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Immunolocalization of venom metalloproteases in venom glands of adult and of newborn snakes of *Bothrops jararaca*

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Abstract. Using immunoelectronmicroscopy we analyzed qualitative and quantitatively the intracellular distribution of bothropasin, hemorrhagic factor 2 (HF2) and hemorrhagic factor 3 (HF3) in the venom secretory cells from adult snakes in the active (7 days after venom extraction) and in the resting (without venom extraction for 40 days) stages of protein synthesis. Glands from the newborn *Bothrops jararaca* were also studied. The results lead to the conclusion that all the secretory cells and the secretory pathway in the cells are qualitatively alike in regard to their content of the three metalloproteases. Secretory cells from the resting glands, unlike the active ones and the newborn glands, did not present immunolabeling in the narrow intracisternal spaces of the rough endoplasmic reticulum (RER). The label intensity for bothropasin was greater than that for the other proteins in the adults. HF3 and HF2 labeling densities in the newborn were higher than in the adults and HF3 labeling was not different from that of bothropasin. Co-localization of the three metalloproteases was detected in the RER cisternae of the active gland secretory cells, implying that mixing of the proteases before co-packaging into secretory vesicles occurs at the beginning of protein synthesis in the RER cisternae.

Keywords: metalloproteases, snake venom gland, immunoelectron microscopy, *Bothrops jararaca*, venom secretion

Introduction

The pit viper *Bothrops jararaca* is the most abundant snake in Brazil where it is responsible for about 90% of snake-bite accidents in the southeastern area (Cardoso & Brando, 1982). Most venoms of Crotalinae and Viperinae are rich in proteolytic enzymes. Some of these proteases cause hemorrhage at the site of envenomation and often disseminate around, affecting the entire appendage. A great number of factors involved in local tissue damage were isolated from this venom, and they were characterized as metalloproteases (Mandelbaum et al., 1982; Assakura et al., 1986; Tanizaki et al., 1989; Maruyama et al., 1992). These enzymes efficiently cleave

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basement membrane proteins allowing the flux of capillary contents to invade the surrounding stroma, causing hemorrhage and the entry of toxins into the circulatory system (Mandelbaum et al., 1998).

Enzymes and other venom components are synthesized and secreted by secretory cells from the venom glands. In viperid snakes, the venom is mainly accumulated in the tubular and gland lumina. The compartment of the secretory vesicles comprises about 4–10% of the venom cell volume (Carneiro et al., 1991). After venom manual extraction (milking) or natural compression of the glands, the venom is depleted from the glandular lumen and the secretory cells initiate a new cycle of synthesis and secretion of the venom (active stage). RNA and protein synthesis steadily increases, reaching a peak between the 4th and 8th days after milking (Rotenberg et al., 1971; De Lucca & Imaizumi, 1972). At the same time, the glandular epithelium increases in size and becomes columnar shaped. The RER intracisternal space distends, and the total cell membrane surface increases (Kochva & Gans, 1970; Ben-Shaul et al., 1971; Oron & Bdolah, 1973; De Lucca et al., 1974; Carneiro et al., 1991). As the synthetic activity decreases, the venom is accumulated in the gland lumen and the secretory epithelium returns gradually to its initial resting stage (Kochva, 1978).

Over the past three decades, some enzymes from snake venom glands have been studied by biochemical, histochemical and immunofluorescence techniques. The few ultrastructural immunocytochemical studies carried out have led to the general hypothesis that all secretory epithelial cells produce all types of venom enzymes and that these are localized in the same secretory vesicles (Schaeffer et al., 1972; Shaham et al., 1974; Sobol-Brown, 1974; Taylor et al., 1986). Asynchronic synthesis of various venom proteins, mainly during the first few days after milking, was also reported (Schenberg et al., 1970; Sobol-Brown et al., 1971, 1975; Oron et al., 1978; Taylor et al., 1986), but the precise relationship of different venom components in the secretory cell was not established.

We have undertaken this study, aiming to verify how the metalloproteases bothropasin, hemorrhagic factor 2 (HF2) and hemorrhagic factor 3 (HF3), isolated from the *B. jararaca* venom (Mandelbaum & Assakura, 1988), are distributed throughout the venom secretory cells. For this purpose, we used venom glands from milked and unmilked adult snakes and from newborn *B. jararaca* snakes. The compartmental localization of these proteases was evaluated by using electron microscopic immunogold labeling techniques.

