Ultrastructure of the accessory venom gland of the puff-adder and effects of nerve stimulation on duct perfusate

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The accessory venom gland of the puff-adder consists of an anterior and a posterior region. The former is comprised of columnar mucus-secreting cells which are abundant, and other cells particularly rich in mitochondria; the latter consists of cuboidal mucus-secreting cells and squamous cells of two kinds. Eruption of the cells in the anterior region was observed in the glands of snakes that had been electrically stimulated prior to fixation. The venom duct and accessory glands were perfused while being stimulated electrically. Total protein, chloride concentration and osmolality of the perfusates were analysed but no differences were found in any of the experiments.

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Die bykomstige gifklier van die pofadder bestaan uit twee dele. Die anterior gedeelte bevat baie silindriese selle wat slym afskei en ander selle wat besonder ryk is aan mitochondria. Die posterior gedeelte bestaan uit kubiese selle wat slym afskei en twee tipes afgeplatte selle. Afskeiding van die selle in die anterior gedeelte is waargeneem na elektriese stimulasie voordat die selle gefikseer is. Die gifklierbuis en die bykomstige gifkliere is uitgespoel terwyl hulle elektries gestimuleer is. Die totale proteïen, die chloriedkonsentrasie en osmolaliteit van die spoelvloeistof is bepaal maar geen verskille is in enige eksperimente waargeneem nie.

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The venom-secreting apparatus of most vipers comprises a posterior main gland, a tapering intermediate neck region containing a primary duct, an anteriorly situated accessory venom gland (surrounding the duct) and a terminal or secondary duct (Kochva & Gans 1965; Gennaro, Gallahan & Lorincz 1963). Relatively little is known about the accessory venom gland (Odor 1965), but it may function in activating primary gland venom when this passes through the accessory gland (Gans & Kochva 1965; Gans & Elliot 1968). In the puff-adder only the histology and histochemistry have been investigated (Lake, Hattingh, King & Trevor-Jones 1983). We have now examined the ultrastructure and carried out preliminary perfusion experiments in an attempt to indicate the function of the accessory apparatus.

Material and Methods

Healthy adult puff-adders (*Bitis arietans*) of both sexes were obtained from the Hartebeespoortdam Snake and Animal Park and housed in glass cages for seven days during which they were fasted, but had free access to water. Three snakes were anaesthetized by intra-peritoneal injection of Ketamine (Parke Davis). One was immediately killed by perfusion with Karnovsky's aldehyde fixative *via* a cannula in the carotid branch of the left aortic arch (Robb 1965). Both accessory glands were subsequently dissected free and immersed in Karnovsky's aldehyde fixative.

One gland in each of the other snakes was stimulated before fixation. Anaesthesia was maintained using a mixture of oxygen and halothane introduced *via* an intra-tracheal tube. The *M. compressor glandulae* (Kochva & Gans 1965) on one side was reflected to expose the subjacent nerve plexus which innervates the venom glands (Kochva & Gans 1965). The nerve plexus on the right side was stimulated in one snake and the left plexus in the other (square wave pulses of 0,1 ms and 20 V at 30 Hz). Stimulation was of 10 s duration followed by 10 s rest and repeated 20 times. These stimuli caused fang erection and venom expulsion. Subsequent to stimulation the snakes were killed and fixed as above.

After an hour in fixative, the glands were separated into posterior and anterior parts distinguished by the dark pigmentation of the posterior part and the non-pigmented anterior portion. The tissues were then washed in a phosphate buffer solution for 30 min, post-fixed in 1% Os_2O_4 for 1 h, dehydrated, cleared in propylene oxide and embedded in araldite for 48 h at 60 °C. Sections were stained with uranyl acetate and lead citrate.

Results

The anterior portion of the accessory gland comprises two distinct cell types: (a) columnar mucus-secreting cells which are the principal cell type; and, (b) narrow, mitochondria-rich cells which are less numerous.

In the resting state, the mucus cells are tall and densely packed with secretory droplets which are connected to one another by slender strands of dense cytoplasm or by discontinuous membranes (Figure 1). The nucleus is situated basally and is flattened or oval with a prominent nucleolus. Mitochondria and profiles of smooth endoplasmsic reticulum are apparent here. Free ribosomes are scarce within the cytoplasm. The apical surface of each mucous cell is covered with irregularly organized microvilli. Some tubules contain secretory material including large and small membrane-bound vesicles and electron-dense granules surrounded by an even darker granular mass (Figure 2).

