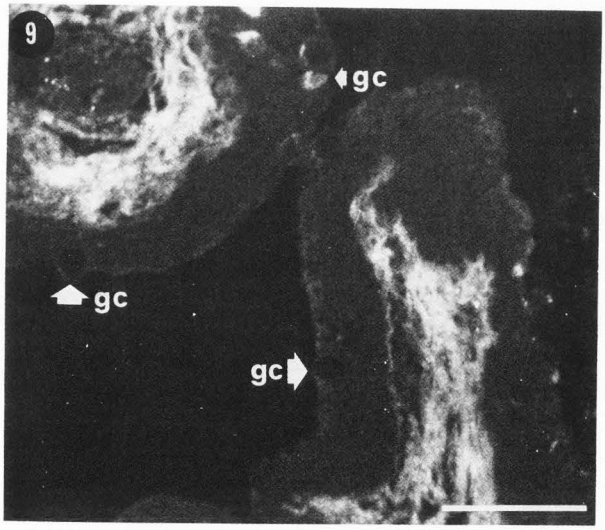
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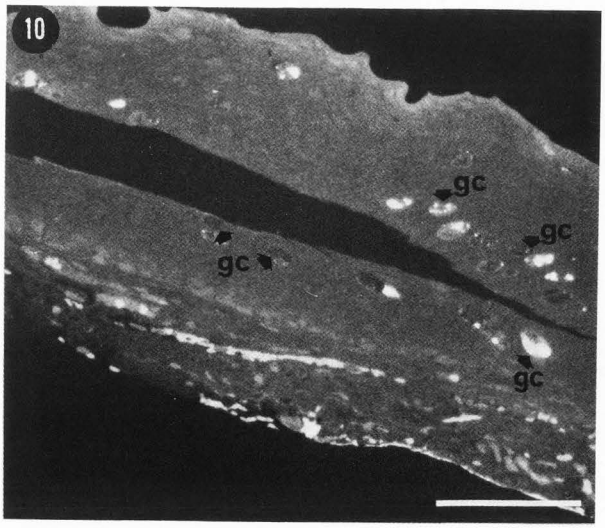
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gc
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Fig. 5. Thick section of group C incubated with FITC-ConA. Goblet cells (gc) are completely negative. Fluorescence is detected in the outer part of the cytoplasm of epithelial cells (arrows). LM Bar = 10 μm

Fig. 6. Semithin section of group B incubated with FITC-ConA. Small dots of fluorescence are seen inside goblet cells (gc). The outer part of cytoplasm in epithelial cells is labelled. Note the better definition as respect to Fig. 5. LM Bar = 10 μm

Fig. 7. Thick section of group C incubated with FITC-PNA. Goblet cells (gc) stain heavily with this lectin. Epithelial cells and corion are unlabeled. LM Bar = 100 μm

Fig. 8. Semithin section of group A incubated with FITC-PNA. The goblet cell (gc) fluorescence is less broad than in Fig. 7. The large arrow indicates an artefactual fluorescence. LM Bar = 100 μm

Fig. 9. Thick section of group C incubated with FITC-SBA. Goblet cells (gc) are completely negative (large arrows) or partially positive (small arrow) for SBA. Epithelial cells are negative, corion is positive for this lectin. LM Bar = 100 μm

Fig. 10. Semithin section of group A incubated with FITC-SBA. The lectin binding pattern is similar to that of Fig. 9 (gc = goblet cells). LM Bar = 100 μm

lial cells rarely stained with FITC-SBA. A few goblet cells showed traces of fluorescence (-/+). In the semithin sections of groups A and B (Fig. 10) goblet cells stained with variable intensity (+/++) but more than in group C. Conjunctival epithelial cells were always negative. Fluorescence disappeared in all the sections by addition of N-acetyl-galactosamine to the FITC-SBA incubation medium.

Colloidal gold-lectins on thick and semithin sections (gold-silver staining).