Material and methods

Animals

Two unfed and unmilked (without feeding and venom extraction for 40 days), two 7-day milked adult *B. jararaca*, and two newborn snakes were obtained from the Instituto Butantan, São Paulo, Brazil.

Venom proteins

The acidic metalloproteases bothropasin, HF2 and HF3 were isolated from a pool of dried crude *B. jararaca* venom according to Mandelbaum et al. (1982) and Mandelbaum and Assakura (1988).

Antibodies

Polyclonal antibodies were prepared against the isolated proteins. According to Mandelbaum and Assakura (1988), 1 mg of each factor in 0.5 ml of 0.15 M NaCl was mixed with an equal volume of Freund's complete adjuvant (Difco) and injected intradermally in four shaved back sites of a white rabbit. After 1 month, boosters of 200 μg of each factor without adjuvant were given weekly for 1 month. A week after, the animal was bled and approximately 40 ml were collected. The animal rested for 1 month and then the scheme of 200 μg injections weekly was repeated. A final bleeding was made 8 days after the last injection.

Preparation of tissue

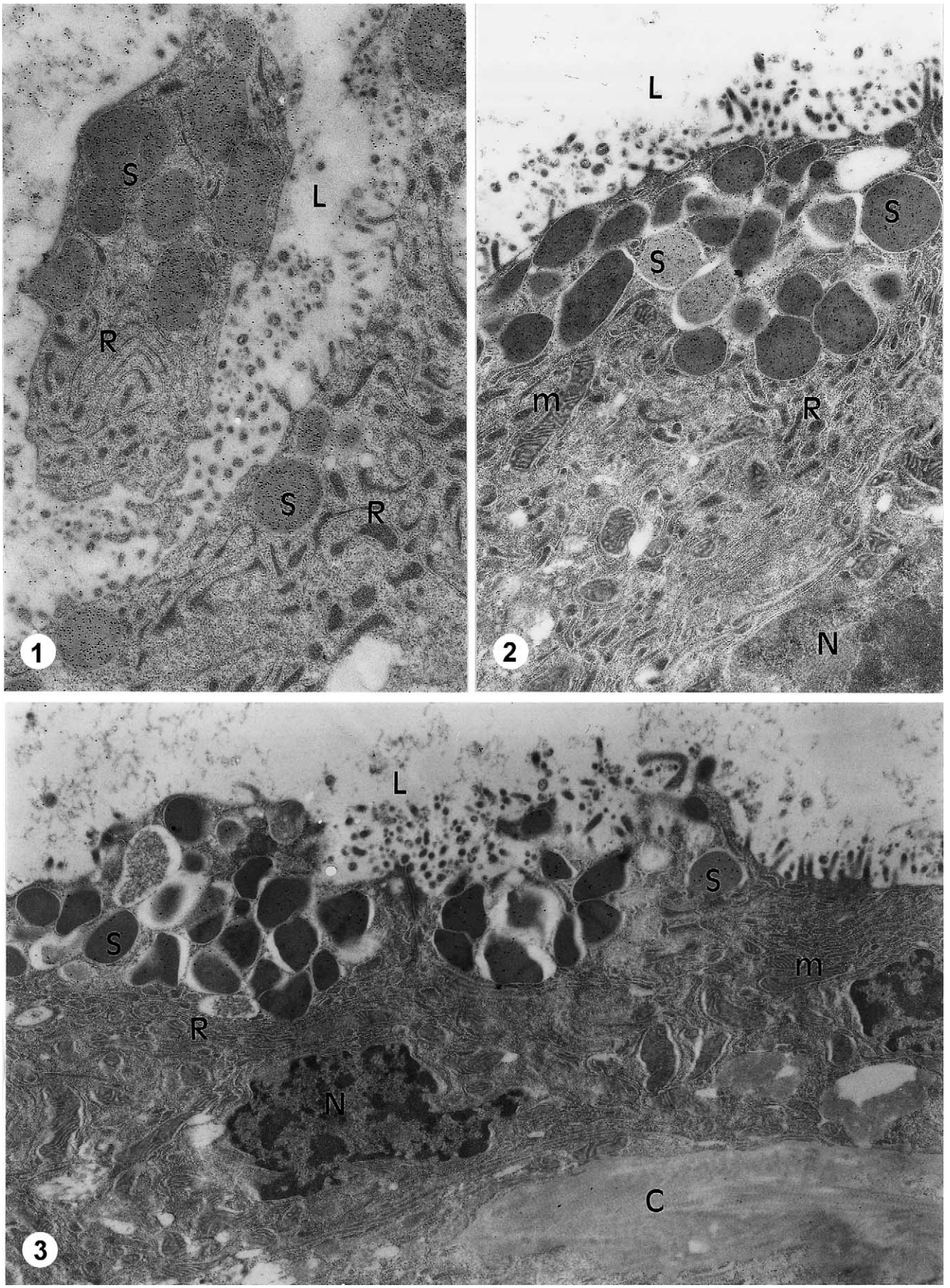
The snakes were sacrificed by decapitation after a brief anesthesia with carbonic gas. Venom glands were excised and pieces with less than 1 mm^3 were immediately immersed in the fixative solution of 4% paraformaldehyde (Sigma), 0.5% glutaraldehyde (Electron Microscopy Sciences—EMS) in 0.1 M cacodylate buffer pH 7.2, for 1 h, at 10 °C.

