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Morphological re-evaluation of the parotoid glands of *Bufo ictericus* (Amphibia, Anura, Bufonidae)

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Abstract

Multicellular glands in the amphibian integument represent a significant evolutionary advance over those of fishes. Bufonids have parotoid glands, symmetrically disposed in a post-orbital position. Their secretion may contribute to protection against predators and parasites. This study provides a re-evaluation of the morphology of the *Bufo ictericus* parotoid glands. The parotoid gland integument of the medial surface shows rounded depressions with small pores that connect with the duct openings of the larger granular glands. Under light microscopic evaluation the integument is constituted by typical epidermis, supported by dermis subdivided into a spongious dermis, a reticular dermis, and a compact dermis. The Eberth-Katschenko layer is identified as a basophilic material scattered throughout the superficial spongious dermis. The parotoid gland is an integument region, in which three exocrine glandular types occur: mixed glands, smaller granular glands and larger granular glands. The mixed gland is formed by mucous and serous cells while the small granular glands contain a homogene acidophilic intake. The larger granular glands produce a basophilic and alcianophilic material, and are responsible for the macroscopic protuberances designed as parotoid glands. Thus, the end product released by the parotoid glands is a mix of secretions produced by the three glands.

Contents

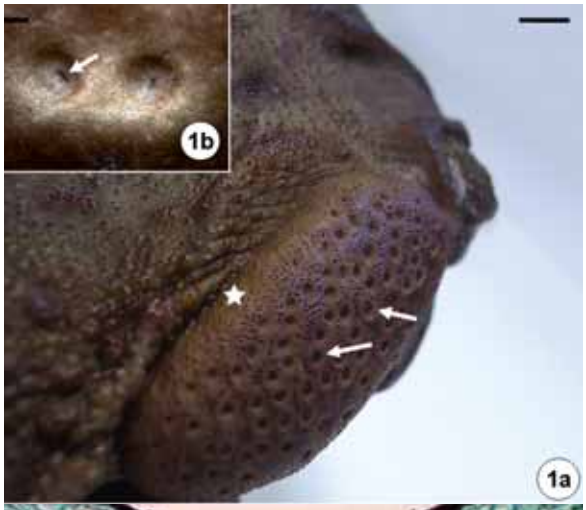
Introduction

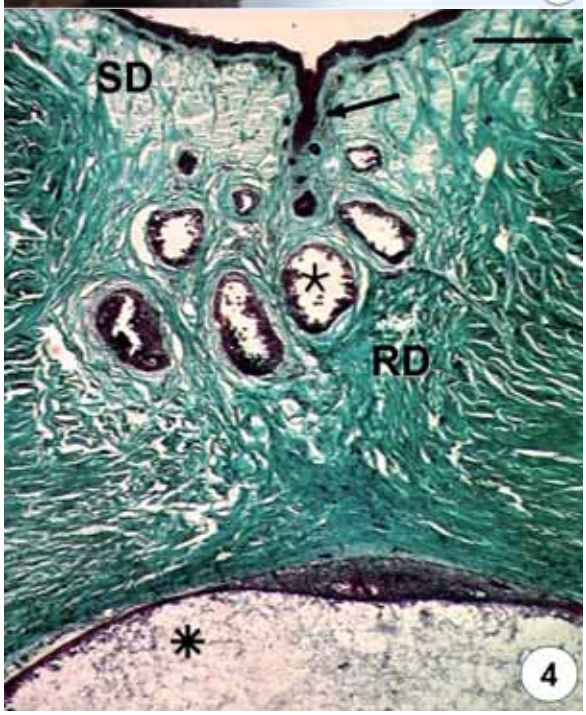
One of the main evolutionary advances of the amphibian integument over that of fishes is the presence of a large number of multicellular alveolar and, in some cases, tubular glands (Porter, 1972). The established histologic classification of exocrine glands is based on different criteria. According to the secretion mechanism, the exocrine gland which releases its secretory product by exocytosis, is classified as a merocrine gland, such as in the case of pancreatic secretion of zymogen granules. When the secretory mechanism involves partial loss of the apical portion of the cell, the gland is named apocrine. The lipid secretion by epithelial cells of the mammary gland is an example of this glandular type. In addition, if the end secretion is constituted by the entire cell and its secretory product, the exocrine gland is designated as an holocrine gland such as the sebaceous glands of the mammalian skin or the avian uropygial gland. Considering the secretory cell type, the merocrine gland is a mucous gland when its product is rich in glycoprotein, which is responsible for the cytoplasm basophilic staining. The merocrine serous gland shows acidophilic cytoplasm and its secretion is enriched with proteins. Thus, the mixed gland contains both mucous and serous cells (Kierszenbaum, 2002; de Brito-Gitirana, 2004).