The lectin-binding pattern for the four lectins first observed at LM level is the same as we have described with the FITC-lectins technique (Fig. 11). No difference in the distribution of the glycosidic residues was found among the sections of the three groups. In addition, reactions performed on semithin sections showed enhanced sensitivity with respect to those applied to thick sections. This was found to be especially true for ConA and SBA.

Observations by SEM in the SE and BSE modes confirmed the glycosidic localization (Figs. 12, 13, 14, 15). Morphological details were enhanced due to increased resolution as compared to LM. In this regard, semithin sections both of groups A and B were found to be better than thick ones. Moreover, in semithin sections the signal seems to come exclusively from the reaction product whereas in thick sections a compositional signal can be observed. This fact is due to the irregular surface of a deparaffinized section. The gold particle diameters did not influence significantly the sensitivity of the reaction.

Colloidal gold-lectins on ultrathin sections.

We tested the lectins conjugated both with Au_5 and Au_{17}. We did not find significant differences in the intensity of the labelling due to gold sizes.

Au_5/Au_{17}-WGA. In Fig. 16 the very specific binding of Au_{17}-WGA on an ultrathin section of group B is shown. The mucus granules of a goblet cell are stained as well as the subsurface mucus vesicles belonging to the "second mucus system". In Fig. 17 an ultrathin section of group A incubated with Au_{17}-WGA is shown. The mucus granules of a young goblet cell and the "second mucus system" vesicles are marked by gold.

Au_5/Au_{17}-ConA and Au_5/Au_{17}-SBA. These two lectins (Figs. 18, 19) slightly labelled all the goblet cells observed in ultrathin sections of both groups A and B. As to SBA, not all the vesicles of the SMS were marked by gold granules. ConA always resulted negative in the SMS vesicles.

Au_5/Au_{17}-PNA. The binding of Au_{17}-PNA on an ultrathin section of group B is shown in Fig. 20. The mucus granules of the goblet cells were well marked also in the sections of group A. Not all the subsurface mucus vesicles of the SMS were marked by gold granules.

Addition of specific haptenic sugars to the incubation medium of the four lectins lead to a drastic reduction of the presence of gold granules (Fig.21).

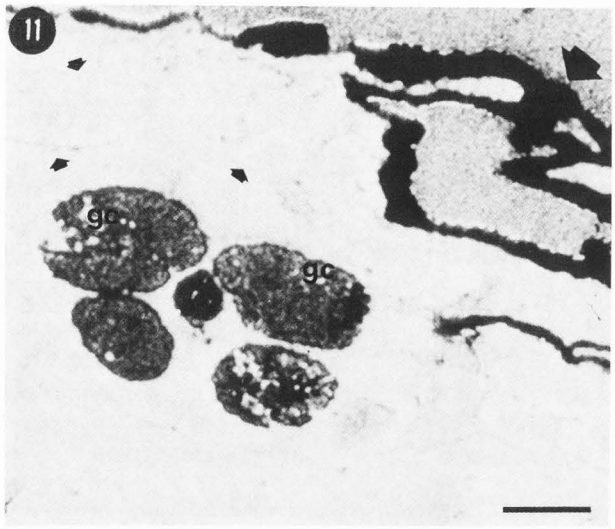


Fig. 11. Semithin section of group A incubated with Au_5-WGA (gold-silver staining). Goblet cells (gc) stain heavily with the lectin-gold-silver complex. The large arrow indicates the gold layer sputtered on the surface of the specimen for the previous SEM observation. The intercellular borders of epithelial cells are only slightly positive (small arrows).
LM Bar = 10 μm

