Histopathological and Ultrastructural Effects of δ-endotoxins of *Bacillus thuringiensis* Serovar *israelensis* in the Midgut of *Simulium pertinax* Larvae (Diptera, Simuliidae)

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The bacterium Bacillus thuringiensis *(Bt) produces parasporal crystals containing δ-endotoxins responsible for selective insecticidal activity on larvae. Upon ingestion, these crystals are solubilized in the midgut lumen and converted into active toxins that bind to receptors present on the microvilli causing serious damage to the epithelial columnar cells. We investigated the effect of these endotoxins on larvae of the* Simulium pertinax*, a common black fly in Brazil, using several concentrations during 4 h of the serovar* israelensis *strain IPS-82 (LFB-FIOCRUZ 584), serotype H-14 type strain of the Institute Pasteur, Paris. Light and electron microscope observations revealed, by time and endotoxin concentration, increasing damages of the larvae midgut epithelium. The most characteristic effects were midgut columnar cell vacuolization, microvilli damages, epithelium cell contents passing into the midgut lumen and finally the cell death. This article is the first report of the histopathological effects of the Bti endotoxins in the midgut of* S. pertinax *larvae and the data obtained may contribute to a better understanding of the mode of action of this bacterial strain used as bioinsecticide against black fly larvae.*

Key words: *Bacillus thuringiensis* serovar *israelensis* - *Simulium pertinax* - midgut - histopathology - ultrastructure

Bacillus thuringiensis (Bt) is a Gram-positive, aerobic bacterium, producing parasporal crystals containing δ-endotoxins responsible for its selective insecticidal activity (Knowles 1994, Schnepf et al. 1998) during the III to V step of sporulation. Upon ingestion by susceptible insects, these crystals are dissolved in the midgut lumen. Under the action of intestinal proteases, the endotoxins are converted into active toxins that bind to receptors present on the microvilli, causing strong damages to the epithelial midgut cells (Gill 1992, Aronson & Shai 2001, Ruiz et al. 2004).

Most of the simuliids are hematophagus insects; they are involved in the transmission of human onchocerciasis, mainly in the North of Brazil. *Simulium pertinax* is the most important species in Southeast region inducing a negative socio-economic impact. The frequent and intensive attacks by *S. pertinax* on the transitory populations in this area helps to reduce tourism during summer months (Gerais & Ribeiro 1986, Araújo-Coutinho 1995, Maia-Herzog et al. 1999, Cavados et al. 2001, Araújo-Coutinho et al. 2003).

After isolation of the *B. thuringiensis* serovar *israelensis* (Bti) by Goldberg and Margalit (1977) and its characterization by De Barjac (1978), several studies were realized confirming the action of Bti as a larvicide mainly for controlling *Simulium* and *Aedes* species (De Barjac 1978, Lacey et al. 1982, Charles & De Barjac 1983, Becker 1990, Araújo-Coutinho 1995, Mardini et al. 1999, Rabinovitch et al. 1999, Cavados et al. 2001, Regis et al. 2001).

Histophatological investigations in larvae of *S. vittatum* infected with *B. thuringiensis* serovar *kurstaki* HD 255 (Lacey & Federici 1979) and *S. variegatum* infected with Bti (Rey et al. 1998) demonstrated morphological lesions in the intestinal epithelium which exhibited swollen cells, degenerated brush borders, disorganized nuclei, enlargement of intercellular spaces and cell lysis.

The present report describes sequential changes in the midgut of *S. pertinax* larvae infected with increasing concentrations of δ-endotoxins of Bti using light and electron microscope methodologies, information relevant to black flies control.

MATERIALS AND METHODS

Bacterial strain - Bti strain IPS-82 (LFB-FIOCRUZ 584), serotype H-14 type strain of the Institute Pasteur, Paris. It was maintained in agar medium with metals-ANM at room temperature (Rabinovitch et al. 1975).

Culture medium - The bacterial biomass was prepared using a fermentation medium based on soya flour and metals (such as Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} and Ca^{2+}) developed in the Laboratório de Fisiologia Bacteriana, Departamento de Bacteriologia, IOC-Fiocruz (Cavados et al.1998, Rabinovitch et al.1998).