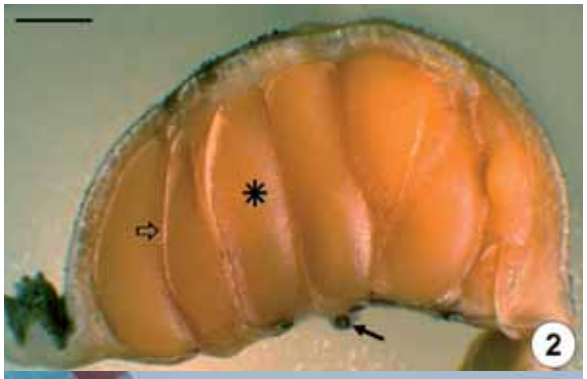
Cutaneous glands of all living amphibians usually belong to four main types located in the spongious dermis: mucous, serous (granular or poison), lipid (or wax), and mixed (seromucous) glands (Duellmann and Trueb, 1994; Brizzi *et al*., 2002).

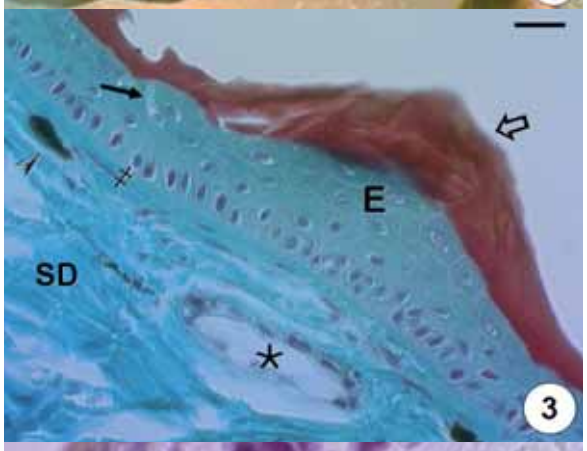
Mucous glands secrete a clear, watery to viscous substance. The mucous secretions keep the integument moist in terrestrial conditions and lubricate it when in water (Porter, 1972). Mucous secretions of amphibian integument glands can also have a bacteriostatic effect (Pough *et al*., 2003), and they possess the ability to mechanically retain microbial and fungal pathogens (Clarke, 1997; Fontana *et al*., 2006).

Granular glands produce a milky secretion that is much more toxic than that of the mucous, thereby providing protection against bacterial and fungal infection as well as defense against predators. In addition, there









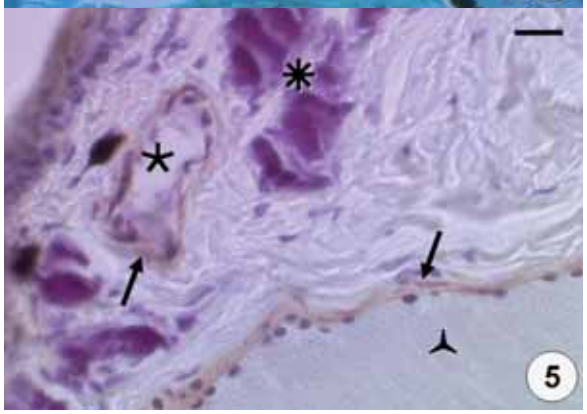
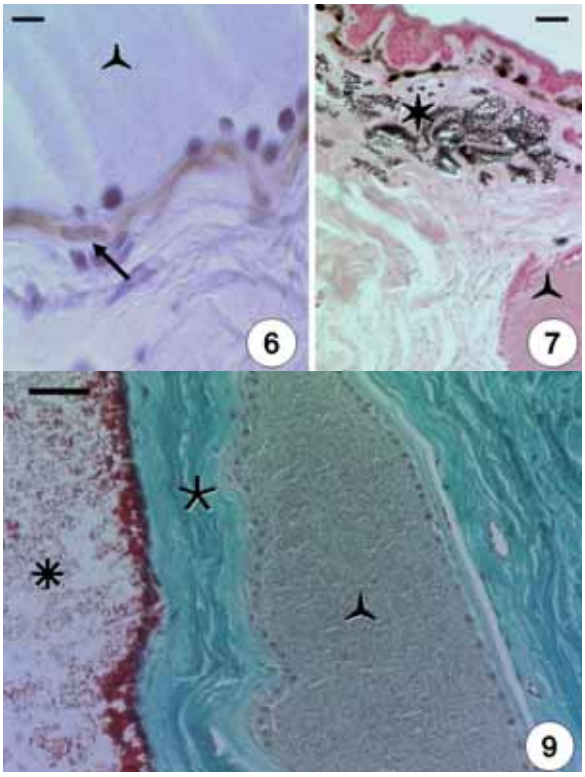


