Histochemical analysis of glycoconjugates in the eccrine glands of the raccoon digital pads

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The distribution and selectivity of complex carbohydrates in the eccrine glands of the digital pads in the North American raccoon (*Procyon lotor*) were studied using light and electron microscopic histochemical methods, particularly lectin histochemistry. In the eccrine glands, the dark cells exhibited neutral and acidic glycoconjugates with different saccharide residues (α-L-fucose, β-D-galactose, β-N-acetyl-D-glucosamine and N-acetyl-neuraminic acid); the clear cells contained numerous glycogen particles and showed a distinct reaction of α-L-fucose. The presence of complex carbohydrates with various terminal sugars was evident in the excretory duct cells. In addition, β-D-galactose and N-acetylneuraminic acid residues were mainly observed in the luminal secretion. The glycoconjugates produced by the eccrine glands of the raccoon digital pads may protect the epidermis against physical damage or microbial contamination. In this way, the normal functioning of the sensory apparatus of the foot pads is ensured.

Key words: eccrine glands, digital pads, glycoconjugates, histochemistry, raccoon, *Procyon lotor*.

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Paper accepted on January 26, 2004

European Journal of Histochemistry
2004; vol. 48 issue 4 (Oct-Dec):393-402

In certain mammals, humans excepted, apocrine glands are primarily observed in the hairy skin, but eccrine glands are confined to specific body regions, as, for example, the foot pads of carnivores, the frog of ungulates, the carpus of pigs, and the nasolabial region of bovines and pigs (Ellis 1968; Calhoun and Stinson 1976; Hashimoto *et al.* 1986). The foot pads possess a remarkable sensory apparatus, and may, additionally, be connected with the improvement of adhesion to the ground during walking as based on the biological functions of their eccrine glands (Meyer and Bartels 1989; Halata 1993; Meyer and Tsukise 1995).

In the cat, dog and wolf, the secretory products of the eccrine glands in the foot pads exhibit high amounts of glycoconjugates, which may be of importance for territorial scent-marking activities and tracking abilities of Canidae, and home orientation, as, for example, of kittens in Felidae (Meyer and Bartels 1989; Meyer *et al.* 1990; Meyer and Tsukise 1995).

Within the Carnivora, the raccoon has been demonstrated to be a valuable model for the study of the somatosensory system, because the primary somatosensory cortex of this species has an evident array of gyri correlating with the glabrous pads (Welker and Seidenstein 1959; Rasmusson 1982; Rasmusson *et al.* 1991). In this connection, several studies have emphasized the specific sensory innervation of the forepaws (Hoggan 1884; Zollman and Winkelmann 1962; Munger *et al.* 1971; Munger and Pubols 1972; Rice and Rasmusson 2000). In the present study, the localization of complex carbohydrates in the eccrine glands of the digital pads of the North American raccoon (*Procyon lotor*) was examined using various light and electron microscopic histochemical methods including lectin staining procedures. Thus, the biologically relevant characteristics of these eccrine glands and the influence of their products on the milieu of the skin surface may be better understood. Such knowledge may also

Table 1. The lectins used and their specific sugar residues.

Lectins		*Sugar binding specificity*	*Inhibitory sugar*
Con A	Concanavalin A	α-D-Man, α-D-Glc	Man, α-methyl-D-glucoside
UEA-I	*Ulex europaeus* agglutinin-I	α-L-Fuc	α-L-Fuc
DBA	*Dolichos biflorus* agglutinin	GalNAcα1-3GalNAc	α-D-GalNAc
RCA-120	*Ricinus communis* agglutinin-120	Galβ1-4GlcNAc	β-D-Gal
PNA	Peanut agglutinin	Galβ1-3GalNAc	β-D-Gal, α-lactose
WGA	Wheat germ agglutinin	β-D-GlcNAc	β-D-GlcNAc
SSA	*Sambucus sieboldiana* agglutinin	Siaα2-6Gal/GalNAc	Sia, sialyllactose
MAM	*Maackia amurensis* agglutinin	Siaα2-3Gal1-4GlcNAc	Sia, sialyllactose

help to better evaluate the normal functioning of the raccoon digital pads as a sensory organ.

Materials and Methods

Experiments were performed under the protocols following the guidelines for the care and use of laboratory animals at the Institute of Experimental Animal Science, College of Bioresource Sciences, Nihon University. Two adult male North American raccoons *(Procyon lotor)* (weight: 5-6 kg), which were carried from the animal care center, were deeply anesthetized with Veterinary Ketalar 50 (ketamine hydrochloride; Sankyo Yell Yakuhin Co. Ltd., Japan) and then exsanguinated from the common carotid arteries. After bloodletting, skin samples were taken from the digital pads of the forelimb.

General histology and cytology

For general structural observation by light microscopy, the tissue specimens were fixed in Bouin's solution for 4 h at room temperature, then washed and dehydrated in a graded series of ethanol. The specimens were then embedded in paraffin wax and cut at a thickness of 5 μm. The paraffin sections were stained with hematoxylin and eosin (H-E) for histological purposes.