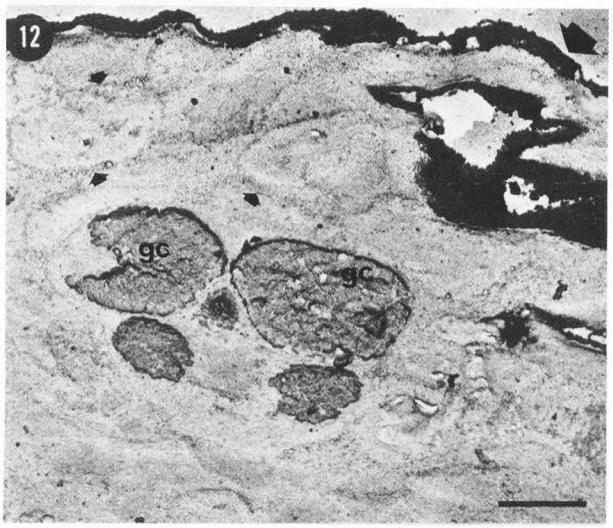
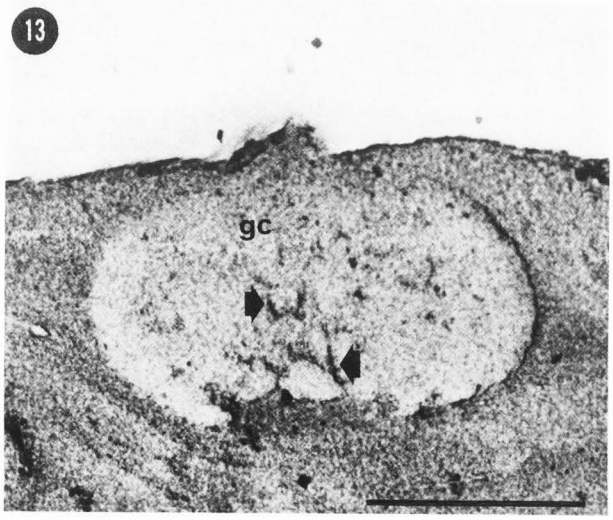


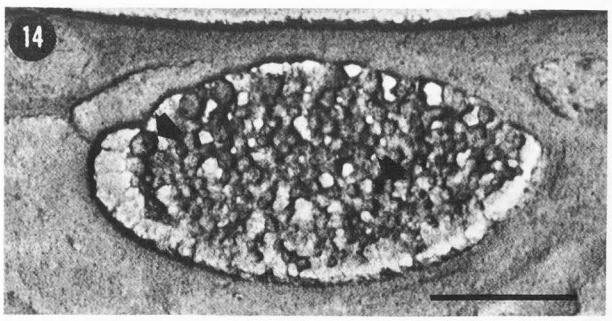
Fig. 12. Semithin section of group A incubated with Au_5-WGA (gold-silver staining). The same cells of Fig. 11 are shown, observed at SEM (SE mode at reverse polarity). Positiveness of reaction is seen in greater detail as respect to Fig. 11, at the level of the goblet cells (gc) and intercellular borders of epithelial cells (small arrows). (sputtered gold layer = large arrow). SEM SE(-) Bar = 10 μm

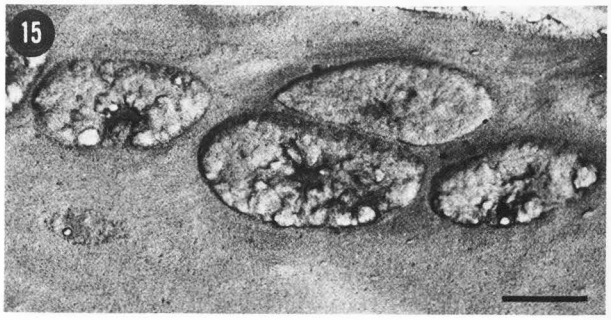
Fig. 13. Semithin section of group B incubated with Au_5-ConA (gold-silver staining). The arrows indicate small zones of the goblet cell (gc) positive for the reaction.
SEM SE(-) Bar = 10 μm

Fig. 14. Semithin section of group B incubated with Au_5-PNA (gold-silver staining). The mucus granules of the goblet cell (arrows) are heavily stained by the PNA-gold-silver complex.
SEM SE(-) Bar = 10 μm

Fig. 15. Semithin section of group B incubated with Au_5-SBA (gold-silver staining). The granules appear to contain a positive core.
SEM BSE(-) Bar = 10 μm