Fig. 1a. Macroscopic view of the cephalic region of *Bufo ictericus* showing one of the parotoid glands. Note the rounded depressions (→) predominate on the medial surface of the parotoid gland. These structures are absent on the lateral surface (★). Scale = 6 mm.
Fig. 1b. Stereoscopic micrograph showing the detail of two elongated glandular openings (→) located on the rounded depressions of the parotoid glands. Scale = 6 mm.
Fig. 2. Macroscopic view of transverse sections through parotoid gland. Note the larger granular glands (✱) forming the central portion of the parotoid gland. Connective septa (⇨) separate secretion portions of the larger granular gland. Note large blood vessels (→) in the hypodermis. Scale = 25 mm.
Fig. 3. Light micrograph of parotoid gland. Note the cornified tubercles (⇨) and a flask cell (➔). Beneath the epidermis (E), the subepidermic region (‡) is separated from the spongious dermis (SD) by pigment cells (➢). A mixed gland (✶) is visualized. Scale = 20 µm. Gomori´s trichrome stain.
Fig. 4. Light micrograph showing the mixed gland (✶) associated to the duct (→) of the larger granular gland (✱). SD = spongious dermis; RD = reticular dermis. Scale = 100 µm. Gomori´s trichrome stain.
Fig. 5. Myoepithelial cells (➔) around the secretory portion of mixed gland (✶) and smaller granular gland (➴). Note the Eberth-Kathschenko layer (✱). Scale = 100 µm. Immunolabeled for smooth muscle α actin.