For general structural observation by electron microscopy, the skin pieces were fixed in a mixture of 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) for 2 h at 4 ˚C. These specimens were postfixed in a 2% osmium tetroxide solution for 2 h. After this double fixation, the tissues were embedded in Epon 812, according to routine techniques (Luft 1961). From these tissue blocks, ultrathin sections were cut on an ultramicrotome (MT-1, Porter-Blum, Sorvall Inc., USA), mounted on copper grids and stained with uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963).

Histochemistry and cytochemistry

For histochemical studies, skin samples were fixed, washed and dehydrated in the same way as histological procedure. The materials were embedded in paraffin wax and cut at a thickness of 5 μm. The sections were stained with various procedures for the histochemical demonstration of glycoconjugates including combined control procedures. In general, the tissue processing as described above seems to be quite appropriate for the lectin binding to tissue sections (Rittman and Mackenzie 1983; Allison 1987; Alroy *et al.* 1988).

The histochemical staining methods employed were periodic acid-Schiff (PAS) (Spicer *et al.* 1967), AB (pH 1.0) (Lev and Spicer 1964), AB (pH 2.5) (Pearse 1968), sensitized high iron diamine procedure (S-HID), sensitized low iron diamine procedure (S-LID) (Hirabayashi 1992), periodic acid-thiocarbohydrazide-silver proteinate-physical development procedure (PA-TCH-SP-PD) (Nakamura *et al.* 1985; Kitamura *et al.* 1988; Yamada 1993). Additionally, sections were treated with different biotinylated lectins (Honen Co. Ltd., Japan) in concentrations of 10-20 μg/mL in 0.05 M PBS (pH 7.2) for 90 min at room temperature, following treatment with Biotin Blocking System (DakoCytomation Co. Ltd., Denmark) for inactivation of endogenous biotin. The lectins used, their specific sugar residues and inhibitory sugars are listed in Table 1 (for lectin specificities see Pearse 1985; Alroy *et al.* 1988; Spicer and Schulte 1992; Danguy 1995).

Afterwards, the sections were soaked with peroxidase-labeled streptavidin (SAB-PO) (Nichirei Co. Ltd., Japan) for 30 min at room temperature. The activity of the peroxidase employed for labeling was determined by a diaminobenzidine (DAB)-hydrogen peroxide system.

Lectin controls were performed by the addition of inhibitory sugars at a final concentration of 0.01 M

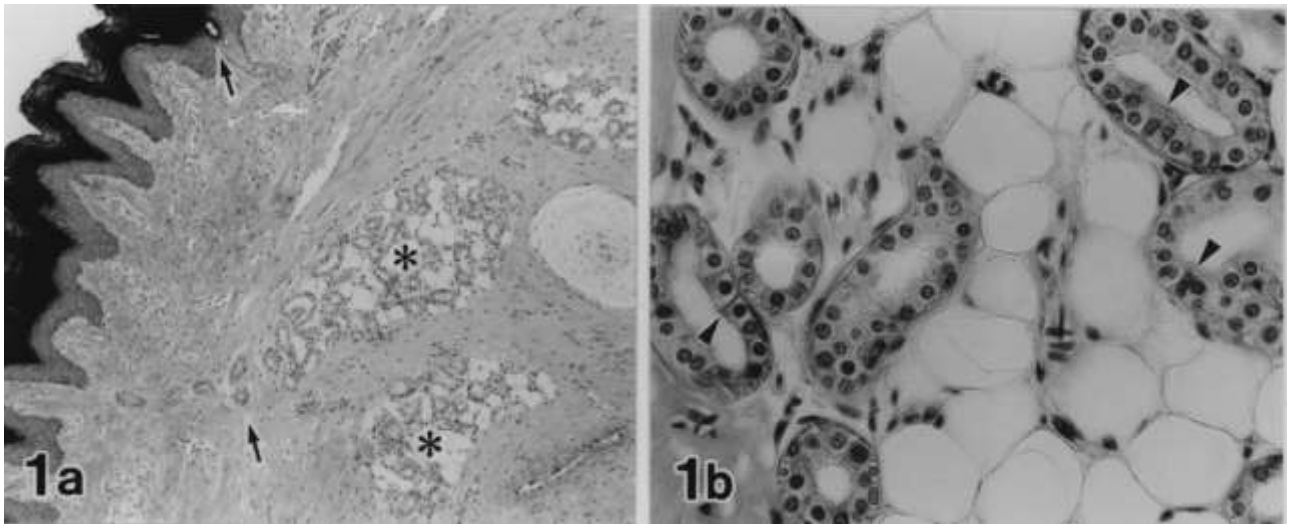

Figure 1. General structure of the digital pads of the raccoon. a) H-E, ×50; b) Higher magnification of the eccrine glands. ×400; arrows: excretory ducts, arrowheads: dark cells, asterisks: secretory portion.