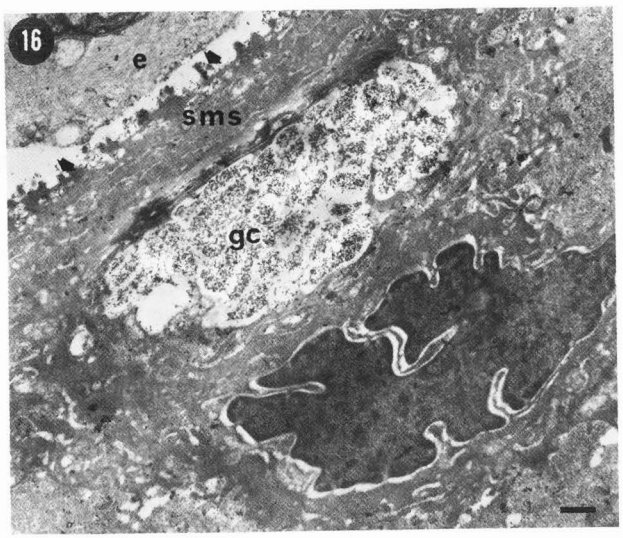


Fig. 16. Ultrathin section of group B incubated with Au_{17}-WGA. Note the extreme specificity of the deposition of gold particles on the goblet cell (gc), mucus vesicles of the SMS and also microvilli (arrows). (e = exfoliated cell). TEM Bar = 1 µm

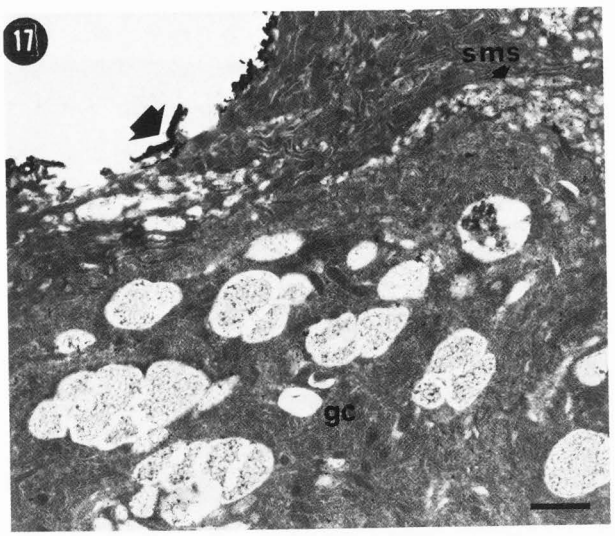


Fig. 17. Ultrathin section of group A incubated with Au_{17}-WGA. The gold particles are located very specifically on the mucus granules of a young goblet cell (gc) and on the vesicles of the SMS. The large arrow indicates the gold layer sputtered on the specimen surface for the previous observation in SEM.
TEM Bar = 1 µm

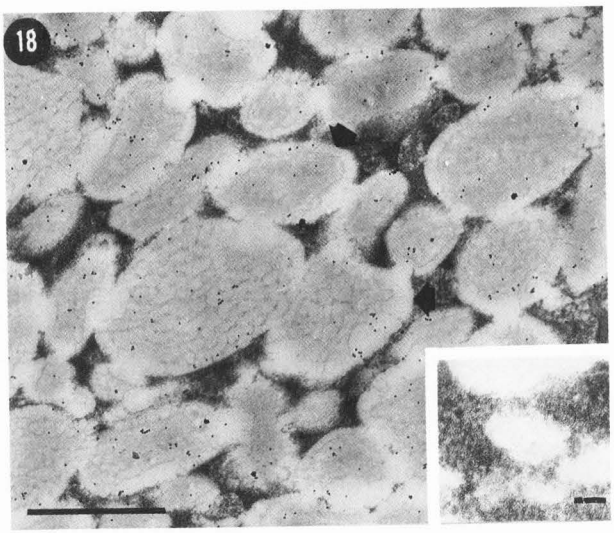


Fig. 18. Ultrathin section of group B incubated with Au_{17}-ConA. Few, but very specifically located, gold particles are seen in the granules of a goblet cell (arrows). Inset: SMS vesicles negative for this lectin.
TEM Bar = 1 µm Inset: Bar = 0.1 µm