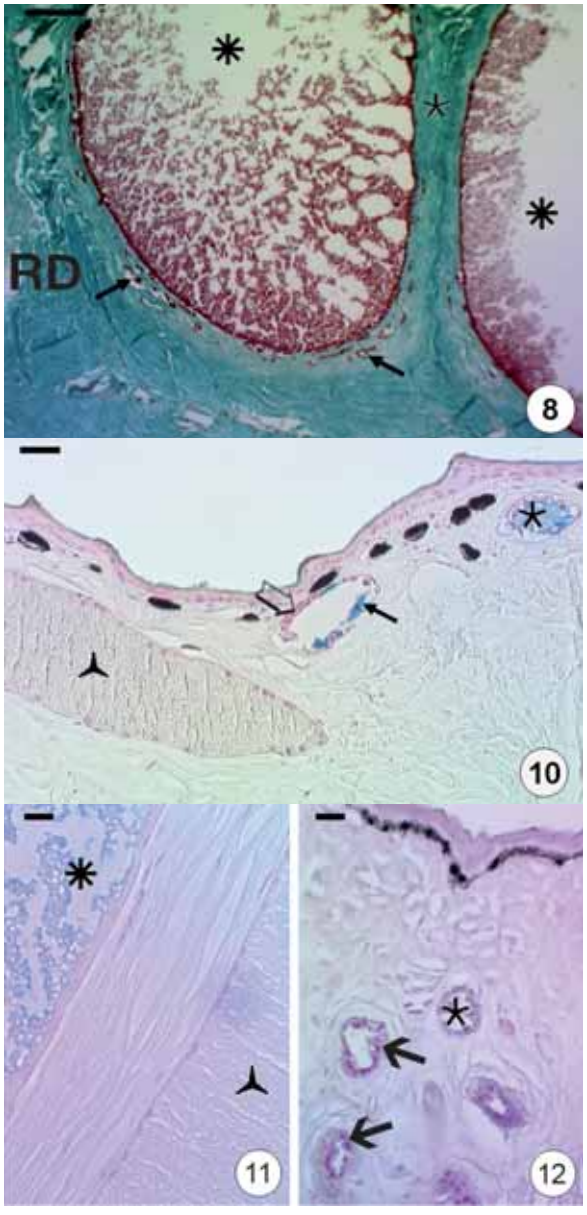


Fig. 6. Detail of a secretory portion of the smaller granular gland (⅄) surrounded by myoepithelial cell (➔). Scale = 20 µm. Immunolabeled for smooth muscle α actin.
Fig. 7. Observe calcium deposits (✶) in the Eberth-Kathschenko layer. Note smaller granular gland (⅄). Scale = 15 µm. Von Kossa method.
Fig. 8. Big alveoli (✳) of the larger granular gland are located in the reticular dermis (RD), and are separated by the septa of dense connective (✶). Note small blood vessels (➔) in the loose connective tissue around the alveoli. Scale = 200 µm. Gomori's trichrome stain.
Fig. 9. Note the clear tinctorial difference between the intake of the smaller granular gland (⅄) and the larger granular intake (✳). Note collagenous fiber of a septum (✶) separating the secretory portions. Scale = 50 µm. Gomori´s trichrome stain.
Fig. 10. Note alcianophilic reaction of the mucous cells (➔) of the mixed gland (✶) and serous cells (⇨) which show no reaction. The smaller granular gland (⅄) demonstrate no reaction to AB-method. Scale = 50 µm. AB- stain.
Fig. 11. The secretion of the larger granular gland (✳) exhibits alcianophilic reaction, and the granular intake of the smaller granular gland (⅄) show no reaction. Scale = 20 µm. AB- stain.
Fig. 12. Note a group of mixed glands (✶); mucous cells (➔) are visualized through PAS-method. Scale = 50 µm.

are other subtypes of serous glands, indicating high morphological variability (Warburg *et al*., 2000). Cutaneous lipids are present in some glands of phyllomedusine anurans (Blaylock *et al*., 1976), hylids (Warburg *et al*., 2000) and in one rhacophorid frog (Lillywhite *et al*., 1997). Cutaneous lipid secretion appears to be the main physiological adaptation of xeric-inhabiting arboreal frogs, enabling them to remain exposed throughout the year, even in the dry season (Warburg *et al*., 2000). For Barbeau and Lillywhite (2005), the lipid secretory glands are related to specialized adaptations of the skin in order to provide resistance to evaporative water loss.

A remarkable glandular type, different from the mu-

cous, serous, and seromucous glands in anuran integument, was described in *Odontophrynus americanus* (Felsemburgh *et al*., 2006). These glands have an acidophilic secretion constituted by a granular intake scattered by a homogeneous secretion. The homogeneous secretion showed continuity to glandular cell cytoplasm, and this continuity characterizes a glandular type with an apocrine mechanism of secretion, which was not previously described for the amphibian cutaneous glands.

Bufonids have peculiar glandular structures symmetrically disposed in a post-orbital position, known as the parotoid glands (Young, 1985; Pough *et al*., 2003). Although toads do not have a venom inoculation system, they are considered poisonous animals due to their cutaneous glandular secretions that contain a variety of compounds such as proteins, peptides, steroids, alkaloids and biogenic amines (Sakate and Lucas de Oliveira, 2000; Maciel *et al*., 2003). These glands are composed of large aggregations of granular glands responsible for the production and storage of a thick and creamy secretion which may contribute to the anuran protection against predators and parasites (Porter, 1972; Croce *et al*., 1973; Duellman and Trueb, 1994; Clarke, 1997; Sakate and Lucas de Oliveira, 2000).

On account of the *Bufo ictericus* parotoid gland relevant role, the aim of this work was to characterize the gross anatomy and the microscopic structure using stereoscopic microscopy, light microscopy, and histochemical methods.