to the respective lectin solutions, by the substitution of unconjugated lectins for biotinylated lectins, and by the exposure of sections to the SAB-PO-DAB system without lectin. To detect endogenous peroxidase activity in the tissue, the reactions of control sections were examined after the application of DAB only. Enzyme digestion with α-amylase (from *Bacillus subtilis*, Seikagaku Kogyo Co., Japan; 1 mg/mL in 0.1 M PBS, pH 6.0, at 37 ˚C for 4 h) (Casselman 1959) was conducted on some sections, prior to the PAS and PA-TCH-SP-PD techniques, and treatment with sialidase (from *Arthrobacter ureafaciens*, Nacalai Tesque, Inc., Japan; 1.0 U/mL in 0.1 M PBS, pH 5.3, at 37°C for 18 h) (Spicer *et al.* 1967) was used before S-LID staining. In the lectin controls, enzyme digestion with sialidase after saponification (0.5% potassium hydroxide-70% ethanol) (Spicer 1960) was performed before the SSA and MAM procedures. For the cytochemical demonstration of complex carbohydrates, the specimens were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde solution in 0.1 M PBS (pH 7.4) for 2 h at 4°C, and embedded in LR-White resin (Newman *et al.* 1983). Ultrathin sections from the LR-White-embedded materials were cut as stated above and placed on nylon grids. The sections on the grids were reacted for PA-TCH-SP-PD (Yamada 1993), followed by counterstaining with uranyl acetate and lead citrate. For the cytochemical identification of glycogen in the cytoplasm, the same ultrathin sections were treated with α-amylase (0.5 mg/mL in 0.1 M PBS, pH 6.0, at 37°C for 30 min) before the PA-TCH-SP-PD procedure. For these enzyme digestion procedures, two types of controls were used; some sections were incubated in buffer solution without the enzymes, and others were kept intact without any incubation procedures.

Results

General histology and cytology

In the skin of the digital pads of the North American raccoon *(Procyon lotor)*, three layers (epidermis, dermis and hypodermis) could be well-differentiated in tissue sections stained histologically with H-E. Eccrine glands were visualized in the connective tissue of the dermis and the hypodermis (Figures 1a, b).

The electron microscopy of the eccrine glands in the raccoon digital pads revealed that the secretory portion was a coiled tubule, consisting of two types of secretory cells with associated myoepithelial cells (Figure 2a). The dark cells were usually located at the luminal side of the glandular acini, and the clear cells at the basal side. Nevertheless, clear cells could also often be observed at the luminal surface of the secretory epithelium. The plasma membrane of the dark cells facing the glandular lumen partially projected into microvilli. In the supranuclear cytoplasm of the dark cells, a well-developed Golgi apparatus and granular endoplasmic reticulum were detected. The supranuclear and apical cytoplasm of the dark