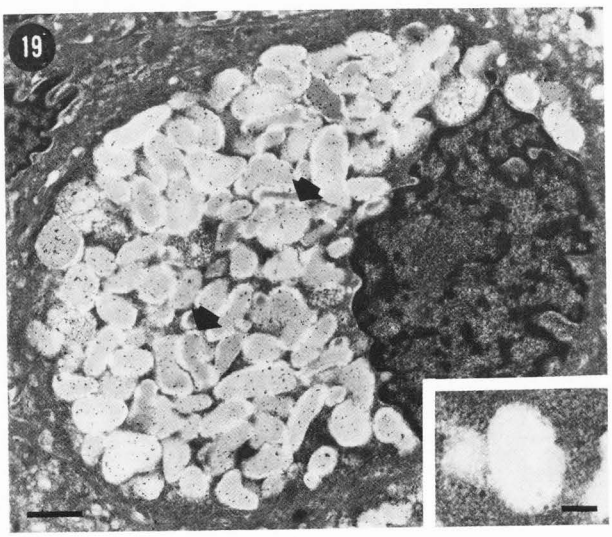


Fig. 19. Ultrathin section of group B incubated with Au_{17}-SBA. A whole goblet cell labelled by SBA is shown. Gold particles are very specifically located on the mucus granules (arrows). Inset: SMS vesicles negative for this lectin.
TEM Bar = 1 µm Inset: Bar = 0.1 µm

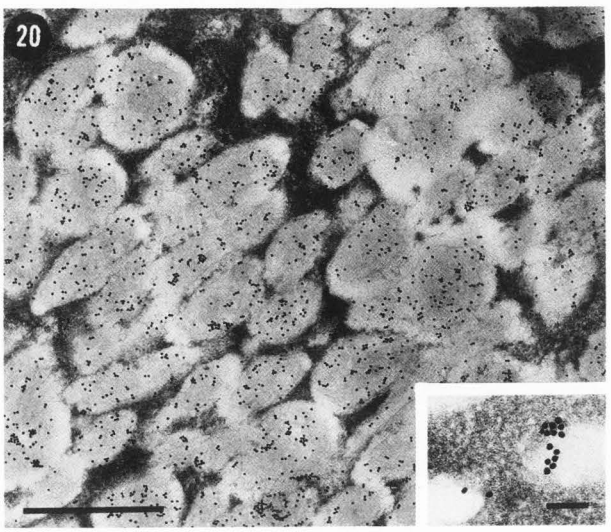


Fig. 20. Ultrathin section of group B incubated with Au_{17}-PNA. The mucus granules of a goblet cell are labelled diffusely with PNA. The gold particles are specifically located.
Inset: SMS vesicles positive for PNA.
TEM Bar = 1 μm Inset: Bar = 0.1 μm

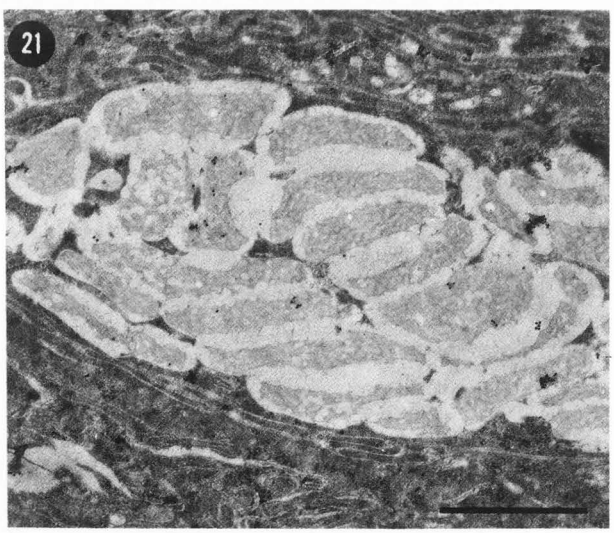


Fig. 21. Ultrathin section of group B incubated with Au_{17}-WGA plus N-acetyl-galactosamine. Note the good inhibition of specific reaction.
TEM Bar = 1 μm

Discussion

FITC-lectins on thick and semithin sections.