Thereafter, the gland fragments were processed according to the method of Berryman and Rodewald (1990) and embedded in LR White (London Resin Co. Ltd., UK), hard grade resin.

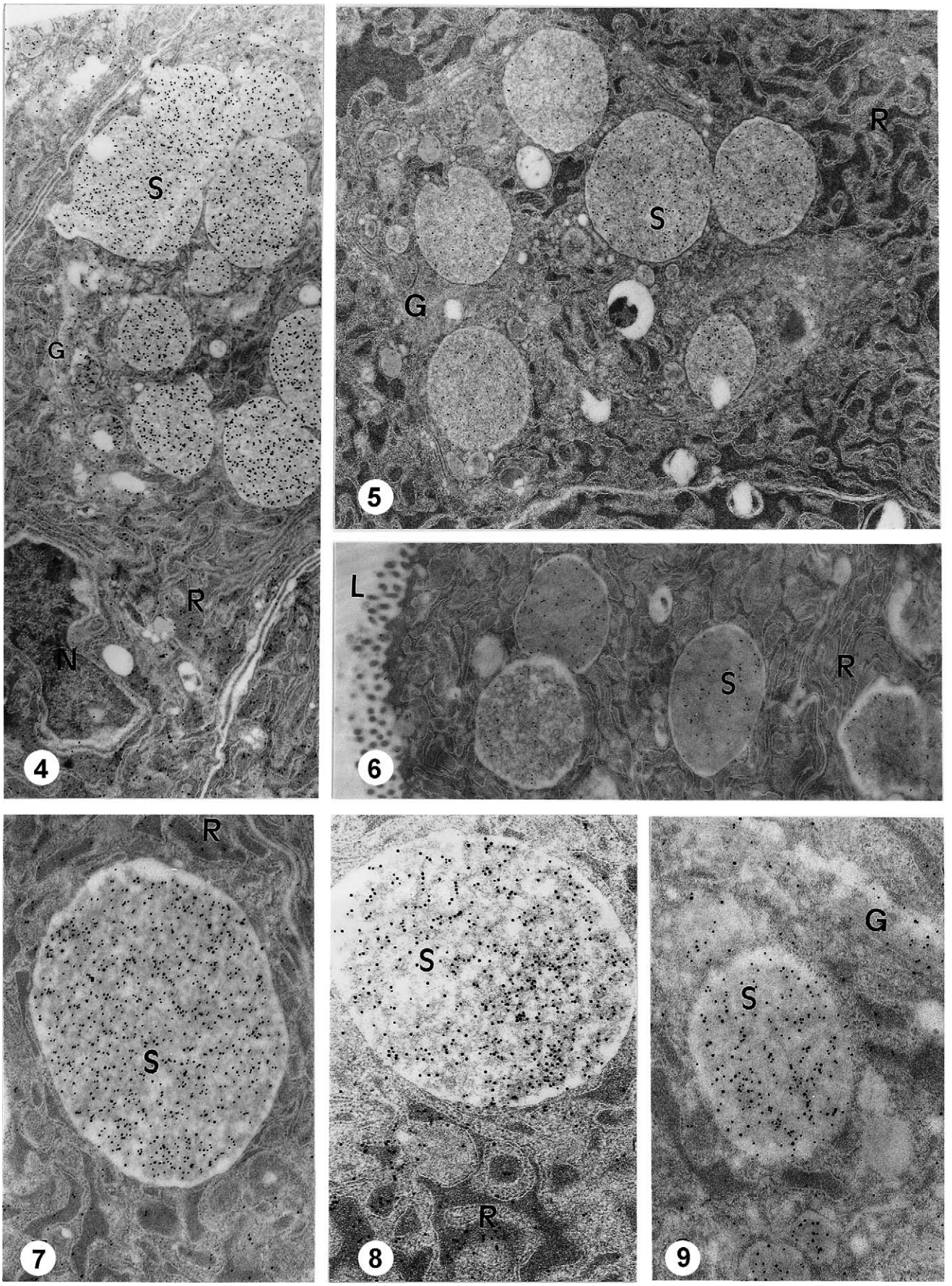
Immunolabeling procedure

Indirect immunolabeling was accomplished using protein A-colloidal gold 15 nm (Amersham). Where double-labeling was done, 10 nm protein A-gold was used to visualize the second antibody. The immunolabeling procedure was carried out on silver sections on nickel grids in the compartments of a grid storage box maintained in a humid chamber at room temperature, according to Souto-Padrón (1998): (a) blocking of non-specific sites was carried out using PBS pH 8.0, 1.5% BSA (Sigma Chemical Co. St. Louis, MO) and 0.01% Tween 20 (Amresco, Solon, OH) for 1 h at room temperature; (b) incubation with