Inoculum and biomass production - Growth started with a pre-inoculum to reduce the duration of the lag-phase of bacterial growth. After inoculation in 125 ml Erlenmeyer flasks containing 50 ml of the medium, the flasks were incubated in a New Brunswick Scientific agitator

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Received 26 February 2004
Accepted 18 June 2004

series 25 D, at 175 opm and 30ºC for 6 h. Subsequently, 3 ml were transferred to 500 ml Erlenmeyer flasks containing 150 ml of the soya flour and metals medium and incubated as previously described for a further 72 h period. Once sporulation had reached a level of 95% of free spores and crystals, each culture was centrifuged (6000 g, 10ºC), the biomasses were kept in an amber container with the pH adjusted to 5.0 with propionic acid and then formulated (Rabinovitch et al. 1998).

Bioassay with S. pertinax larvae - *S. pertinax* larvae were collected in the Soberbo river in the municipality of Guapimirim, state of Rio de Janeiro. Field-collected larvae were maintained in chambers where the water was aerated by a continuous stream of air bubbles. Biological insecticide doses equivalent to 2, 4, 6 mg/l were applied to the different groups of larvae. The exposure times employed ranged from 1 to 4 h. Only live larvae were examined. At the end of each time period the larvae were observed under a stereoscopic microscope and the head and anal region were dissected and discarded (Cavados 2000). The remainder of the larval body was fixed and processed for observation using light and electron microscopy.

Light microscopy (LM) - Semi-thin sections were made from intestine samples previously embedded in Epon, stained with a methilene blue-azure II solution in phosphate buffer 0.2 M, pH 6.9 (Richardson et al. 1960, Humprey & Pittman 1974) and observed in a Zeiss Axiophot microscope.

Electron microscopy (TEM) - Samples of the intestine were fixed in 2.5% glutaraldehyde in 0.2M cacodylate buffer, pH 7.2, then washed in cacodylate buffer containing 7.2% sucrose, post-fixed in 1% osmium tetroxide for 1:45 h, dehydrated in graded acetone and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds 1963) and examined with a Zeiss EM-900 electron microscope.

RESULTS

The non-infected *Simulium* control midgut shows a well-preserved layer of epithelial cells. The ovoid shaped nuclei are located in the center of the cell (Fig. 1). Long and regularly placed microvilli border the midgut lumen (Fig. 2).

The midgut of the larvae exposed to Bti using 2 mg/l shows some cells presenting an irregularly structured brush border within 1 to 2 h (Fig. 3). The cells begin to be swollen by a slight vacuolization and increasing of secretion vesicles. (Fig. 4). This feature is confirmed when observed by ultrastructure (Fig. 5). From 3 to 4 h after applying the endotoxin, structural changes occur in some of the epithelial cells (Fig. 8), whereas other ones maintain its morphology (Fig. 7). In addition, cell groups observed at the basis of the epithelium suggest a beginning of tissue recovery (Fig. 6).

When the midgut of larvae exposed to 4 mg/l of the endotoxin is analyzed after 1 to 2 h, it shows increased morphological changes of the epithelium with most of the cells swollen, vacuolated, with an increased number of secretion vesicles and an irregularly disposed brush border (Fig. 9). After 3 to 4 h, the pathological effects are observed in nearly all of the intestinal cells. The epithelium presents detached cells also with bubble shape tips (Fig. 10) and cells with short and thick (Fig. 11), irregularly and modified microvilli (Fig. 12).

When 6 mg/l of the bioinsecticide are employed, the midgut can only be analyzed during the first hour of exposure to the endotoxin, since after this time all the larvae are dead. The structural disorganization of the intestinal epithelium is evident, showing cells without the characteristic morphology (Fig. 13), becoming elongated, presenting destroyed tips and sometimes budding into the intestine lumen (Figs 14, 15).

DISCUSSION

In our experiments, which were stopped after 4 h of exposure to the Bti endotoxin, *S. pertinax* larvae could survive when a low concentration of Bti (2 mg/l) was applied. Histological alterations of some columnar cells of the midgut epithelium during the exposure to the endotoxin were observed. Nevertheless, after 4 h of toxin action well preserved groups of cells located at the base of the epithelium next to the basal membrane (Fig. 6) indicated that cell recovery was in progress. Increasing the endotoxin concentration (4 mg/l) nearly all columnar-cells were affected after 3 h of exposure to Bti endotoxin and no preserved cell groups next to the basal membrane of the midgut could be detected, though the larvae had not yet died. When 6 mg/l of the endotoxin were applied, all the larvae died after 1 h.