Recently, Kawano et al. (1984) used FITC conjugated lectins applied to frozen or paraffin-embedded conjunctival biopsies, at the LM level. They found that N-acetyl-glucosamine, galactose, N-acetyl-galactosamine and sialic acid are present in goblet cells, while fucose and mannose are absent. In our study, we obtained the same results with the four lectins we utilized, on the thick sections of group C. In particular, in goblet cells WGA and PNA appear always positive, ConA is always negative, SBA is usually negative, only sometimes slightly positive.

When applying the reactions to semithin sections, the distribution of the glycosidic residues was the same as found in thick sections, except for ConA and SBA. With these lectins, fluorescence was present in traces in some goblet cells. This fact could be explained as an increased sensitivity of the reaction due to a more suitable fixation and embedding of the material. The intensity of the fluorescence was evaluated on slides jointly by two (P.V.; MC.M.) observers; a decrease of intensity was observed in the different reactions performed on semithin sections as compared to the thick ones. This result is probably a consequence of the difference in section thickness. In fact, a fluorescent dye is present in greater quantity in a thicker section when compared to a thinner section. In this way, the intensity of fluorescence, observed at the same magnification, will be higher in a thick section than in a semithin section. On the other hand, semithin sections are more reproducible in thickness and give increased resolution. So, on the basis of our data, we can say that, when performing reactions with FITC-lectins, semithin sections are preferable for better resolution and sensitivity.

No difference was found between semithin sections of groups A and B. This demonstrates that the previous schedule applied for the observation in SEM of the group A specimens does not inhibit or reduce the lectin binding.

Colloidal gold-lectins on thick and semithin sections (gold-silver staining).

This technique gives the opportunity to observe the same thick or semithin section stained by cytochemical reaction both at LM and SEM levels. It overcomes the gap between LM and TEM because of the wide range of magnification at ones's disposal.

In our work, by this technique, a similar lectin binding pattern as in fluorescence micro-

scopy was found. Also in this case semithin sections make it possible to detect the reaction products not evidenced in thick sections. Sections from reprocessed specimens recovered from SEM gave the same results of those originally processed for TEM.

Observation in SEI and BEI modes of the deparaffinized thick sections allows a good three-dimensional view of the goblet cell granules, due to the presence of structures free from the embedding medium. However, the irregularity of the surface may cause an artefactual BEI signal, so providing a compositional image. On the contrary, the surface of the semithin sections is quite regular and so the BSE signal comes only from the silver deposited on the lectin-colloidal gold complexes.

Colloidal gold-lectins on ultrathin sections.

In their study, Kawano et al. (1984) did not show any evidence for lectin binding in the mucus vesicles of the non-goblet epithelial cells, because of LM resolution. The contribution of the SMS is important, even in normal conditions. For this reason, we have studied also at ultrastructural level the lectin binding, by applying colloidal gold conjugated lectins to ultrathin sections.

We tested both Au_5 and Au_{17}, but in this case gold particles of 17 nm size were preferred because we needed to take micrographs at relatively low magnification in order to obtain the image of the largest part of a goblet cell.

The glycosidic receptors for WGA and PNA are well evidenced by a large number of gold particles present in a very specific distribution at the level of goblet cell granules. ConA and SBA receptors are slightly present in all the goblet cells observed. The subsurface vesicles of the non-goblet epithelial cells are always positive for WGA; they are negative and only in a few cases are they positive for SBA and PNA; they are always negative for ConA. We obtained the same results applying the cytochemical reactions on ultrathin sections both of groups A and B.

