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Effects of *Bacillus thuringiensis kurstaki* on Malpighian Tubule Cells of *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae) Larvae

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In this study effects of *Bacillus thuringiensis kurstaki* (*Btk*) on Malpighian tubule cells of *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae) larvae was investigated by electron microscopy. 3 mg/l *Btk* was given with food. After *Btk* administration, the Malpighian tubule cells were investigated and compared with a control group. 3 and 6 hrs after *Btk* administration swelling in Malpighian tubule cells was observed. Swelling of mitochondria and separation of their cristae was seen after 12 hrs. After 24 hrs dissolution of the basal cytoplasm, swelling and vacuolization of all mitochondria, partial dissolution of the nucleoplasm, and swelling and separation of microvilli was documented. A membrane-body in the nucleus was seen after 48 hrs. The nucleoplasm was completely dissolved after 72 hrs and after 96 hrs large vacuoles appeared in the cytoplasm and shortening of microvilli was observed.

Key words: Malpighian tubules, *Bacillus thuringiensis kurstaki*, *Thaumetopoea pityocampa*, ultrastructure, transmission electron microscope.

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Thaumetopoea pityocampa, the pine processionary caterpillar, is the most important endemic pine pest in the Mediterranean area, not only because of its high defoliating power, but also due to human health problems caused by the urticating hairs of the larvae (RAUSELL *et al.* 1999). Thaumetopoein, a protein found on the hairs of *T. pityocampa* larvae, produces allergic reactions in humans and animals (NOVAK *et al.* 1987; REBOLLO *et al.* 2002). Although there is an increasing demand for environmentally friendly alternative methods, efforts to control this lepidopteran insect involve mainly the use of chemical insecticides, particularly insect growth inhibitors (RAUSELL *et al.* 1999). Insect viruses, fungi, pheromones, parasites and especially bacteria have been investigated for the biological control of *T. pityocampa* (BATTISTI *et al.* 1998). *Bacillus thuringiensis* (*Bt*) is a rod-shaped 1-1.2 micron, gram-positive, facultatively anaerobic, spore forming bacterium. During sporulation, it produces insecticidal crystal proteins or delta-endotoxins. *Bt* is very target specific, therefore field application can conserve beneficial predators, parasitoids, pollinators, birds, fishes, humans and ecosystems in general. More than 50 insect species belonging to the orders Lepidoptera, Diptera and Coleoptera are known to be susceptible to *Bt* (BURGES 1982).

The aim of the present study was to investigate the effects of *Bacillus thuringiensis kurstaki* (*Btk*) on the Malpighian tubules of *T. pityocampa* larvae.

Material and Methods

Insects

Larvae of *T. pityocampa* were collected in Kahramanmaras, Turkey. The larvae were fed pine needles (*Pinus nigra*) in the laboratory. Larvae were individually reared in the laboratory at 25 ±1 °C, 60 ±10% r.h. under a 12:12 photoperiod.

Bacillus thuringiensis

The commercial preparation which was selected is called MVP Bioinsecticide (Mycogen Corporation, USA). MVP is made of *Bacillus thuringiensis kurstaki* and has already been used for several years against Lepidoptera (KALENDER *et al.* 1999). Its concentration is 10000 IU/mg.

Treatment of insects

Tests were done on fourth instar larvae of *T. pityocampa*. *T. pityocampa* larvae are active at night, therefore experiments were initiated at night. Larvae were put on a diet 2 days before the beginning of the experiments. Larvae were divided into a control and test groups. 50 larvae were present in each group. *Btk* was diluted in distilled water and 3 mg/l was given with food to larvae. Fresh *P. nigra* needles were dipped in a suspension of *Btk*, air dried and placed in 10 cm plastic dishes. Larvae were placed into the plastic dishes. Fresh *P. nigra* needles were given to the control group. 3, 6, 12, 24, 48, 72, 96 h after *Btk* administration, larvae were dissected and prepared for electron microscopy.

Electron microscopy

For electron microscopic examinations of Malpighian tubules, primer fixation was made in 3% glutaraldehyde (Agar Sci. Ltd., Essex, England) in sodium phosphate buffer (200 mM, pH 7.4) (Merck, Alfred Paluka Co., Turkey) for 3 h at 4 °C. The material was washed with the same buffer and postfixed in 1% osmium tetroxide (Agar Sci. Ltd., Essex, England) in sodium phosphate buffer pH 7.4 for 1 h at 4 °C. Tissue samples were washed with the same buffer for 3 h at 4 °C and dehydrated in a graded ethanol series (Agar Sci. Ltd., Essex, England) and embedded in Araldite (Agar Sci. Ltd., Essex, England). Thin sections were cut with a Reichert OM U3 (Leica Co., Austria) ultramicrotome. Samples were stained with 2% uranyl acetate and lead citrate. The sections were viewed and photographed on a Jeol 100 CX II transmission electron microscope (TEM) (Jeol Ltd, Japan) at 80 kV.