Using Bti at a very low concentration (0.4 mg/l) during routine field application against *S. variegatum*, Rey et al. (1998) observed that 72 h after the beginning of the treatment all black flies died, but only 15.7% after 24 h of treatment. Lacey and Federici (1979) using a concentration of 10 mg/l of *B. thuringiensis* serovar *kurstaki* against *S. vittatum* larvae noted that mortality was increased by temperature elevation. In another experiment, Charles and de Barjac (1983) using 0.08 mg/l of purified Bti crystals against the larvae of *Aedes aegypti*, reported that all were dead after 10 h. Lahkim-Tsor et al. (1983) used 10 mg/l of Bti for *Ae. aegypti* larvae feading and found that the larvae died between 37 and 120 min after the beginning of exposure to the endotoxin. Regarding these experiments, the cytopathic effects observed in larvae midguts were proportional to Bti endotoxin concentrations applied and inversely proportional to the time of exposure. The endotoxin concentrations used in our experiments gave a clear idea of *S. pertinax* larvae resistance.

Ultrastructural observations showed that the first cell damages due to the Bti endotoxin in the *S. pertinax* larvae midgut were related to brush border microvilli degeneration. As illustrated in Percy and Fast (1983) using purified Bt crystal toxin (1 g/l) against silkworm larvae, the dissolution of cytoskeleton structures inside and at the basis of the microvilli were responsible for its decrease in size and further disappearance, when bubbles of cytoplasmic substances protrude into the midgut lumen as in *S. pertinax* (Figs 4, 7, 14). At this stage, before the cell death, the columnar cells appeared more elongated in light microscope observations (Fig. 9).

The use of *B. thuringiensis* endotoxins originated vacuolization of the midgut epithelial cells in the different