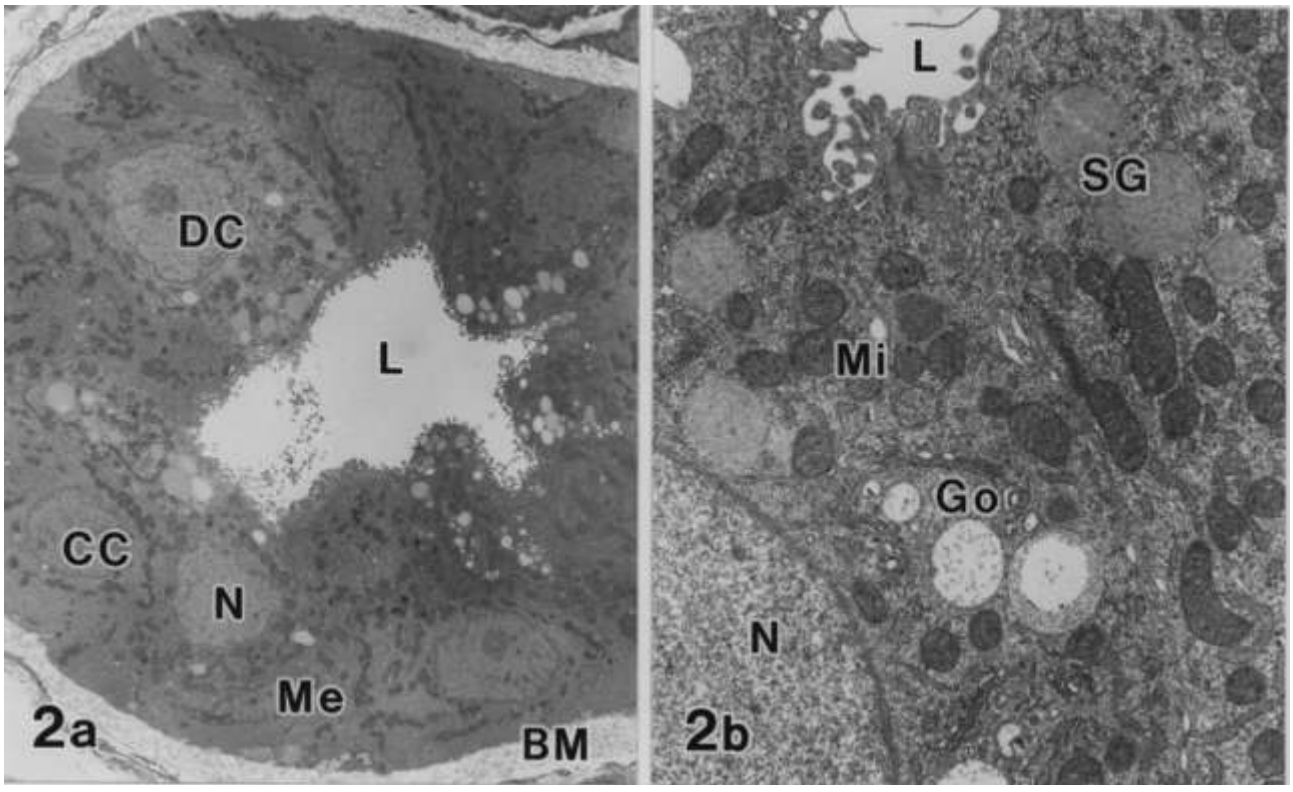

Figure 2. Ultrastructure of the eccrine glands in the digital pads stained with uranyl acetate and lead citrate. a) The eccrine glandular acini consist of two types of secretory cells. ×3,000. b) Part of the supranuclear and apical cytoplasm of the dark cells. ×16,000. BM: basal membrane, CC: clear cell, DC: dark cell, Go: Golgi apparatus, L: lumen, Me: myoepithelial cell, Mi: mitochondria, N: nucleus, SG: secretory granule.

cells contained varying numbers of secretory granules of medium density. In addition, several immature granules were presented near the dictyosomes of the Golgi apparatus (Figure 2b). In the cytoplasm of the clear cells, the Golgi apparatus and distinct amounts of glycogen particles were observed. Both cell types of the eccrine glands had one oval-shaped nucleus in a more or less central position.

Throughout the cytoplasm, mitochondria of varying morphology with long cristae were scattered among the above-mentioned ultrastructures.

Histochemistry and cytochemistry

The results obtained for complex carbohydrates in the eccrine glands of the raccoon digital pads are summarized in Table 2.

When the skin sections were subjected to the PAS procedure, the dark cells of the eccrine glands showed a strong to moderate positive reaction, whereas the clear cells exhibited a very weak positive reaction, and the reaction intensities of the excretory duct cells and luminal secretion were moderate to weak. The intensity of the PAS reaction was only slightly reduced after α -amylase digestion. It was evident that the reactions for AB (pH 1.0 and

Table 2. Light microscopic histochemical reactions in the eccrine glands of the digital pads in the raccoon.

Reactions	*Secretory cells*		*Excretory duct cell*	*Luminal secretion*
	Dark cells	*Clear cells*		
PAS	3-4	1	2	2-3
α-Amyl-PAS	3	0-1	1	2-3
AB (pH1.0)	2	0-1	1	1-2
AB (pH2.5)	3	1	1	1-2
S-HID	3-4	0-1	1-2	2-3
S-LID	4-5	2	2-3	3
Sial-S-LID	3-4	1	1-2	2
PA-TCH-SP-PD	5	3-4	3-4	4
α-Amyl-PA-TCH-SP-PD	4	1-2	3-4	4
Con A	3-4	3	3	4
UEA-I	4-5	4-5	4-5	3
DBA	3	3	3-4	2
RCA-120	4	2	3-4	3-4
PNA	4	1	3	2
WGA	4	2-3	3-4	3
SSA	4-5	0-1	2-3	3-4
MAM	4-5	1-3	3-4	3

Reaction intensities: 0: negative; 1: very weak; 2: weak; 3: moderate; 4: strong; 5: very strong. The reaction intensities are based on subjective observations.*

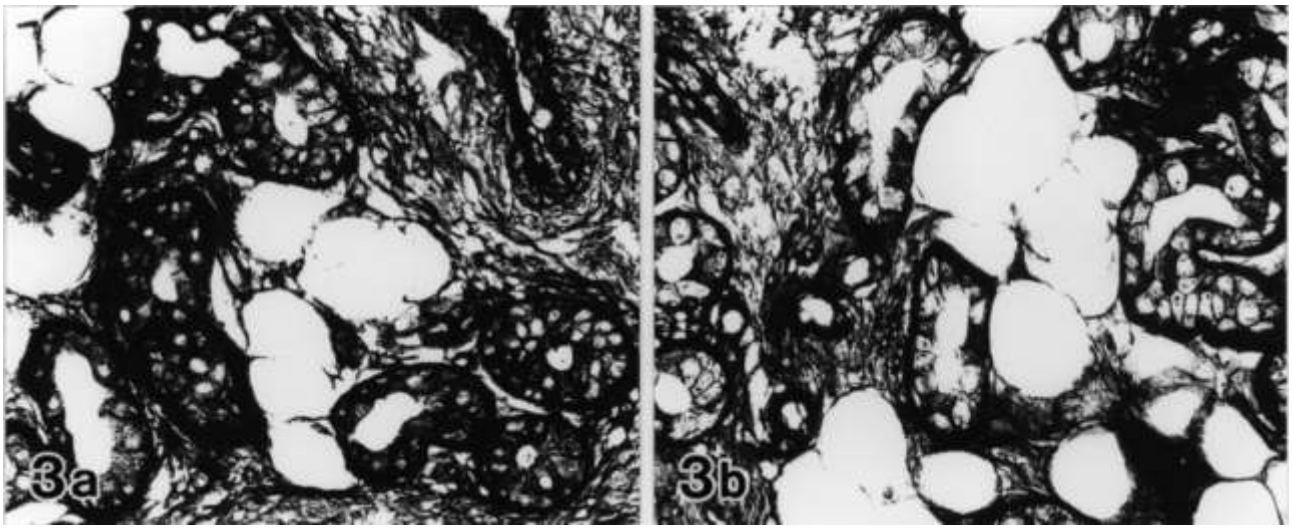

Figure 3. Carbohydrate histochemical appearance of the eccrine glands in the digital pads. a) PA-TCH-SP-PD, ×400; b) α-Amylase digestion followed by PA-TCH-SP-PD reaction. ×400.