Results

A single pair of Malpighian tubules is present in *T. pityocampa* larvae. Each tubule consists of three moniliform segments, which fuse proximally, forming a short common duct. This duct joins the alimentary tract at the junction of the midgut and hindgut. Each segment consists of proximal and distal regions. The proximal region extends alongside the midgut and hindgut, while the distal region lies freely in the haemocoel on the rectum.

In cross sections of Malpighian tubules of *T. pityocampa*, 5-8 cells can be found around the lumen. Malpighian tubule (Mt) cells, responsible for urine metabolism, are squamous (Fig. 1). Nuclei of Mt cells are near the basal region. A chromatin clump is scattered in the nucleus. On the luminal surface, the cytoplasm is evaginated forming closely packed microvilli. Mitochondria extend into most of the microvilli. Mitochondria, endo-

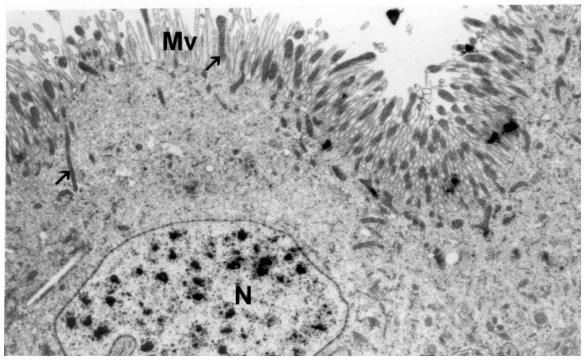

Fig. 1. Electron micrograph of a Malpighian tubule cell of control *T. pityocampa* larvae. Mv – Microvilli, N – Nucleus, → – Mitochondria, x 5500.

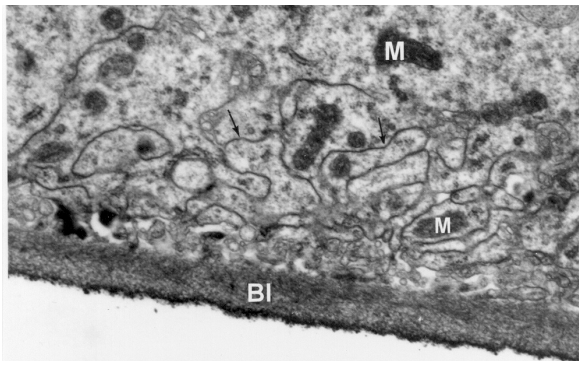

Fig. 2. Electron micrograph of basal plasma membrane (→) invaginations of Malpighian tubule cells of control *T. pityocampa* larvae. M – Mitochondria, Bl – Basal lamina, x 20000.

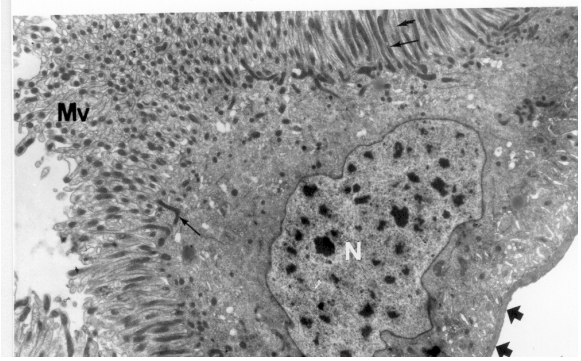

Fig. 3. Electron micrograph of weak swelling in Malpighian tubule cells of *T. pityocampa* larvae at 3 h after *Btk* treatment. Mv – Microvilli, N – nucleus, → – Mitochondria, ➡ – Basal lamina, x 5500

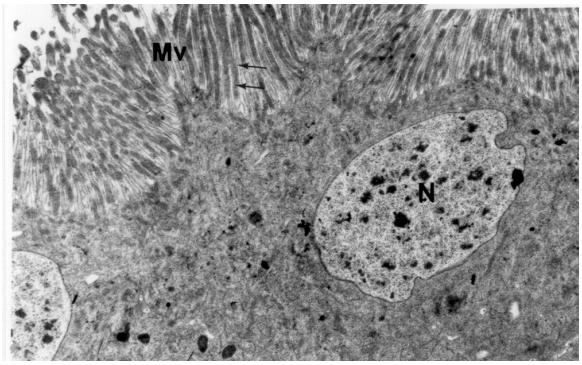

Fig. 4. Electron micrograph of weak swelling in Malpighian tubule cells of *T. pityocampa* larvae at 6 h after *Btk* treatment. Mv – Microvilli, N – Nucleus, → – Mitochondria, x 5500.