These results state that the glycoproteins secreted by goblet cells are different from those derived from non-goblet epithelial cells. This could be expected by the fact that epithelial cells are not specifically devoted to secreting mucus and thus, probably, do not have the same enzymes of goblet cells. The consequence is a different contribution from these two sources to the glycosidic content of mucus layer in tear film as Srinivasan et al. (1977) also concluded by using more conventional histochemistry. In some pathological conditions, such as Giant Papillary Conjunctivitis due to contact lens wear (Greiner et al., 1980; Greiner and Allansmith, 1981) and vernal conjunctivitis (Takakusaki, 1969), it was reported an increase in the number of the vesicles of the SMS. The surface morphological appearance of this fact, evidenced by SEM, was described to be an aggregation of microvilli in clusters cemented by mucus. We demonstrated in a previous paper (Versura et al., 1985) that an enhanced activity of the SMS in mucus secretion exists also in adenovirus follicular conjunctivitis and hay fever conjunctivitis. In these diseases an alteration of the physical properties of tear film may be objectively observed. Wright and Mackie (1977) in a biochemical study suggested that in keratoconjunctivitis sicca a change in glycoprotein content of mucus does occur. On the basis of our data, we can suggest that in all these conditions mucus layer can change its biophysical characteristics due to the SMS contribution.

Conclusions

The normal character of tear film is due to both the structural integrity of the mucus layer and to the normal presence and distribution of microvilli of the conjunctival epithelial cells. In this report, we have demonstrated that it is now possible to get correlative information of these two parameters from the same specimen. In fact, the sensitivity of lectin binding was unchanged between the sections from the blocks processed for TEM and those from the blocks recovered from SEM and reprocessed for TEM.

The possibility of applying cytochemical reactions at different levels on serial sections, gives the opportunity to better characterize the glycosidic content of a cell. FITC-lectin staining is a simple and rapid method which gives best results when applied to semithin sections even if the semiquantitative assignment of the intensity is based on subjective evaluation. Gold-silver staining is a method which links light microscopic to ultrastructural observations. It is easily performed, extremely sensitive and permits one to stock the glass slide without appreciable loss in staining intensity, in order to carry out retrospective studies. The best way to detect glycosidic residues is the application of colloidal gold conjugated lectin technique in TEM. In fact, only with this method the subsurface vesicles of the SMS can be observed. Morphological quantization of the specific glycoproteins can be performed by simply counting the number of gold particles in each square unit, so avoiding subjective evaluations.

In the present work we have characterized the glycoconjugates in the vesicles of the non-goblet epithelial cells. Moreover, we have demonstrated that a difference in mucus glycoprotein secretion exists between goblet cells and non-goblet epithelial cells. This fact may be particularly important when considering the mucus alteration in conjunctival disease in which an increase of the SMS activity occurs.

References

Bonvicini F, Versura P, Caruso F, Maltarello MC, Caramazza R, Laschi R (1983). Lectin receptors on human corneal epithelium: visualization by fluorescence microscopy and electron microscopy. Cornea 2, 237-241.

Bonvicini F, Gasbarrini G, Caruso F, Ercoli C, Maltarello MC, Zoli G, Laschi R (1984). Lectin receptors on small intestine epithelium. A histochemical study in normal subjects and coeliac patients. Ital J Gastroenterol 16, 70.

Danscher G, Nörgaard JOR (1983). Light microscopic visualization of colloidal gold on resin-embedded tissue. J Histochem Cytochem 31, 1394-1398.

Debray H, Decont D, Strecke RG, Spik G, Montreuil J (1981). Specificity of twelve lectins towards oligosaccharides and glycopeptides related to N-glycosylproteins. Eur J Biochem 117, 41-56.

Essner E, Schreiber J, Griewski P (1978). Localization of carbohydrate components in rat colon with fluoresceinated lectins. J Histochem Cytochem 26, 452-458.

Freeman HJ, Lotan R, Young SK (1980). Application of lectins for detection of goblet cell glycoconjugate differences in proximal and distal colon of the rat. Lab Invest 42, 405-412.

Frens G (1973). Controlled nucleation for the regulation of the particle size in monodisperse gold suspension. Nat Phys Sci 241, 20-22.

Goldstein IJ, Hayes CE (1978). The lectins: carbohydrate binding proteins of plants and animals. Adv Carbohydr Chem Biochem 35, 127-158.

Gorelick FS, Sarras MP, Jamieson JD (1982). Regional differences in lectin binding to colonic epithelium by fluorescent and electron microscopy. J Histochem Cytochem 30, 1097-1108.