Material and methods

Five adult males of *Bufo ictericus* Spix, 1824 were collected in Teresópolis, Rio de Janeiro State, according to the Brazilian laws (no. 191/2005 - IBAMA). The average weight of the toads was 120 grams and the average length (snout-vent) was 11 centimeter. For stereoscopic microscopy (SM) the entire parotoid gland region was excised (in toto), cut into 5 millimeter sections with a sharp blade, and fixed in 10% buffered neutral formaldehyde solution. The sections were analyzed under a Stemi SV11 Zeiss. The images were captured by the AxioVision 2.05 system. This procedure was used to characterize the gross anatomy of the internal arrangement of the parotoid gland region. For light microscopic (LM) analysis, parotoid gland fragments were fixed with 10% buffered formaldehyde and/or Bouin's liquid, and processed according to standard histological techniques for paraffin embedding. Five-micrometer thick serial slices were stained with haematoxylin-eosin (HE) and Gomori's trichrome (Lillie and Fulmer, 1976). Staining with 0.1% Alcian Blue (AB) 8GX at pH 2.5 (Kiernan, 1990) was employed for demonstration of sulfated and carboxylated forms of acid mucosubstances. The periodic acid Schiff (PAS) staining was employed to detect neutral glycoproteins (Mowry, 1963). The von Kossa method (Prophet *et al*., 1994) was used to detected calcium. The localization of smooth muscle α-actin positive cells was performed using the standard avidin biotin conjugate (ABC) immuno-assaying procedure. Paraffinized sections were de-waxed, dehydrated and then washed in phosphate buffered saline (PBS). Nonspecific binding sites were blocked with 3% hydrogen peroxide solution for 30 minutes. The sections were incubated in a humid chamber for 30 minutes with 1% bovine serum albumen (BSA) at room temperature. The sections were then incubated with monoclonal antibody anti-smooth muscle α-actin (Dako, HHF35 code no. M0635) diluted to 1:50 overnight. After washing with PBS, the sections were incubated for 1 hour at room temperature with the biotinylated secondary antibody (1:100) (Dako, Multilink swine anti-goat, mouse, rabbit immunoglobulins/biotin, code no. E0453). The sections were then incubated with ABC complex (extravidin 1:100) for 30 minutes. The sections were washed in PBS and revealed by treatment with a 3´3-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.1% hydrogen peroxide. The sections were counterstained with haematoxylin, and permanent preparations are made as usual for standard technique. As control procedure, the treatment with the primary antibody was omitted. Slices were observed under an AxioVision 2 Zeiss microscopy, and the images were captured using the AxioVision 2.05 system.

Results

Macroscopically, the parotoid glands of *Bufo ictericus* are paired, elongated protuberances in post-orbital position (Fig. 1a). The parotoid glands are, on average, 3.5 ± 0.2cm in length, 1.5 ± 0.2cm in wide and 0.7 ± 0.2cm in thickness. On the external surface, numerous and slightly rounded concave depressions (0.7 ± 0.1mm in diameter) occur, containing elongated glandular openings (0.3-0.4mm) (Fig. 1b). The rounded depressions predominate on the parotoid medial surface, but they are absent on the lateral surface (Fig.

1a). In the middle of the rounded depressions, small pores are visible and these correspond to the duct openings of the larger granular glands.

Parotoid gland transverse sections reveal that the largest secretory units of exocrine glands are separated by connective tissue septa (Fig. 2). These glandular alveoli (4 ± 1mm in wide) occupy the middle portion of the parotoid gland and become smaller towards the periphery. Light microscopic analysis shows that the parotoid gland region is covered by an epidermis which is supported by the dermis. The epidermis shows similar features of the dorsal integument, i.e., the keratinocytes are organized in four to six cellular layers and they form sporadic conical projections that correspond to the cornified tubercles. In the epidermis, flask cells are also visualized (Fig. 3).

Just beneath the epidermis, there is a thin region in which the connective tissue is looser. This subepidermic region is separated from the spongious dermis by a pigment cell layer (Fig. 3). The spongious dermis of the parotoid gland region consists of loose connective tissue which contains small mixed glands (Figs. 3, 10).

Due to the collagenous fiber organization, an additional layer of irregular dense connective tissue is evidently present below the loose connective tissue. This region is characterized by coarse, thick, and intertwined bundles of collagenous fibers randomly organized (Fig. 4). This dermic region, reticular dermis, contains alveoli of smaller and larger granular glands. From the reticular dermis, septa of connective tissue containing blood vessels separate the larger glandular units (Fig. 8). Below the reticular dermis, there is a thin compact dermis. However, the collagenous fibers do not exhibit the criss-crossed arrangement.

In the integument of the parotoid gland region, the Eberth-Katschenko (EK) layer is evident through its typical basophilia (Fig. 5). Using the von Kossa method, calcium deposits are visualized in the EK-layer of the parotoid gland integument (Fig. 7).