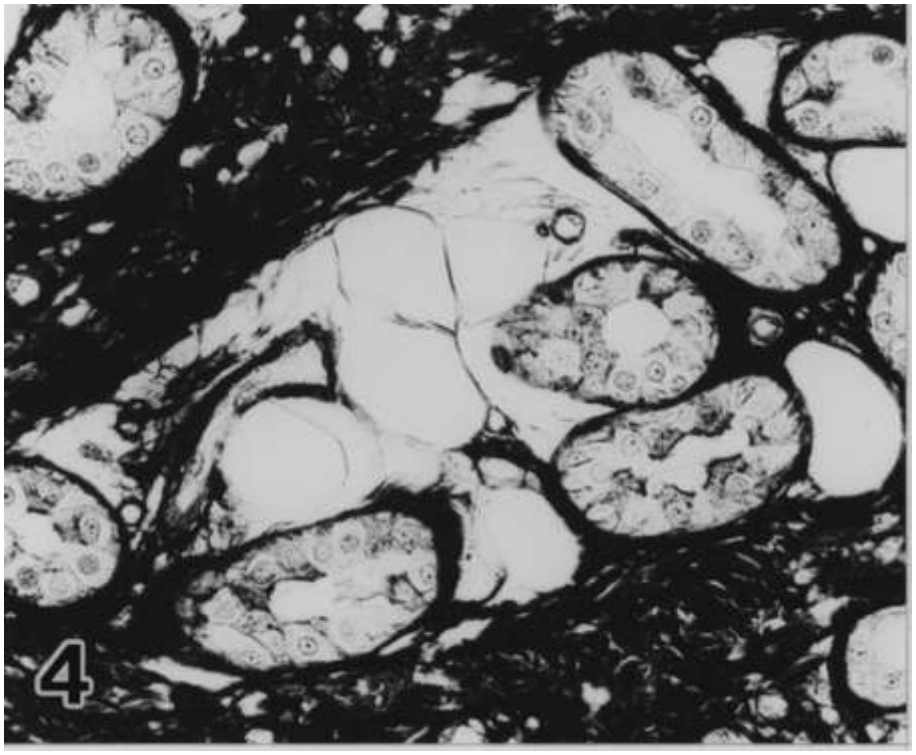

Figure 4. S-HID reaction of the eccrine glands in the digital pads. ×540.

2.5) were generally moderate to very weak in the eccrine glandular acini, excretory duct cells and luminal secretion. With regard to the PA-TCH-SP-PD procedure, the dark cells of the glandular acini reacted very strongly, and strong to moderate reactions could also be observed in the clear cells, the duct cells and the luminal secretion (Figure 3a). The digestion procedure with α-amylase failed to alter the PA-TCH-SP-PD staining intensity, except for the clear cells (Figure 3b). In tissue sections reacted for S-HID and S-LID, the dark cells were stained very strongly (S-LID) to moderately (S-HID), while the reaction intensities of the clear cells were rather weak compared with those of the dark cells (Figures 4, 5). Digestion with sialidase caused a weaker S-LID reaction in all parts of the glands.

When incubated with Con A, UEA-I and DBA, both cell types of the secretory portion were found to exhibit strong (UEA-I) or moderate (Con A, DBA) positive staining (Figures 6a, b). In contrast,

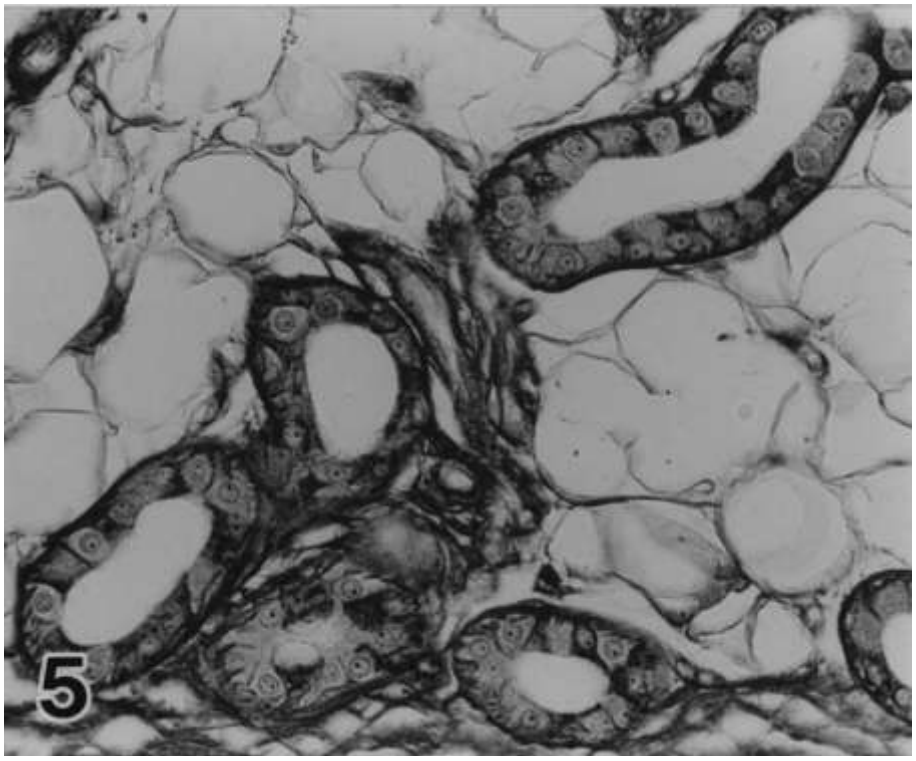

Figure 5. S-LID reaction of the eccrine glands in the digital pads. ×540.