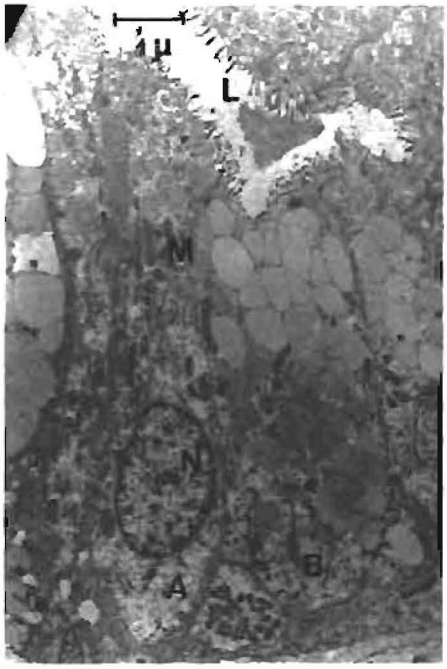

Figure 1 Mitochondria-rich (A) and mucous cells (B) in anterior portion. N = nucleus; M = mitochondria and L = lumen.

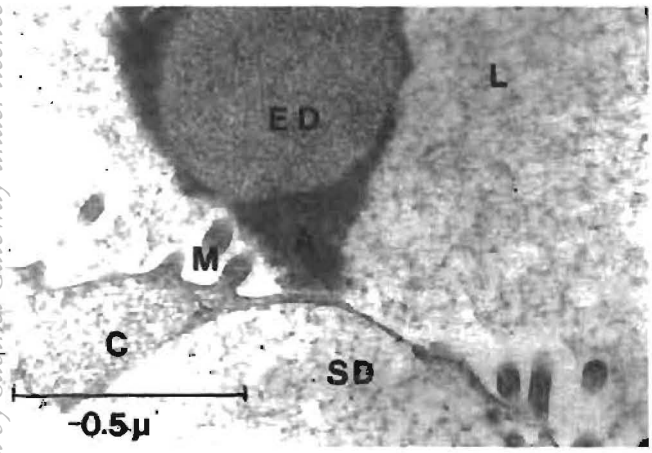

Figure 2 Secretory products in duct lumen (L). ED = electron-dense granule; A = extravesicular aggregations; M = microvilli; C = cytoplasm and SD = secretory droplet.

Narrow mitochondria-rich cells lie interspersed amongst the mucous cells (Figure 1). The basal and lateral cell membranes possess numerous and complex interdigitations and desmosomal attachments to neighbouring mucous cells. The cell surface is covered with microvilli. The cytoplasm of these cells lacks free ribosomes and a Golgi complex is seldom visible.

The posterior portion of the accessory gland also comprises two cell types: (a) cuboidal mucous-secreting cells with oval to flattened nuclei of low electron density; and, (b) flattened or squamous cells with horizontally elongated electron-dense nuclei.

The cuboidal cells (Figure 3) contain electron-dense secretory vesicles and also secretory droplets of low electron density. Mitochondria and profiles of both rough and smooth endoplasmic reticulum are visible. The apex of each cell is again covered with numerous microvilli and the base rests on a prominent basement membrane. Possible myoepithelial cells are occasionally visible between the secretory cell base and the basement membrane.

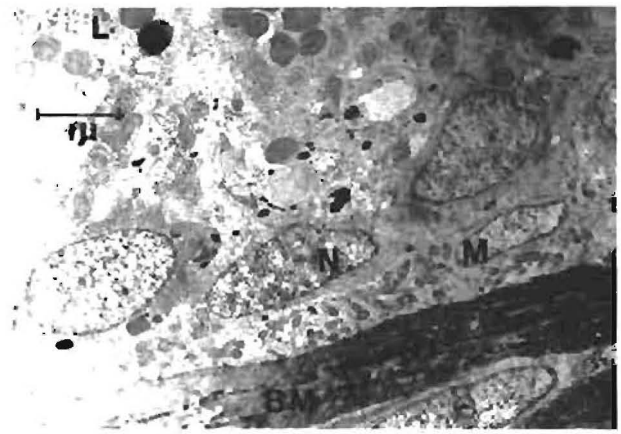

Figure 3 Cuboidal cells of the posterior region. N = nuclei; L = lumen; BM = basement membrane and M = myo-epithelial cell?