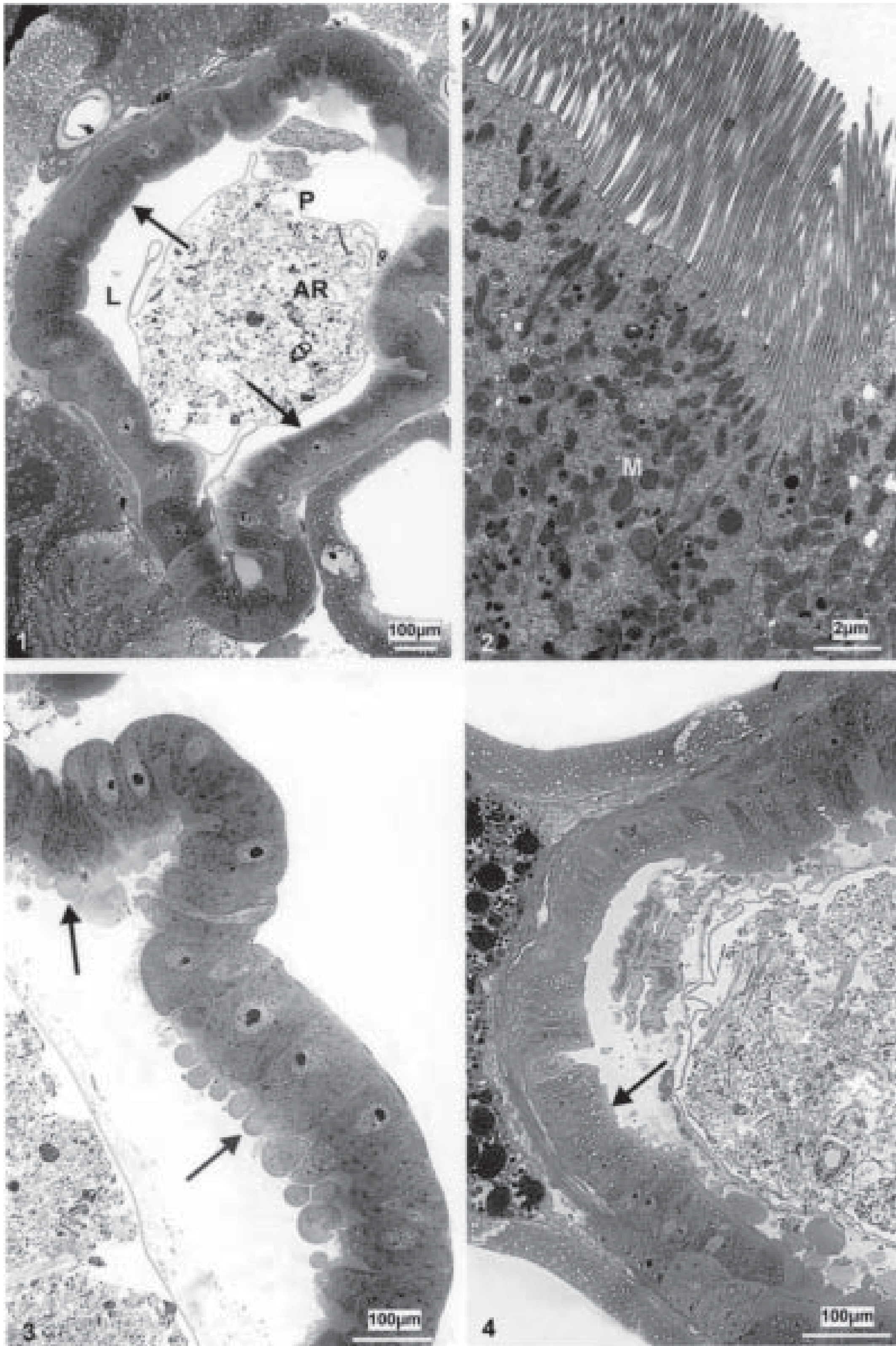

Fig. 1: the continuous brushborder (arrows) characterizes the midgut epithelium in control larvae of *Simulium pertinax*. L: intestinal lumen; P: peritrophic membrane; AR: alimentar residues; semithin section. Fig. 2: longitudinal section of microvilli of a columnar cell of the midgut in a control larva of *S. pertinax*. M: mitochondria; transmission electron micrograph. Fig. 3: columnar cells of the midgut of a larvae after treatment of 2 h with 2 mg/l of *Bacillus thuringiensis* serovar *israelensis* (Bti) suspension. Note cell swelling and strong emission of secreted bubbles (arrows). Fig. 4: columnar cells of the midgut of a larva after treatment of 4 h with 2 mg/l of Bti suspension. Note the strong vacuolization of columnar cells, partially non-altered brush borders (arrow) and strongly altered columnar cells with secreted bubbles.