the RCA-120 and PNA reactions showed distinct differences in intensity between the dark cells and the clear cells. The two above-mentioned lectin histochemical techniques resulted in strong reactions of the dark cells, and in weak (RCA-120) to very weak (PNA) reactions of the clear cells (Figure 6c). In the skin tissue sections stained with WGA, strong staining was confined to the dark cells, and the clear cells showed a moderate to weak positive reaction (Figure 6d). A distinct reaction intensity of the dark cells was obtained after the application of SSA, while the other type of secretory cells was stained very weakly (Figure 6e); the MAM staining pattern was similar to that one of WGA or SSA (Figure 6f). The reactions for all the biotinylated lectin procedures were generally moderate in the excretory duct cells, except for a strong UEA-I staining. Additionally, several lectins (DBA, PNA) showed weak reactions to the luminal secretion, whereas the other lectins produced moderate reactions.

In the control tissue sections stained with the biotinylated lectin-SAB-PO-DAB staining procedure (i.e. adding a particular monosaccharide to the respective biotinylated lectin solutions, substituting unconjugated lectins and exposing the sections without lectins), all the histologic structures in the eccrine glands were seen uniformly to exhibit negative reactions.

Throughout the tissue, endogenous PO activity could not be detected at all, except for the erythrocytes in the lumen of blood vessels. The SSA and MAM staining intensities were negative with sialidase digestion after saponification.

In the secretory cells stained with the PA-TCH-SP-PD procedure, a series of ultrastructures exhibited strongly or moderately positive reactions, such as secretory or immature granules in the dark cells and glycogen particles in the clear cells (Figure 7 a, b). Moreover, PA-TCH-SP-PD reactive ultrastructures, including certain elements of the Golgi complexes and the surface coat of the plasma membranes in both types of the secretory cells, showed positive reactions. Digestion with α-amylase abolished PA-TCH-SP-PD reactive glycogen particles in the cytoplasm of the clear cells.

The two control procedures for the above-mentioned enzyme digestions did not alter the reactive structures.

Discussion

In the digital pads of the North American raccoon *(Procyon lotor)*, the eccrine glands were observed as coiled tubules in the connective tissue of the dermis and the hypodermis. As in humans, this gland type consisted of two different cell types within the