The squamous cells appear to be subdivided into two different cell types. One type contains electron-dense and electron-translucent vesicular bodies within the cytoplasm. The other type contains extensive smooth endoplasmic reticulum. These cells appear to elaborate and secrete an assorted array of secretory granules of variable density and size. The possibility does exist that these cells are either necrotic or that the cells are being replaced. Both types of squamous cells have microvilli on their luminal surfaces.

The main duct passing through the centre of the accessory gland is lined by a tall columnar secretory epithelium. Its cells contain both electron-dense and electron less-dense secretory granules and numerous large mitochondria (Figure 4). These cells are largely devoid of microvilli, but contain minute cytoplasmic projections, which appear to be merely 'budding off' of small amounts of secretory material. Interspersed amongst these cells are occasional ciliated columnar cells.

Certain changes are noticeable in stimulated glands. Some mucous cells in the anterior portion of the gland appear to 'erupt' on stimulation (Figure 5). The vesicular content of these cells appears as an almost homogeneous mass, within which the delineations of each secretory droplet are obliterated. In addition, the apices of many of the cells appear to bulge into the lumen as if preparing forcefully to release their contents.

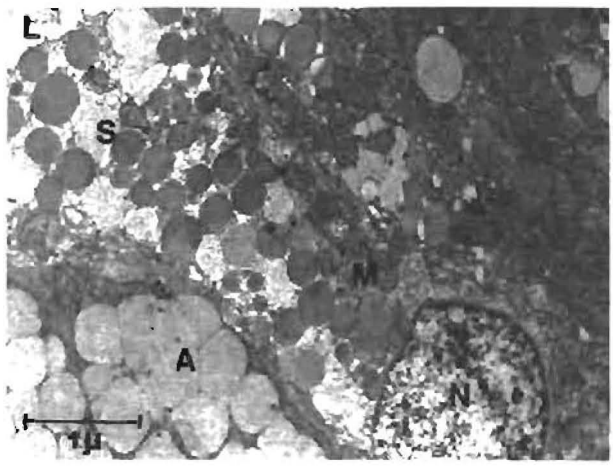

Figure 4 Tall secretory cell lining the main duct. N = nucleus; L = lumen; M = mitochondria; S = secretory vesicles and A = adjacent mucous cell.

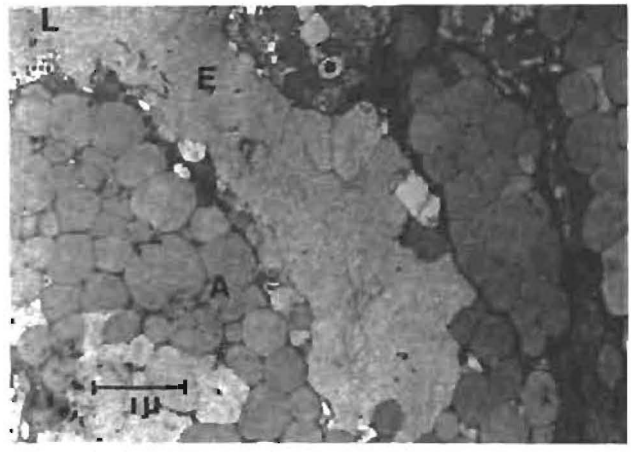

Figure 5 Anterior region stimulated accessory gland. E = eruption of cell contents; L = lumen and A = adjacent mucous cells. Note loss of membranes of cytoplasmic contents.

The lumina of some tubules contain large quantities of secretory material. Cells in the posterior portion of the stimulated gland also show 'eruption' and bulging of cytoplasmic contents into the tubule lumen. Some cells do not show signs of secretory activity and the active cells lack the homogeneous cytoplasm shown in cells of the anterior part of the gland.

Two additional snakes were anaesthetized with Ketamine followed by halothane as described and prepared for perfusion of the venom gland ducts. The *M. compressor glandulae* was reflected on both sides to expose part of the main gland and the accessory gland whilst retaining the blood circulation intact. The primary duct was ligated as it emerged from the main gland and cannulated below the ligature so that the duct and accessory gland could be perfused and the perfusate collected.

Each gland-duct system was perfused 3 times with 1 ml Ringer-Locke solution, at a flow rate of 0,25 ml/min, to rinse residual secretion from the system. Each gland was then perfused for 4 min while the nerve supply to one gland was stimulated as described. Following a 5 min rest period the process was repeated with stimulation of the opposite gland.