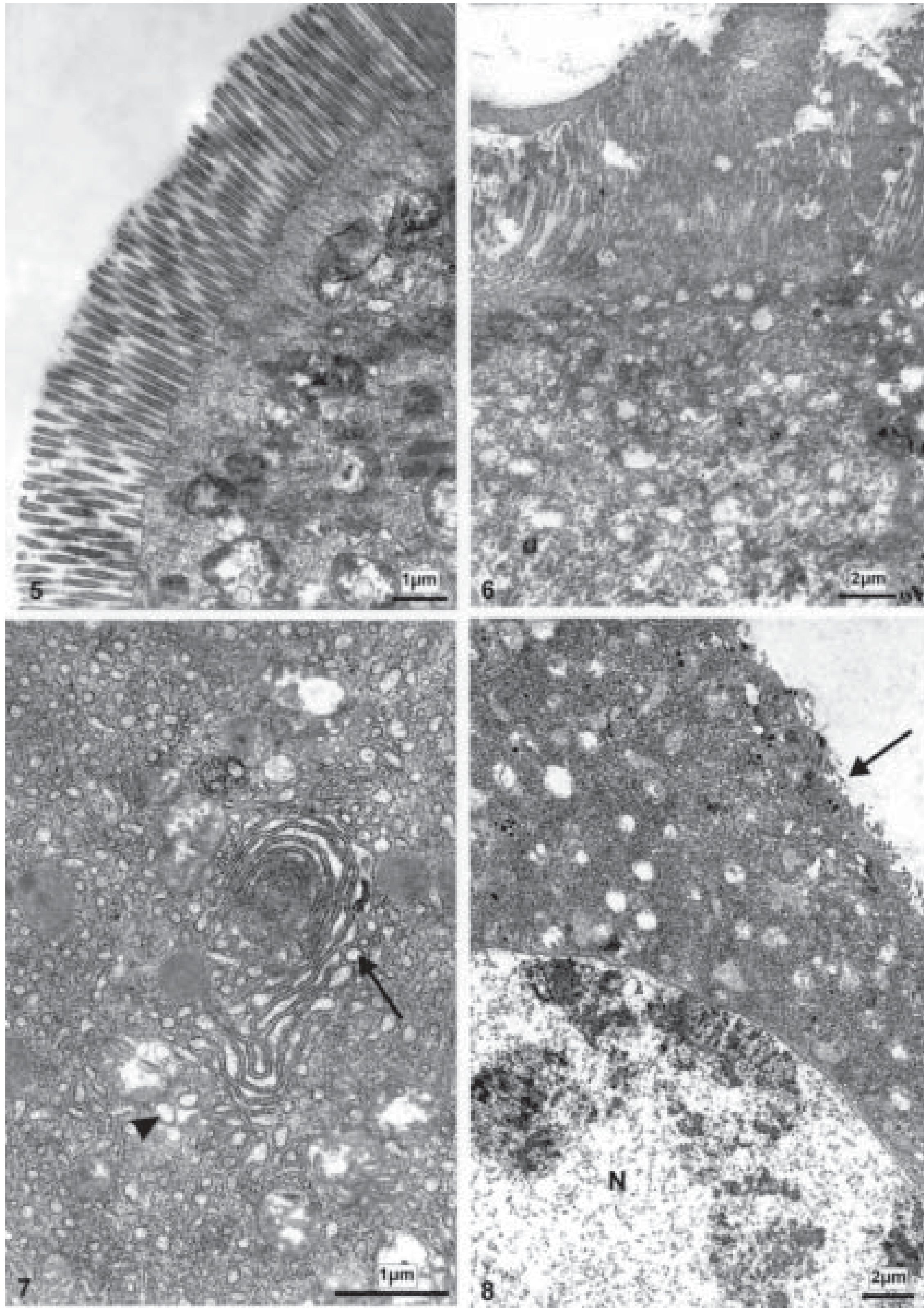

Fig 5: columnar cell of the midgut of a larva after treatment of 3 h with 2mg/l of *Bacillus thuringiensis* serovar *israelensis* (Bti) suspension. Note non-altered microvilli but strong alterations of cytoplasmic structure and organelles; transmission electron micrograph. Fig. 6: columnar cell of the midgut of a larva after treatment of 4 h with 2mg/l of Bti suspension. Note strongly altered microvilli and secreted substances between and also cell vacuolization. Fig. 7: columnar cell of the midgut of a larva after treatment of 2 h with 2 mg/l of Bti suspension. Note proliferation of rough endoplasmic reticulum derived vesicles (arrow) that in sequence lost their ribosomes (arrowhead). Fig. 8: columnar cell of the midgut of a larva after treatment of 2 h with 2 mg/l of Bti suspension. Note the fragmented chromatin inside the nucleus (N), vacuolization of the cytoplasm and destroyed brush border (arrow).