specific antisera diluted: 1:400 (bothropasin) or 1:50 (HF2, HF3) in blocking solution for 1 h at room temperature; (c) rinsing in blocking solution; (d) incubation in protein A-gold 15 nm, 1:50 in PBS pH 7.2, 1.5% BSA and 0.01% Tween 20, for 1 h, at room temperature; (e) rinsing with distilled water; (f) staining with uranyl acetate and lead citrate. For double-labeling experiments, after step (e) the grids were returned to the blocking solution (a). They were then incubated in the second specific antisera and subsequently steps (c) to (e) were used.

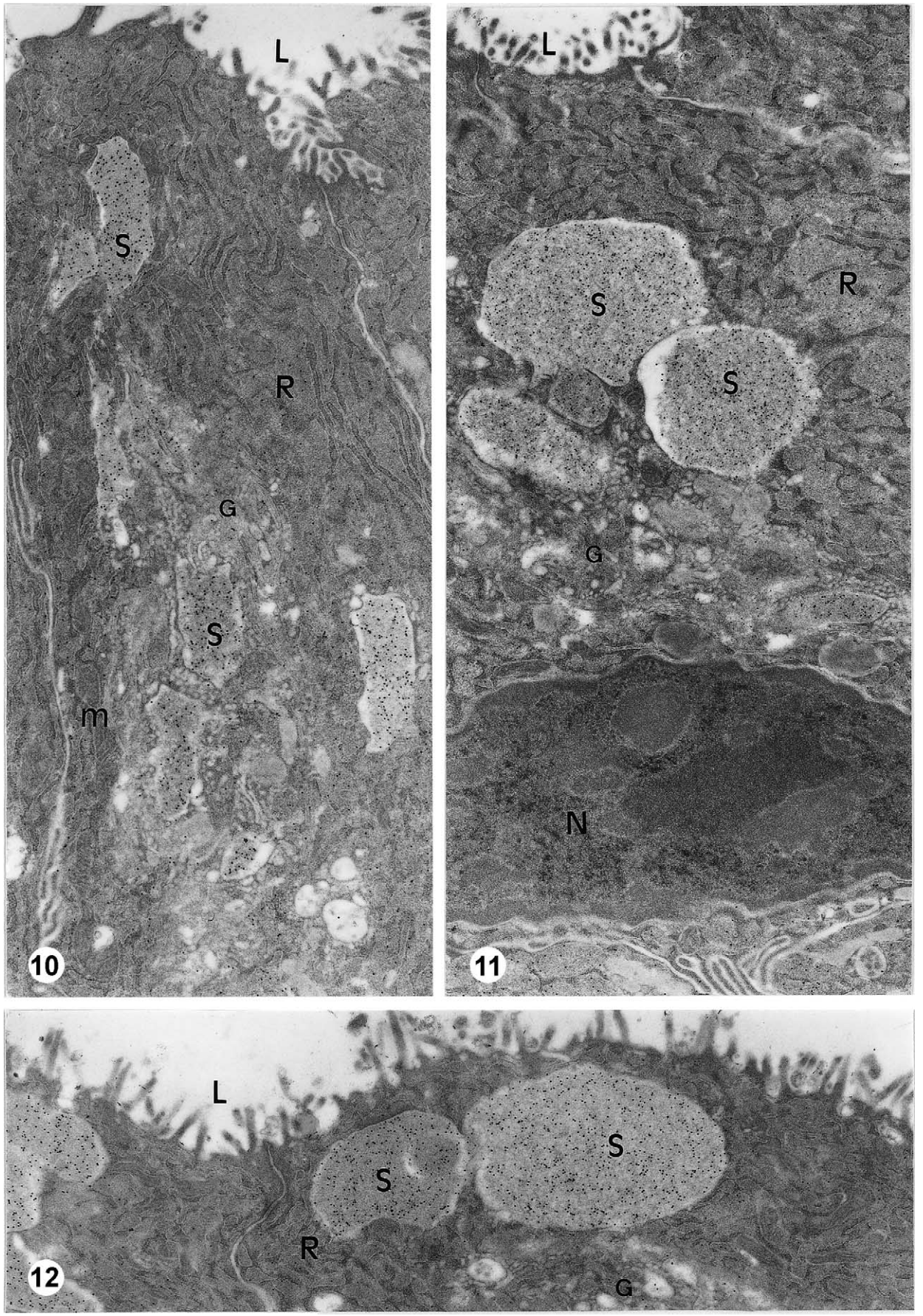
Controls were performed by using normal sera in place of the specific antibodies. The titres of anti-HF2, anti-HF3 and bothropasin determined by enzyme-linked immunosorbent assay (ELISA) were: 1:32 000; 1:2 048 000 and 1:256 000, respectively.