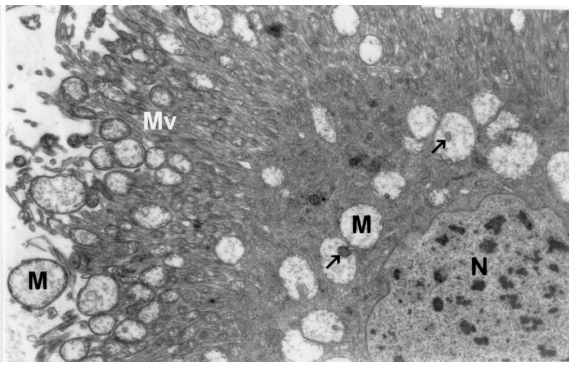

Fig. 5. Electron micrograph of swelling of mitochondria (M) and separation of their cristae (→) in Malpighian tubule cell of *T. pityocampa* larvae at 12 h after *Btk* treatment. Mv – Microvilli, N – Nucleus, x 7500.

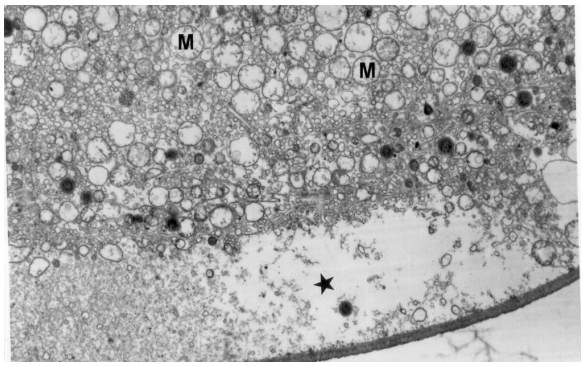

Fig. 6a. Electron micrograph of dissolution of the basal cytoplasm (★), swelling and vacuolization of mitochondria (M) in Malpighian tubule cells of *T. pityocampa* larvae at 24 h after *Btk* treatment, x 5000.

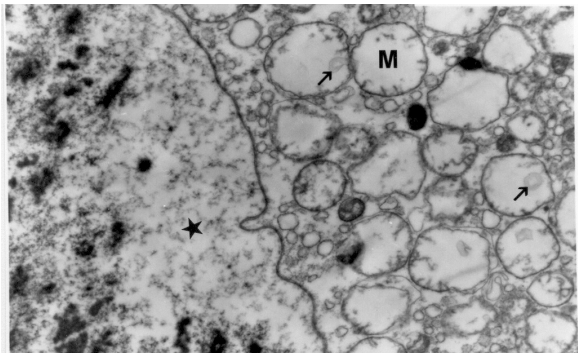

Fig. 6b. Partial dissolution of nucleoplasm (★) and swelling and vacuolization of mitochondria (M) and separation of their cristae (→) in Malpighian tubule cells of *T. pityocampa* larvae at 24 h after *Btk* treatment, x 12500.

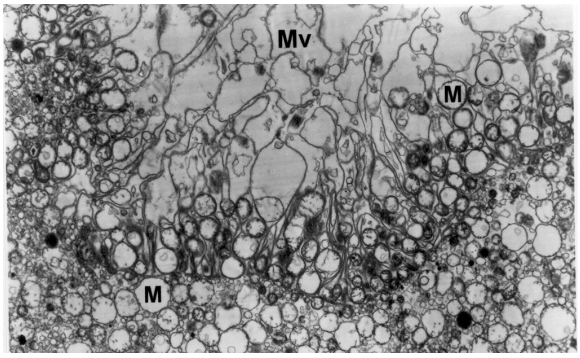

Fig. 6c. Electron micrograph of swelling and separation of microvilli (Mv) of Malpighian tubule cells of *T. pityocampa* larvae at 24 h after *Btk* treatment. M – Mitochondria, x 5000.