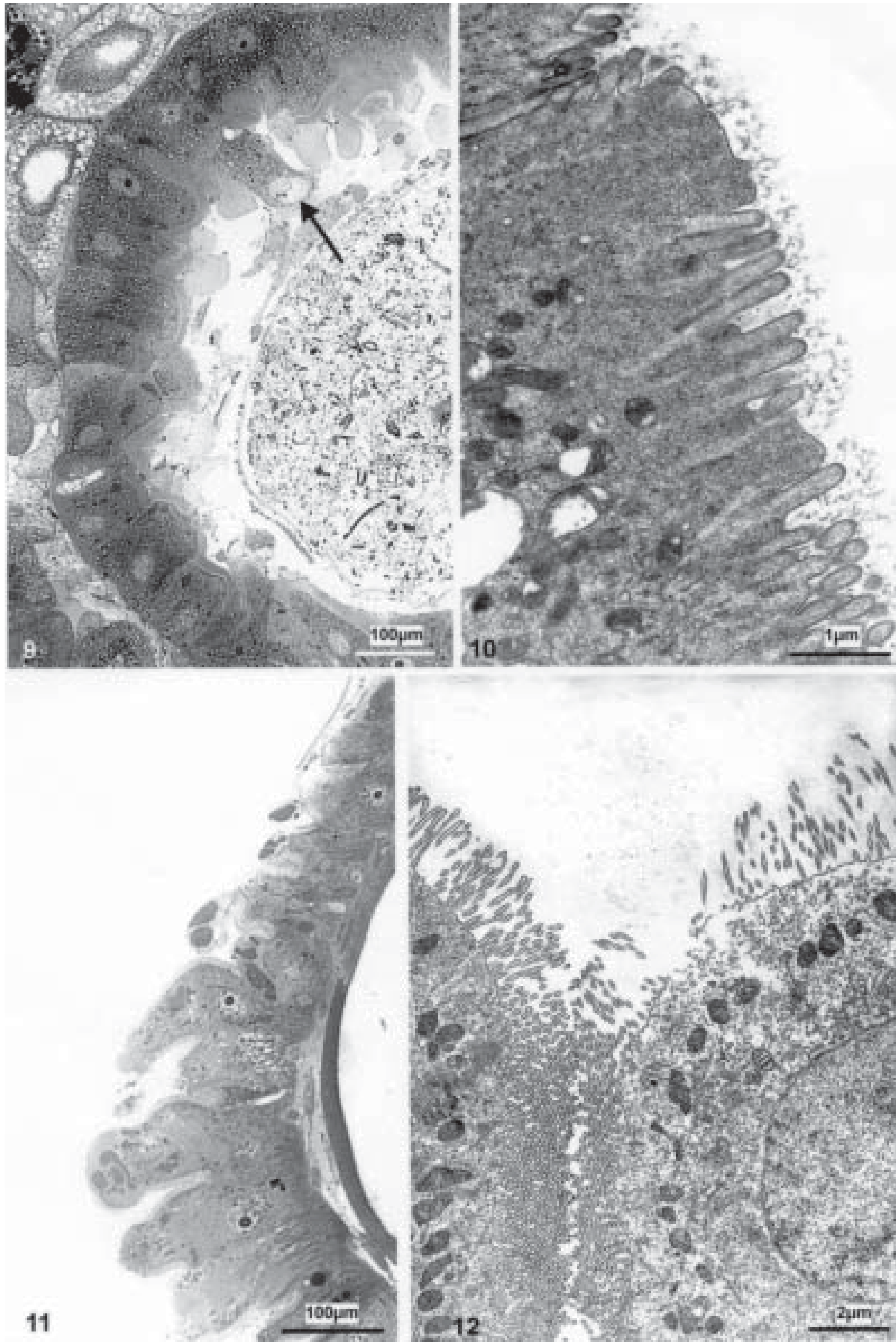

Fig. 9: columnar cells of the midgut of a larva after treatment of 1 h with 4 mg/l of *Bacillus thuringiensis* serovar *israelensis* (Bti) suspension. Note one elongated and detached columnar cell (arrow) and also disorganized nuclei and cytoplasmic structures; semithin section. Fig. 10: columnar cell of the midgut of a larva after treatment of 4 h with 4 mg/l of Bti suspension. Note swollen mitochondria, absence of plasmalema, disorganized and shortened and confluent microvilli membranes; transmission electron micrograph. Fig. 11: columnar cells of the midgut of a larva after treatment of 1 h with 6 mg/l of Bti suspension. Note that all cells are strongly modified, elongated and no continuously disposed brush border can be identified. Fig. 12: two adjacent columnar cells of the midgut of a larva after treatment of 1 h with 6 mg/l of Bti suspension. Note disorganized chromatin, alterated mitochondria and microvilli and cell individuation.

experimental models (Percy & Fast 1983, Charles & Barjac 1983, Rey et al. 1998), as also in *S. pertinax* (Figs 5, 8). These vacuoles proceeded from enlarged rough endoplasmic cisterns that had lost their ribosomes (Percy & Fast 1983).

This article is the first report of the histopathological effects of the Bti endotoxins in the midgut of *S. pertinax* larvae and the data obtained may contribute for better understanding the mode of action of this bacterial strain used as bioinsecticide against black fly larvae.

ACKNOWLEDGEMENTS

To Dr OM Barth, head of the Laboratory Electron Microscopy, Department of Virology, Instituto Oswaldo Cruz, for helpfull electron microscope utilities and critical review of the manuscript.

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