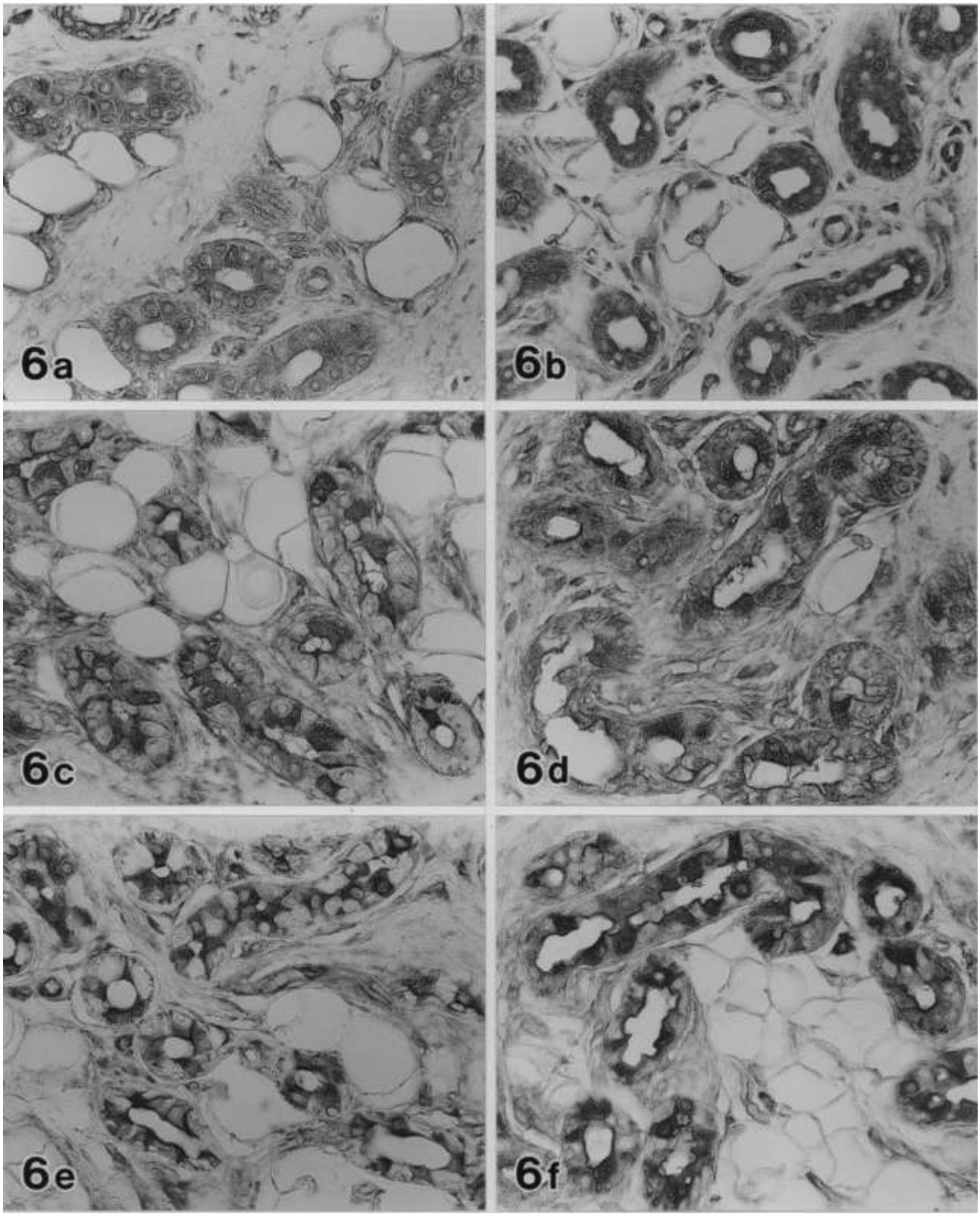

Figure 6. Lectin histochemical staining in the eccrine glands of the digital pads. a) Con A, ×400; b) UEA-I, ×400; c) RCA-120, ×400; d) WGA, ×400; e) SSA, ×400; f) MAM, ×400.

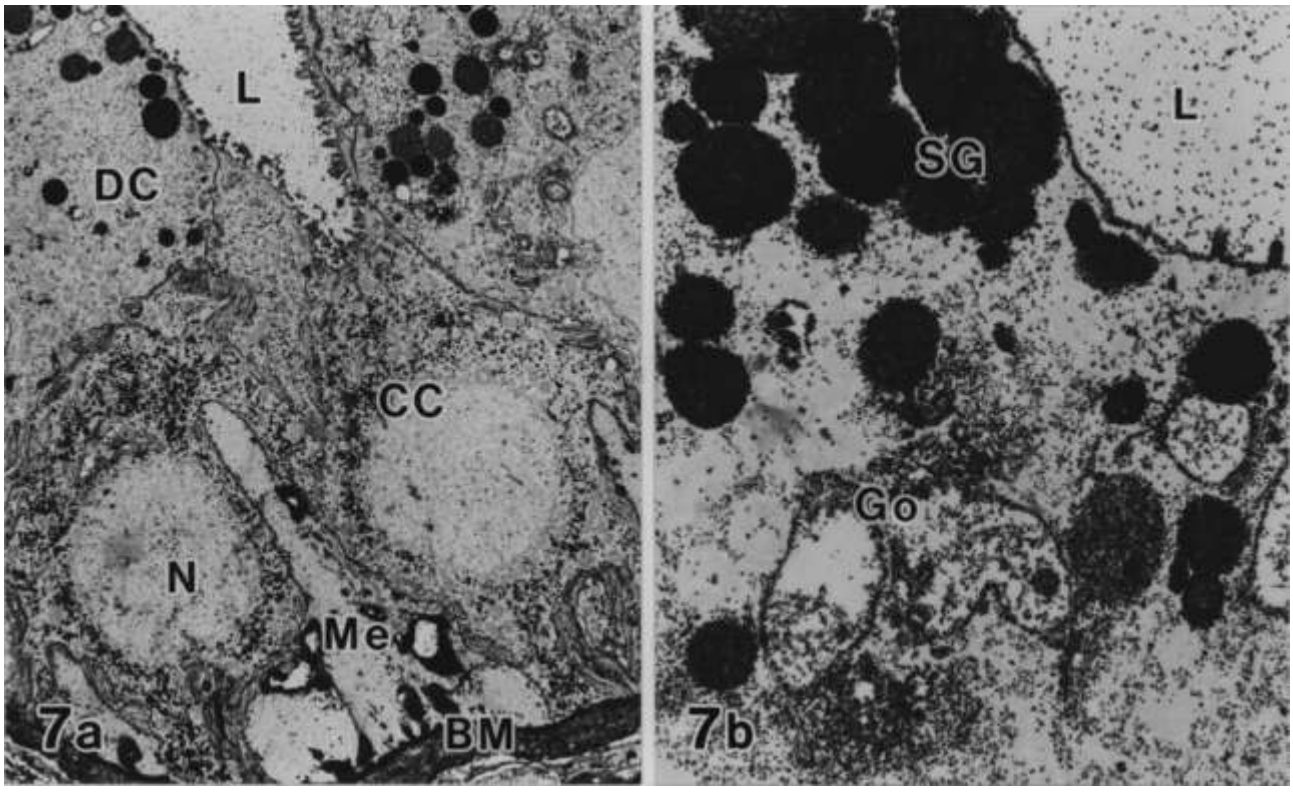

Figure 7. Cytochemical PA-TCH-SP-PD staining of the eccrine glandular acini. a) A series of ultrastructures exhibiting positive reactions. ×4,800. b) Positive reactions of secretory and immature granules are observed near the Golgi apparatus. ×16,000. BM: basal membrane, CC: clear cell, DC: dark cell, Go: Golgi apparatus, L: lumen, Me: myoepithelial cell, N: nucleus, SG: secretory granule.