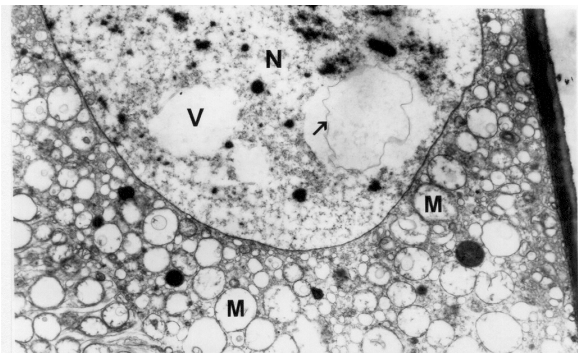

Fig. 7. Electron micrograph of swelling of mitochondria (M), membrane-body (→) and vacuole (V) in nucleus (N) of Malpighian tubule cells of *T. pityocampa* larvae at 48 h after *Btk* treatment, x 8000.

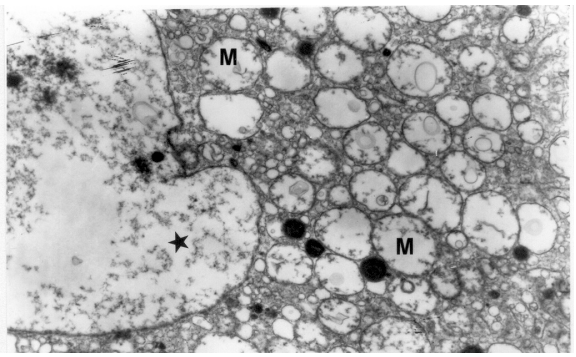

Fig. 8. Electron micrograph of completely dissolving nucleoplasm (★) of Malpighian tubule cells of *T. pityocampa* larvae at 72 h after *Btk* treatment. M – Mitochondria, x 10500.

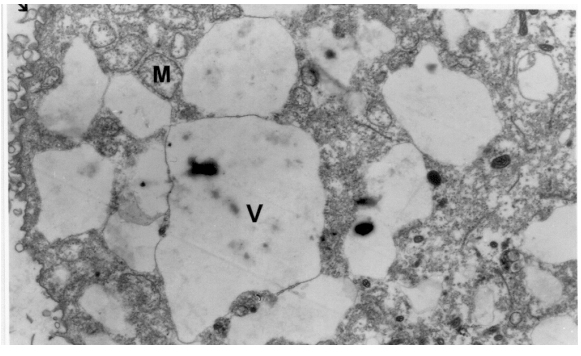

Fig. 9. Electron micrograph of large vacuoles (V) in the cytoplasm and shortened microvilli (→) in Malpighian tubule cells of *T. pityocampa* larvae at 96 h after *Btk* treatment. M – Mitochondria, x 7500.

plasmic reticulum and other organelles are found in the cytoplasm. The basal membrane of the cells is infolded with mitochondria among these infoldings. Mt cells are surrounded with a basal lamina from outside (Fig. 2).

3 and 6 h after *Btk* administration, swelling in Mt cells was observed. No pathology was detected in organelles (Figs 3 & 4). 12 h after *Btk* administration, swelling of mitochondria and separation of their cristae were observed (Fig. 5). 24 h after *Btk* administration, the basal cytoplasm of Mt cells dissolved, the swelling and vacuolization of all mitochondria (Fig. 6a), partial dissolution of the nu-

cleoplasm (Fig. 6b) and the swelling and breaking off of microvilli were observed (Fig. 6c). 48 h after *Btk* administration a membrane-body and vacuole in the nucleus was observed. Extreme swelling of mitochondria was recorded after 72 h (Fig. 7), and the nucleoplasm was completely dissolved (Fig. 8). 96 h after treatment, large vacuoles appeared in the cytoplasm and microvilli became shorter (Fig. 9). The color of larvae began to darken and their length shortened, they also become insensitive. Larval mortality was observed approximately after 72 h.