Figs 1–3 Adult *B. jararaca*: venom gland 40 days after venom extraction. Immunogold particles are almost exclusively located over the secretory vesicles (S). There is no specific labeling over the rough endoplasmic reticulum (R), nucleus (N), mitochondria (m) and connective tissue (C). Some gold particles are seen over the tubular lumen (L). Labeling pattern of bothropasin. **Fig. 1** ×21 500; HF3. **Fig. 2** ×21 000, HF2. **Fig. 3** ×12 900.



Figs 4–6 Adult *B. jararaca*: venom gland 7 days after venom extraction. Immunogold labeling present over secretory vesicles (S), Golgi complex (G) and rough endoplasmic reticulum (R). Labeling pattern of bothropasin. **Fig. 4** ×11 400; HF3. **Fig. 5** ×12 900; HF2. **Fig. 6** ×15 500. **Figs 7–9** Adult *B. jararaca*: venom gland 7 days after venom extraction. Double-labeling experiments. Immunogold particles of 10 and 15 nm are co-localized over secretory vesicles (S) and rough endoplasmic reticulum cisterns (R). **Fig. 7** Bothropasin (10 nm gold) and HF3 (15 nm gold) ×25 800. **Fig. 8** HF2 (15 nm) and bothropasin (10 nm) ×32 000. **Fig. 9** HF3 (10 nm) and HF2 (15 nm) ×32 000.



Figs 10–12 Newborn *B. jararaca* venom secretory cells. Immunolabeling present over the large irregular secretory vesicles (S), rough endoplasmic cisterns (R) and Golgi complex (G). There is no specific labeling over the nucleus (N). L, tubular lumen; m, mitochondria. Labeling pattern of bothropasin. **Fig. 10** ×20 150, HF3. **Fig. 11** ×20 930, HF2. **Fig. 12** ×20 150.

Grids were viewed at the transmission electron microscope LEO 906E from the Laboratory of Cell Biology of Instituto Butantan.

Quantitative evaluations

Quantitative evaluations of gold particles were performed on eletronmicrographs enlarged at a final magnification of ×43 000. Profiles of 30 secretory vesicles for each studied protease were analyzed for each animal condition and for the control. After the evaluation of the areas occupied by the secretory vesicles by the point-counting method (Weibel, 1969), the number of gold particles (N_i) were counted over each secretory vesicle, and then, the number of gold particles per μm^2 (N_S) of secretory vesicle area (S_a) was evaluated: $N_S = N_i/S_a$ (Bendayan, 1984). The labeling present over mitochondria in the same cells, indicative of background was also evaluated. The statistical analysis were performed by applying the Tuckey–Kramer multiple comparisons test to the quantitative results.