secretory epithelium and peripheral myoepithelial cells. These cell types are called dark and clear cells, based upon their staining profiles (Ellis 1968; Montagna and Parakkal 1974). However, a distinct difference in their stainability could not be recognized in the raccoon digital pads after H-E staining. Ultrastructurally, the apical cytoplasm of the dark cells in humans contains a number of dense secretory granules and some lipid droplets. These dense secretory granules arising from the Golgi apparatus, are considered to include complex carbohydrates and/or glycoproteins (Ito and Shibasaki 1966; Ellis 1967, 1968; Kurosumi *et al.* 1982). In the raccoon, the secretory granules were of medium density, and lipid droplets could not be observed within the apical portion of the dark cells. While two different mechanisms, apocrine-like and exocytosis, have been proposed to account for the secretory process of the dark cells in humans (Ito and Shibasaki 1966), such mechanisms could not be verified by the results of the present study.

The histochemical techniques applied demonstrated reaction intensities for different glycoconjugates in the two cell types of the secretory epithelium, the luminal secretion, and in the excretory duct cells of the eccrine glands of the raccoon digital pads. The secretion produced indicated neutral glycoproteins, as supported by the general staining abilities of the PAS- as well as the PA-TCH-SP-PD procedures applied (Pearse 1985). In particular, the PA-TCH-SP-PD technique enhances the demonstration of neutral glycoproteins and glycogen more than the PAS procedure (Yamada 1993). Additionally, the dark cells contained acidic glycoproteins with sialyl residues and/or sulfate ester groups. These findings were corroborated by the results of the AB (pH 1.0 and 2.5), S-HID and S-LID techniques. The more sensitive biotinylated lectin procedure revealed a spectrum of particular saccharide residues in the glycoprotein masses found. According to previous carbohydrate histochemical studies on the similar gland type in the foot pads of the cat (Meyer and Bartels 1989), dog and wolf (Meyer and Tsukise 1995), it has been shown that glycoconjugates of the eccrine glandular acini are more neutral rather than acidic in nature. However, these glands in the

fore paws of the raccoon exhibited relatively stronger reactions of α-L-fucose residues and acidic glycoconjugates containing sialic acid than those of the mammals referred to above.

With regard to specific functions of the eccrine glands, our results demonstrated that in the raccoon they contained α-L-fucose, β-D-galactose, and β-N-acetyl-D-glucosamine, especially in the dark cells. All these terminal sugars are liberated on the skin surface by bacterial and fungal activities, and have the ability to inhibit the adherence of various bacteria and fungi to epidermal cells; thus, general antimicrobial properties are obvious (Meyer *et al.* 2000, 2001), which may be completed by several respective peptides (see e.g. Nizet *et al.* 2001; Meyer *et al.* 2003).

The intracellular inclusion of α-L-fucose, β-N-acetyl-D-galactosamine, β-D-galactose, β-N-acetyl-D-glucosamine or sialic acid residues indicated that mucin-type glycoproteins are stored in the secretory granules and secreted to protect epithelial surfaces (Strous and Dekker 1992; Sames *et al.* 1999). The more serous secretion of the basally located clear cells passes through narrow intercellular channels and may be mixed with the mucus dark cell products, similarly to secretion production in Gianuzzi's/von Ebner's demilunes of salivary glands (Sames *et al.* 1999). The existence of distinct amounts of glycogen in the clear cells may be closely related to high energy demands during secretion production (Meyer and Bartels 1989). The localization of α-L-fucose and sialyl residues is of particular interest within the terminal sugar group obtained by our results. Sialoglycoconjugates have been reported to be involved in physiologic processes in normal and transformed cells by functioning in intercellular recognition and protection (Mandal and Mandal 1990). Moreover, sialic acid, which has the ability to establish crosslinks between glycoprotein molecules (Guslandi 1981), seems to determine the regulatory factor of mucus viscosity with sulfate groups (Kasinathan *et al.* 1991; Slomiany *et al.* 1991; Majima *et al.* 1999). Additionally, both sialylation and sulfation may generally increase the resistance of mucus to bacterial degradation (Rhodes *et al.* 1985). Furthermore, the occurrence of α-L-fucose as a relatively hydrophobic sugar could indicate specific functional properties of the eccrine gland secretion. The role of fucosylated glycoconjugates may be especially significant in determining the coefficient of viscosity and elasticity of mucus (Majima *et al.* 1999), whereby fucose seems to provide the viscoelasticity of the eccrine gland secretion. The presence of hydrophobic sugars may also be involved in the fluidity and rheological properties in the excretory duct systems (Schulte *et al.* 1985; Meyer and Tsukise 1995).

It has to be emphasized, finally, that eccrine gland secretions of the digital pads in the raccoon may play an important role in the prevention of physical damage to the epidermis and the protection against bacterial invasion, so that the important specific sensory functions of the digital pads can be performed without any problems.

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