Total protein determinations were carried out on the perfusates by chemical means (Lowry, Rosenbrough, Farr & Randall 1951). The chloride concentration was measured using a CMT 10 chloride titrator (Radiometer), and the osmolality measured with a 5120 B Vapour Pressure Osmometer (Wescor). The sodium and potassium content was measured using a FLM 3 Radiometer Flame Photometer. No change in the composition of the perfusate could be measured in any experiment and only negligable amounts of protein were added to it. Mucopolysaccharide concentrations were not measured because it was assumed that any function of the gland relating to venom activation would be reflected more in the ionic composition of the perfusate.

Discussion

The ultrastructural findings reported are in keeping with the observations of other authors (Kochva & Gans 1965) who have reported the presence of mucopolysaccharides in the secretory epithelium of posterior and anterior gland tubules. It has been postulated (Gans & Elliot 1965) that the secretion of the accessory glands could affect some inhibitory mechanism and/or unmask active sites during the passage of venom. The mucopolysaccharides may protect the gland tissues (surface coat) after venom activation (see below).

It has not been proved, although it is generally accepted, that the secretion of the glands (main gland and accessory) is not regulated by extrinsic factors, but by the glands themselves (Bdolah 1979; Kochva 1978). A resorption process possibly involving volume control by intracellular digestion and lysosomal action would thus be necessary because:

(i) Continously secreting glands probably cannot retain all the venom produced for indefinite periods; an outlet (or control) system is necessary as in mammalian salivary glands where there exists an excretory duct system or in the testis where a resorption process is present, and/or

(ii) If the hypothesis of Gans & Elliot (1965) is true, that the accessory gland functions in activation of main gland venom, then it is unlikely that it can store activated toxic venom for extended periods.

Mitochondria-rich cells have been reported (Odor 1965; Marshawsky, Haddad, Goncalves, Valeri & de Lucca 1973) but only in the main venom gland. Kochva (personal communication) has suggested that they are involved in the absorption of water and electrolytes. We observed no change in perfusate composition during stimulation but we did not study resorption. These cells in the anterior portion may function in volume control (under resting conditions); if they absorb water other substances may be absorbed with it due to 'osmotic-drag'. The presence of microvilli on the mitochondria-rich cell apex possibly supports this.

The posterior portion of the accessory gland has two different cell types and this would suggest a function different from that of the anterior region. It is postulated that this region either secretes or activates a component(s) of the venom, or modifies the venom before it reaches the anterior part of the gland. The presence of mucus-secreting cells within the epithelium, suggests that protection is already necessary in this part of the gland, thus assigning a possible activating function to the posterior region.

However, further studies of the function of the squamous cells is necessary; ultra-structurally these cells appear to be secretory in nature. It is apparent that tight junctions are present at the apical intercellular borders of the secretory cells in the duct and glandular epithelium and would function to prevent passage of fluid into the intercellular spaces. In addition numerous desmosomes and other junctions are present between

adjacent cells of the posterior and anterior regions of the accessory gland and may function in intercellular communication. This would support the hypothesis of Bdolah (1979) that the control mechanisms involved in venom synthesis are found in the venom glands themselves and depend on the amount of venom present in the lumina of the glands.

Section of the main nerve supply to the venom glands does not affect venom production, protein concentration or enzymic activity (Allon 1973). The absence of any detectable change in the composition of the perfusate after electrical stimulation suggests that neural activity is not important in controlling the composition of the secretion (at least during the time course of the present experiments). It may be that the venom from the main gland could control the secretion of the two regions of the accessory gland (chemically and/or physically). This could occur through the effect on duct membrane receptors coupled with an intercellular communication system of gap junctions. A more informative experiment might involve perfusion of the accessory gland with a solution containing components of main gland venom.

The unstimulated glands still contain 'erupting' and 'bulging' cells that are apparent in the ultrastructure of the stimulated glands (although less than noted in stimulated glands). In addition, some cells within the stimulated tubules are affected, while others are not. This finding agrees in part with the report of Kochva (1978) that only some of the tubules of the main gland secretes at any one time. (We, however, never observed all cells of a given stimulated tubule erupting or bulging.) This is more efficient than drawing a little venom from each of the tubular lumina and provides the snake with a rather simple method of venom replenishment and a local means of regulating venom production at the level of each secretory tubule. However, the possibility cannot be excluded that the apparent response of the secreting cells to stimulation might be due to the passage of venom through the gland during stimulation; the primary duct was not ligated as during the perfusion investigation. As the main gland was not isolated from the accessory gland this aspect requires further investigation.

Acknowledgements

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