Greiner JV, Allansmith MR (1981). Effect of contact lens wear on the conjunctival mucus system. Ophthalmology 88, 821-832.

Greiner JV, Henriquez AS, Weidman TA, Covington HI, Allansmith MR (1979). "Second" mucus secretory system of the human conjunctiva. Arvo Abstracts Invest Ophthalmol Vis Sci Suppl. p. 123a.

Greiner JV, Kenyon KR, Henriquez AS, Korb DR, Weidman TA, Allansmith MR (1980). Mucus secretory vesicles in conjunctival epithelial cells of wearers of contact lenses. Arch Ophthalmol 98, 1843-1846.

Greiner JV, Weidman TA, Korb DR, Allansmith MR (1985). Histochemical analysis of secretory vesicles in nongoblet conjunctival epithelial cells. Acta Ophthalmol (Kbh) 63, 89-92.

Horisberger M (1981). Colloidal gold: a cytochemical marker for light and fluorescent microscopy and for transmission and scanning electron microscopy. Scanning Electron Microsc.1981; II: 9-31.

Kawano K, Uehara F, Sameshima M, Ohba N (1984). Application of lectins for detection of goblet cell carbohydrates of the human conjunctiva. Exp Eye Res 38, 439-447.

Kornfeld S, Kornfeld R (1978). Use of lectins in the study of mammalian glycoproteins. In: The Glycoconjugates, Horwitz MJ, Pigman W (eds.), Academic Press, NY, 437-459.

Lemp MA, Holly FJ, Iwata S (1970). The precorneal tear film: I. Factors in spreading and maintaining a continuous tear film over the corneal surface. Arch Ophthalmol 83, 89-94.

Lis H, Sharon N (1981). Lectins in higher plants. In: The biochemistry of plants, Marcus A (ed.), Academic Press, NY, 371-419.

Matsumoto K, Mimura Y (1974). Histochemical studies on the conjunctival goblet cells. Conn Tissue 6, 85-90.

Pasquinelli G, Scala C, Martegani F, Laschi R (1985). A new approach for studying semithin sections of human pathological material: intermicroscopic correlation between light microscopy (LM) and scanning electron microscopy (SEM). Scanning Electron Microsc.1985; III: 1133-1142.

Roth J (1978). The lectins. Molecular probes in cell biology and membrane research. Exp Pathol 3, Suppl, 1-12.

Spicer SS, Meyer DB (1960). Histochemical differentiation of acid mucopolysaccharides by means of combined Aldehyde-Fuchsin-Alcian Blue staining. Am J Clin Pathol 33, 453-460.

Srinivasan BJ, Worgul BV, Iwamoto T, Merriam GR (1977). The conjunctival epithelium: histochemical and ultrastructural studies on human and rat conjunctiva. Opthalm Res 9, 65-79.

Takakusaki I (1969). Fine structure of the human palpebral conjunctiva with special reference to pathological changes in vernal conjunctivitis. Arch Histol JPN 30, 247-282.

Thomopoulos GN, Schulte BA, Spicer SS (1983). The influence of embedding media and fixation on the post-embedment ultrastructural demonstration of complex carbohydrate. I Morphology and periodic acid-thiocarbohydrazide-silver proteinate. Staining of vicinal diols; Histochem J 15, 763-783.

Versura P, Bonvicini F, Caramazza R, Laschi R (1985). Scanning electron microscopy study of human cornea and conjunctiva in normal and various pathological conditions. Scanning Electron Microsc.1985; IV: 1695-1708.

Wright P, Mackie IA (1977). Mucus in healthy and diseased eye. Trans Ophthalmol Soc UK 97, 1-7.

Discussion with Reviewers

R.C. Tripathi: Incubation with specific inhibitor sugar is a control for the specificity of the FITC lectin binding. What control did the authors use for non-specific background staining?
Authors: We treated the sections with 1% periodic acid for 20 minutes before labelling, as suggested by Roth J (1983). Application of lectin-gold complexes for electron microscopic localization of glycoconjugates on thin sections. J Histochem Cytochem 31, 987-999.