Results

The metalloproteases bothropasin, HF3 and HF2 isolated from *B. jararaca* venom were localized by Protein A immunogold labeling at the electron microscope in the venom secretory cells of adult *B. jararaca* at the stages of high and low activity of protein synthesis and in the newborn gland.

The specific labeling for the three metalloproteases was similar and the pattern of labeling did not vary from cell to cell in the secretory epithelium. In the resting glands, immunolabeling was restricted to the electron dense secretory vesicles, (about 0.60 μm in diameter) localized at the apex of the cell and to the tubular lumina. The RER cisterns and the Golgi complex showed no significant labeling (Figs 1–3). Unlike the resting glands, the specific immunolabeling in the active glands was localized over the dilated lumina of RER cisterns as was the labeling over all the secretory vesicles (about 1–2.5 μm in diameter), Golgi complex and tubular lumina (Figs 4–6). Double-labeling experiments indicated co-localization of the three metalloproteases in the secretory vesicles, Golgi complex and in the RER (Figs 7–9). The newborn snakes presented venom glands with well developed RER, secretory vesicles with moderate electron dense content and Golgi complex, all of them labeled for the three metalloproteases, suggesting active protein synthesis. Some of the secretory vesicles (1–3 μm in diameter) are very irregularly shaped. As in the adult snakes, they were the most highly labeled compartment (Figs 10–12).

The results of quantitative evaluations of labeling densities over the secretory vesicles, obtained for each metalloprotease in the unmilked, 7-day milked and in the newborn glands are presented in Table 1. These quantitative evaluations demonstrate that the intensity of labeling was greater for bothropasin in the unmilked and 7-day milked glands followed by HF3 ($P < 0.001$). In the new born, values for HF3 and HF2 were significantly higher ($P < 0.001$) than those of the unmilked and 7-day milked glands, and HF3 labeling did not differ significantly from bothropasin. Few gold particles were seen over the control condition as well as over mitochondria or nuclei (<1 μm^{-2}).

Table 1 Intensities of labeling obtained for bothropasin, HF3 and HF2 over secretory vesicles from venom glands of *B. jararaca*, unmilked (40 days after milking), 7-day milked and from the newborn

	Bothropasin	HF3	HF2
Unmilked and unfed	439 ± 23.20	155 ± 10.10	21 ± 1.65
7-day milked	127 ± 5.71	52 ± 4.24	27 ± 4.07[a]
Newborn	259 ± 30.37	228 ± 7.76[b]	158 ± 38.16[c]

Gold particles/μm^2; mean values ± SEM. [a] Not significantly different from unmilked ($P > 0.05$). [b] Not significantly different from bothropasin ($P > 0.05$). [c] Not significantly different from HF3 ($P > 0.05$).

Discussion

Bothrops jararaca venom secretory cells are structurally similar to those of other viperids such as *Vipera palaestinae* (Ben-Shaul et al., 1971; Oron & Bdolah, 1978), *Vipera ammodytes* (Oron & Bdolah, 1973), *Crotalus durissus terrificus* (Warshawsky et al., 1973), *Crotalus viridis oreganus* (Mackessy, 1991) and *Bothrops jararacussu* (Carneiro et al., 1991). As in these snake species, during the first week after milking, the cisternae of the RER of *B. jararaca* secretory cells expand, resulting in an increase in cell volume and height. Secretory vesicles are seen at this stage at the trans-Golgi side. Thereafter, up to the 30th–60th days after milking, the venom is gradually accumulated in the gland lumen, the RER cisternae become narrowed, the secretory vesicles, when present, are located at the apex of the cell and the cell volume and height decrease (Carneiro et al., 1991).