The parotoid gland region contains three exocrine glandular types: mixed glands, small granular glands and large granular glands (Figs. 3, 4, 5, 8, 9). The glandular duct of the mixed and small granular glands is lined with bi-stratified cubic epithelium and the epidermal horny layer penetrates into the initial portion of the glandular duct. The duct of the larger granular glands is formed by stratified epithelium, because of the epithelial invagination during glandular development. Secretory portions of these three glandular types are surrounded by myoepithelial cells (Figs. 5, 6).

The mixed glands are located in the spongious dermis as single glands (Figs. 3, 4, 5). They can also be seen in small groups around the duct of the larger granular gland (Fig. 4). They are constituted by two cellular populations: mucous cells and serous cells. The mucous cells of the mixed glands are PAS- (Fig. 12) and AB-positive (Fig. 10). The serous cells are acidophilic and show no reaction to neither PAS nor AB methods (Figs. 10, 12).

Smaller granular glands are located in the outermost region of the reticular dermis. The secretory portion is made up of acidophilic cells and contains a homogeneous acidophilic granular intake (Figs. 5, 7, 9, 10). They are identified preferentially in the lateral region of the parotoid gland integument and are PAS- and AB-negative (Figs. 10, 11). Besides, the secretory cells constitute a syncytium.

The alveoli of the larger granular glands are macroscopically visualized (Fig. 2) and contain both basophilic and alcianophilic granular content (Fig. 11). Their secretory cells are represented by an acidophilic cellular syncytium with rounded nuclei at the basal region (Fig. 11). Large blood vessels are observed below the larger granular glands in a connective tissue layer which corresponds to the hypodermis (Fig. 2).

Discussion

The parotoid gland of bufonids has received different designations. Firstly, the parotoid gland was designated as parotid gland; later it was named parotoid gland by Boulanger in order to differentiate it from the mammal salivary gland (Vital-Brazil and Vellard, 1925). However, diverse denotations are still observed, such as paratoid gland (de Assis *et al*., 1985), parotid gland (Santa Coloma *et al*., 1984; Fox, 1986), parotoid gland (Mahan and Biggers., 1977; Cannon and Hostetler, 1976; Pasquarelli *et al*., 1987; Duellman and Trueb, 1994; Hutchinson and Savitzky, 2004), and parotoid organ in *Bufo* (Delfino *et al*., 1999). In spite of the different designations, researchers agree that these glands represent an aggregate of granular glands in the post-orbital region (Cannon and Hostetler, 1976; Toledo *et al*., 1992; Duellman and Trueb, 1994; Hutchinson and Savitzky, 2004). In the present work, the term parotoid gland is used to designate large elevations on both sides of the toad body in post-orbital position.

In *Bufo ictericus*, the parotoid gland epidermis is similar to that of the dorsal integument as already described by de Brito-Gitirana and Azevedo (2005). Toledo *et al*. (1992) described rounded depressions on

the parotoid gland surface as pores. In this work, we showed that the pores are located at the bottom of the rounded depressions. De Brito-Gitirana and Azevedo (2005) described rounded orifices on the dorsal and ventral integument, which corresponded to the openings of the glandular ducts. Thus, the pores in the bottom of the depressions are the excretory duct openings through which the milky venom is extruded.

In the spongious dermis of the anuran integument, two exocrine glands occur: mucous gland and granular (venom) gland (Porter, 1972; Delfino *et al*., 1987; Duellman and Trueb, 1994; de Brito-Gitirana and Azevedo, 2005; Felsemburgh *et al*., 2006).

In *B. ictericus*, the present work shows that the parotoid gland is a specialized region in the integument in which an additional glandular type also occurs. The glandular units are well-developed and occupy the entire dermis. Their secretory portion can be macroscopically visualized, and is microscopically characterized by larger granular glands. Besides the larger granular glands in the parotoid gland of *B. ictericus*, mixed and smaller granular glands also occur, and they are visualized only through light microscopy.

Mixed glands are constituted by mucous and serous cells. According to the established histology which uses histochemical and structural parameters (Weiss and Greep, 1977; Kierszenbaum, 2002), mixed secretory units are made up of mucous cells and serous cells; they produce a seromucous secretion delivered into the same lumen. The cytoplasm of mucous cells is basophilic provided that they contain neutral glycoproteins and acid mucosubstances. The nucleus is flattened, and appears at the basal portion of the cell (Kierszenbaum, 2002). The serous cells have a round nucleus centrally placed and its cytoplasm is acidophilic.