Discussion

In both humans and animals, *T. pityocampa* larvae cause symptoms such as atopy, urticarial dermatitis, oedema, conjunctivitis, dyspnea and anaphylactic reactions (ETKIND *et al.* 1982; EVERSON *et al.* 1990; VEGA *et al.* 1999; VEGA *et al.* 2003; KALENDER *et al.* 2004). Such symptoms are severe especially in people who already have allergic reactions. But so far, no death caused by caterpillars has been recorded (BURNETT *et al.* 1986). Two persons in our team have shown allergic reactions during the study. Blisters were observed especially on hands, arms and necks and hence they were kept in the hospital for two days. In addition, the same types of allergic reactions appeared on their family members. This occurred because hair of larvae can be transferred with clothes. Insect viruses, fungi, pheromones, parasites and especially bacteria (*Bt*) have been investigated for the biological control of *T. pityocampa* (BATTISTI *et al.* 1998). *Bt* is preferred because of its non-toxic activity to mammals and other organisms. *Bt* is a microbial insecticide that has been used for insect control with positive results (MATHAVAN *et al.* 1989; KALENDER & KALENDER 1995, 1997; RAUSELL *et al.* 2000). Researchers generally investigated effects of *Bt* on the midgut of insects (DELELLO *et al.* 1984; SINGH *et al.* 1986). Studies on Malpighian tubules are very scarce. Malpighian tubules are responsible from homeostasis in insects. The primary function of Malpighian tubules is the excretion of nitrogenous wastes from the haemolymph. They discharge a nearly isoosmotic secretion into the hindgut where this fluid may be modified by the selective absorption of useful substances (WIGGLESWORTH 1972; SOHAL 1974). Reabsorption of useful substances may also be augmented by the activity of cells located in the Malpighian tubules (WIGGLESWORTH 1972). The distal region of the Malpighian tubule in these insects transports water and solutes from the haemolymph to the lumen, whereas in the proximal region, some of the substances are reabsorbed from the lumen (KALENDER *et al.* 2001). *Btk* toxins cause paralysis, hinder feeding, induce changes in pH of the haemolymph, and alter the transport of K+ between the midgut and haemolymph. The cellular actions of the toxin have been related to its effect on membranes, either the plasma membrane or the mitochondria (DELELLO *et al.* 1984). In this study feeding of larvae stopped 12 h after administration of *Btk* because of the effects of *Btk* toxins. In the authors' opinion, pathological changes occurred in Malpighian tubules cells because of alterations of ion concentration between haemolymph and Malpighian tubules. In this study no ultrastructural changes were observed in plasma membranes of Malpighian tubules cells. However pathology was observed in organelles with cell membranes.

Bt produces a parasporal inclusion body during sporulation usually referred to as a crystal. This crystal is made of proteins. A large number of related crystal proteins are known and more than one protein type can co-assemble in one crystal. In an effort to overcome a somewhat confused situation, a classification of crystal proteins and their genes was proposed (HÖFTE & WHITELY 1989). This classification is based on the crystal protein structure and on the host range. More than 14 distinct crystal protein genes have been described, and recently additional insecticidal proteins have been identified (LERECLUS *et al*. 1993; JOUNG & COTE 2000). The crystal proteins exert their effect on the host by causing lysis of midgut epithelial cells, which leads to gut paralysis. The insect stops feeding and if it does not recover, it eventually dies. Upon ingestion, the crystals dissolve in the alkaline environment of the host insect midgut. The solubilized crystal protein or protoxin is proteolytically processed to produce the actual toxic fragment. The toxin binds to specific receptors present on the membranes of epithelial midgut cells. Finally the membrane-bound toxin induces the formation of pores in the midgut epithelial cell membrane. As a result of pore formation the cells die, eventually leading to death of the larvae (ARONSON *et al*. 1986; HÖFTE & WHITELEY 1989; LERECLUS *et al*. 1989; ADANG 1991; GILL *et al*. 1992; SULUDERE *et al*. 1992; BAUER 1995; JOUNG & COTE 2000). *Bt* endotoxin has been shown by light microscope immunofluorescence staining to bind to cells of the insect midgut and Malpighian epithelium (RYERSE *et al*. 1990).

Similar findings were observed in cell ultrastructure when *Bacillus thuringiensis* delta endotoxin was isolated and given to insects orally. The effect occurred after a short time, i.e. 60 min (SINGH *et al*. 1986; REISNER *et al*. 1989). Death, connected with toxin administration, was observed early. *Btk* that was used in this study is available commercially at a concentration of

10000 IU/mg. The excessive effect is observed 24th h after treatment. Ultrastructural changes were observed 3-6 h after. In conclusion, *Btk* not only affects the midgut epithelium of insects, but also causes pathologic changes in cells of Malpighian tubules. It disturbs the mechanism of oxidative phosphorilation and causes the dissolution of nucleoplasm, disordering of microvilli, swelling of mitochondria and vacuolization.

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