Using immunofluorescence techniques it was noticed that venom antigens had a ubiquitous distribution over the secretory cells of venom glands from viperid snakes (Shaham & Kochva, 1969; Sobol-Brown, 1974; Shaham et al., 1974). In *B. jararaca*, using immunoelectron microscopic techniques, it was possible to find the proteases bothropasin, HF2 and HF3 in each of the secretory vesicles from all secretory cells examined. It was also possible to accurately localize these proteases throughout the entire classic secretory pathway (Palade, 1975). In the resting glands, no specific labeling was observed in the narrow intracisternal spaces of the RER. Studies of immunoprecipitation carried out by Oron and Bdolah (1973) in *V. palaestinae* showed that in the first day after venom extraction only 25% of the synthesized proteins correspond to venom proteins, while by the 8th day, almost all synthesized proteins are venom proteins. It was suggested that during the resting stage, secretory cells mainly synthesize structural proteins, which are necessary for the organelles expansion, while venom proteins are intensively synthesized in the active stage.

The consistent labeling of the three proteases over all the secretory vesicles in the three animal conditions, and over the RER cisternae and the Golgi complex in both newborn and

unmilked animals are evidence of the simultaneous presence of the three studied proteases in these cellular compartments. In the *B. jararaca* 7-day milked venom gland, at least bothropasin, HF2 and HF3 are also intermixed in the RER and in the Golgi, before co-packaging in the secretory vesicles, as observed in the double-labeling experiments. Co-packaging of secretory products in all secretory granules was observed in several mammalian exocrine glands (Kraehenbul et al., 1977; Herzog & Miller, 1976), differing in this way from endocrine cells and other cells like PMN leukocytes where different contents are distributed in different population of granules (Bainton & Farquhar, 1968). Recently we have detected acid phosphatase acivity, with the TEM, in secretory vesicles from the venom glands of *B. jararaca*, when cytidine 5′-monophosphate was the substrate but not when the substrate was β-glycerophosphate. The observed distribution pattern is compatible with the occurrence of co-packaging of acid phophatase with the above studied proteases in secretory vesicles (Carneiro et al., 2001). Lysosome enzymes have been localized to mature and immature pancreatic zymogen granules of several species (Tooze et al., 1991; Beaudoin & Grondin, 1992; Grondin & Beaudoin, 1996); they are secreted in parallel with digestive enzymes in a regulated pathway or they are sorted from the proteins of this pathway by the pinching off of clathrin coated vesicles occurring at the periphery of the maturing condensing vacuoles and involving the receptor for mannose 6-phosphate (Sesso et al., 1980; Beaudoin & Grondin, 1992; Grondin & Beaudoin, 1996; Sesso & Ferreira, 1997; Klumperman et al., 1998). In the very initial stage of maturation of the condensing vacuoles in the pancreatic acinar cell of the rat, the acid phosphatase positive material is distributed throughout the vesicle mixed with the non lysosomal secretory proteins (Fig. 13 of Carneiro & Sesso, 1987). In more advanced maturation stages the reaction for acid phosphatase is progressively concentrated at the periphery of the maturing granule (Sesso et al., 1990). This material is also seen inside budding vesicles at the condensing vacuole periphery (Sesso & Ferreira, 1997). The acid phophatase material observed in the secretory vesicles of *B. jararaca* venom glands is seen scattered throughout these organelles (Carneiro et al., 2001) reminiscing what occurs in the very early stage of maturation of the secretory granule in the rat pancreatic cell. In this aspect the venom secretory vesicle seems to correspond to an earlier stage of maturation of the mammalian pancreatic immature granule. On the other hand, there is accumulating evidence in the literature that lysosomes have capacity for regulated exocytosis after determined stimuli (Andrews, 2000). It remains a matter of speculation to imagine that the venom gland secretory vesicles could be a lysosomal compartment that have acquired capacity for regulated exocytosis.

Enzymes in digestive glands, are usually stored as non-active pro-enzymes (zymogens), and in the venom glands, no activation of venom enzymes was reported in gland homogenates. One of the interesting questions is how the proteins within the crude venom remain stable during storage in the venom gland in the presence of many proteolytic enzymes and yet are intact and functionally active immediately upon envenomation. Freitas et al. (1992) showed the presence of citrate in high concentrations, of about 140–160 mM, in snake venom. Later Francis et al. (1992) demonstrated that citrate is an endogenous inhibitor of snake venom enzymes in that it forms complexes with divalent metal ions. The presence of citrate may represent an important mechanism by which snakes protect themselves against the toxic effects of their own venom. Furthermore, in the crude venom of most crotalid snakes pyro-glutamic tripeptides are found in concentrations estimated to be in the millimolar to micromolar range. These pyro-glutamate peptides were observed to inhibit '*in vitro*' metalloproteases present in the venom (Robeva et al., 1991; Francis & Kaiser, 1992). On the other hand, Zhang et al. (1994) demonstrated the structural interaction of pyro-glutamate tripeptide (pyro–Glu–Asp–hhTrp) with the atrolysin c, a metalloprotease isolated from the venom of the diamond rattlesnake *Crotalus atrox*, through atomic resolution by X-ray crystallographic methods. Thus, we can suppose that, in the gland, the venom enzymes are in enzymatically active, but in an inhibited form due to the presence of such inhibitors. When the venom is injected into prey, enzymes may dissociate from inhibitors due to the venom dilution and this fact may allow their activation.