In the *B. ictericus* parotoid gland, the small mixed glands have the same morphology as those distributed through the *B. ictericus* integument (de Brito-Gitirana and Azevedo, 2005). Although they are formed by two cell types, they are usually named mucous glands. Here, we prefer the designation mixed gland to emphasize this glandular type. In addition, the histochemical methods revealed that the end product of the mixed gland is enriched with proteins produced by serous cells and neutral glycoproteins as well as sulfated and carboxylated acid mucosubstances produced by mucous cells.

Toledo *et al*. (1992) pointed out that the secretion of granular glands is PAS-negative and AB-positive. However, they did not relate structural differences between the smaller granular gland and the larger granular gland. For Toledo *et al*. (1992), the granular glands produce an alcianophilic secretory product. Their results are in contrast to our histochemical data once the tinctorial affinities of the secretory intake of both glandular types are different, i.e., the smaller granular glands produce an acidophilic secretion but the larger granular glands have a predominant granular basophilic intake. Thus, the histochemical methods provide evidence that both granular glands differ in the chemical nature of their secretion.

Our results show that the mixed glands are also associated to the duct of the larger granular glands. So, the final secretory product is a mixture of secretions of both glandular types. Besides, we can not discard the fact that the secretory product of the mixed gland is important for the effectiveness of the venom released by the larger granular gland.

In *B. ictericus*, myoepithelial cells occur around the secretory portions of all gland types, and probably play a role in extrusion of secretory product. Mills and Prum (1984) and Delfino *et al*. (1987) related that myoepithelial cells are important in the structural organization of the glandular secretory portion.

In this work, the EK layer in the parotoid gland integument region was also visualized. Toledo *et al*. (1992) did not mention the EK layer in the parotoid gland integument of *B. ictericus*, but they commented that the EK layer occurs in *Bufo cruficer*, and it is absent in *Bufo typhonicus*. The water retention capacity of the EK layer is pointed out by several authors (Kobelt and Linsenmair, 1986; Toledo and Jared, 1993). Azevedo *et al*. (2006a) indicated that the entire spongious dermis is the cutaneous region responsible for the hydric reservoir in the *B. ictericus* integument. Their study was based on the localization of hyaluronic acid, a non-sulfated glycosaminoglycan with a water-binding quality.

In the dermis of the parotoid region, the collagenous fibers are visualized as irregularly oriented bundles. Since the collagenous fiber organization is different from that of the compact dermis of the *B. ictericus* body integument (Azevedo *et al*., 2006b), we introduced the designation reticular dermis to characterize this organization of the connective tissue in this region.

The cutaneous secretory products of bufonids have been characterized, and different compounds are described and divided into four main categories: biogenic amines, bufodienolides (bufogenines and bufotoxins), alkaloids, peptides and proteins (Daly *et al*., 1987; Toledo and Jared, 1995; Erspamer, 1994). Due to the structural glandular arrangement of the parotoid gland,

its secretory product represents the cutaneous secretions elaborated by the smaller and the larger glandular glands as well as by the mixed glands. The mucous secretion participates in the animal defense due to its noxious or toxic compounds (Phisalix, 1923; Sawaya, 1940; Noble and Noble, 1944). Clarke (1997) and Fontana *et al*. (2006) suggested that mucus possesses a bacteriostatic effect, and it represents a potential mechanical trap for microbial and fungal pathogens. Eggert-Kruse *et al*. (2000) demonstrated that the human cervical mucus has a considerable antimicrobial activity. Many antimicrobial compounds have been isolated from mucous secretions of different tissues/organs (Brogden, 2005). Hutchinson and Savitzky (2004) commented that the large vessels provide the precursor molecules necessary to formation of toxins contained in the secretion of the parotoid gland. In *B. ictericus*, large vessels occur in the hypodermis of the parotoid gland region.

This work showed that the parotoid gland is a specialized region of the integument, in which three gland types occur: the mixed, the smaller granular, and the larger granular glands. The larger granular glands are the main glandular component, and are responsible for the macroscopic protuberances known as parotoid gland. Thus, the end product released by the parotoid gland, know as venom, represents a mix of secretions which are elaborated by these three glandular types.

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