The quantitative results, in this study, indicated a non-parallel accumulation of the three proteases in the secretory vesicles of the adult glands since the intensity of labeling for bothropasin was significant higher than for HF3, and HF3 higher than HF2. In fact, bothropasin is a major component of the *B. jararaca* venom corresponding to about 2.1% of the total venom protein, while HF2 and HF3 are less than 0.5% each one (Mandelbaum et al., 1982; Mandelbaum & Assakura, 1988). The differences in protein composition of the venom in various viperid species is thought to be due to the asynchronic synthesis of secretory proteins that occurs during the first 2 days after venom extraction (Oron et al., 1978; Sobol-Brown et al., 1971, 1975; Taylor et al., 1986). The regulation of the production and secretion of venom in viperid snakes was thought to be somehow due to the amount of venom stored in the gland lumen (Oron & Bdolah, 1973). Recently, it was demonstrated that the sympathetic nervous system also plays an important role in the production and secretion of venom in *B. jararaca* and that stimulation of both α- and β-adrenoceptors is involved in protein secretion (Yamanouye et al., 1997).

In mammalian exocrine glands non-parallel secretion that occurs (occurring) in response to different physiological or pharmacological stimuli (Beaudoin & Grondin, 1992), was attributed to different populations of granules within cells (Mroz & Lechene, 1986), to different concentrations of various enzymes in the same zymogen granule (Bendayan et al., 1980) or to content variations in different acini or areas of the gland (Bendayan & Ito, 1979; Posthuma et al., 1986; Gingras & Bendayan, 1992). In our study we could not disclose any difference in labeling distribution pattern either among secretory vesicle populations nor in different areas of the venom gland.

Immunofluorescence studies on the carpet viper *Echis carinatus* (Taylor et al., 1986) demonstrated specific labeling within some horizontal cells, located at the basal epithelium, when antiserum against whole venom was used. These cells were thought to be secretory cell precursors (Shaham & Kochva, 1969). In our experiments, however, no specific labeling of the studied venom proteases was observed in the horizontal cells.

The venom gland of the newborn snake is fully differentiated. Its RER and Golgi complex are well developed. Most of the secretory vesicles are large, irregularly-shaped and electron-transparent. The three studied metalloproteases were distributed in the same pattern as in the adult active gland. The presence of immunolabel in the RER and Golgi complex indicates that active protein synthesis is occurring in that early stage of life. Some tubular lumen areas are filled with venom, showing that secretory vesicles stored during the late fetal period were exocytosed soon after birth, perhaps due to a natural compression of the gland. In the glands of the later embryonic stages, no venom is present in the lumen and the cytoplasm is replete with secretory vesicles (unpublished results). Similar irregularly-shaped electron-transparent zymogen granules have been reported in fetal pig pancreases (Lainé et al., 1996). This fact was attributed to a particular composition of the matrix associated to the absence of GP-2, a major membrane protein from secretory granules (Dittié & Kern, 1992). These light granules could be considered immature granules since they lack normal quantities of other enzymes with the exception of chymotrypsin.

In the *B. jararaca* newborn, the intensities of labeling for the hemorrhagic factors are increased in comparison to that of the adult specimens and are similar to that of bothropasin. Although there is no references about comparative hemorrhagic activities in *B. jararaca* adult and juveniles, Gutierrez et al. (1980) and Castro (1999) related an increased hemorrhagic activity in juveniles *Bothrops asper* and in the newborn *Bothrops neuwiedi*, respectively. In fact, marked differences on venom composition, immunological and enzymatic activities and local effects between adult and newborn or juvenile snakes were reported by several authors (Furtado et al., 1991). These ontogenetic variations were attributed at least in part to different feeding habits between juvenile and adult snakes (Daltry et al., 1996).

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