Mode of Action of *Bacillus thuringiensis* δ-Endotoxin: Changes in Hemolymph pH and Ions of *Pieris*, *Lymantria* and *Ephestia* Larvae

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The general pathology in terms of larval behavior and hemolymph physiology following the administration of *Bacillus thuringiensis* δ-endotoxin was studied on the so-called Type II insects, *Pieris rapae*, *Lymantria dispar* and *Ephestia cautella*.

After the impact of the toxin, larvae of the three species stopped feeding and died in 2–7 days. *Pieris* became gradually sluggish, suffering from vomiting and diarrhea, while *Lymantria* and *Ephestia* looked normal except for occasional vomiting and diarrhea in the former.

When the columnar cells of midgut epithelium showed the obvious histopathological changes corresponding to those of the paralyzed *Bombyx*, hemolymph pH changed from 6.8 to 7.4 accompanied by a rise in K⁺ level up to 2 times that of the normal hemolymph in *Pieris*. On the other hand, in both *Lymantria* and *Ephestia* there was no change of pH and rises of K⁺ levels of 1.4 times in *Lymantria* and 1.1 times in *Ephestia* were observed. Thus, the degree of rise in the pH and K⁺ concentration of hemolymph seemed to be associated with intensities of symptoms as well as cytoplasmic condensation which appeared in the goblet cells as follows: *Bombyx* > *Pieris* > *Lymantria* > *Galleria* > *Ephestia*.

INTRODUCTION

In previous papers, we have reported a sequential observation of ultrastructural changes in the larval midgut of *Bombyx mori* (ENDO and NISHIITSUTSUJI-UWO, 1980), *Pieris rapae*, *Lymantria dispar*, *Ephestia cautella* (ENDO and NISHIITSUTSUJI-UWO, 1981) and *Galleria mellonella* (NISHIITSUTSUJI-UWO and ENDO, 1981) following the administration of *Bacillus thuringiensis*. Sequential changes which appeared in the columnar cells were essentially the same among species: cells swelled losing microvilli and burst. On the other hand, two different changes among species were observed in the goblet cells: condensation of the cytoplasm, or/and disintegration or disappearance of cytoplasmic projections lining the goblet cavity. The former appeared strongly in *Bombyx* and *Pieris* and weakly in *Lymantria* and the latter was characteristic to *Lymantria*, *Galleria* and *Ephestia*.

The goblet cells are thought to be the principal agent of active potassium transport from hemolymph to the lumen of the midgut (ANDERSON and HARVEY, 1966). Therefore, we wondered if the different histopathological responses might reflect differences among the species in their physiological responses to the toxin. And yet,
there is little information about the changes in hemolymph pH and ion concentrations following the administration of the toxin except for silkworms and our Galleria. This paper describes the general pathology in terms of larval behavior and hemolymph physiology of so-called Type II insects following the administration of crystals of this bacterium.

MATERIALS AND METHODS

We used three species, the common cabbageworm, Pieris rapae, the gypsy moth, Lymantria dispar and the almond moth, Eiphestia cautella. The main work has been done with Pieris. Method for rearing the insects was previously described (Nishitsutsujii-Uwo and Endo, 1980b). Methods for cultivation of a sporeless mutant strain of B. thuringiensis subsp. aizawai and purification of crystals were reported elsewhere (Nishitsutsuji-Uwo et al., 1979).

Pieris rapae. A pure crystal suspension (25 µl containing 5 µg of crystals) was injected per os into a fifth-instar larva. Blood and gut juice were collected from four larvae each at 0, 15, 30, 60 and 120 min. Forced injection per os resulted in LD₅₀ for 24 hr 2.0 µg, for 48 hr 0.2 µg and for 72 hr 0.06 µg per larva.

Lymantria dispar. A pure crystal suspension (50 µl containing 50 µg of crystals) was injected per os into a fourth instar larva. Blood and gut juice were collected and pooled from two larvae at 0 and 120 min after the injection. Forced injection per os resulted in LD₅₀ for 24 hr 25 µg, for 48 hr 5 µg and for 72 hr 1.3 µg per larva of sixth instars.

Eiphestia cautella. Twenty-four hr after the administration of toxin-diet (2 mg crystals per gram of rice-bran), blood was collected and pooled from 100–120 living larvae of fifth instars. Under the microscope, a glass capillary was inserted into a wound made on an abdominal leg of each larva and blood was obtained by capillary attraction. As controls, normal blood from 100–120 larvae was collected. Gut juice was also obtained after dissection of the midguts of 100–120 normal larvae. Feeding test resulted in LC₅₀ for 3 days 1 mg, for 5 days 600 µg and for 7 days 175 µg per g diet (fourth instars).

Since blood has strong buffering action (Nishitsutsuji-Uwo and Endo, 1980a), use of N₂ gas seemed unnecessary when blood was collected. However, blood samples were prevented from oxidation by adding phenylthiourea (2.5 mg/ml) at 0°C. After the centrifugation, hemolymph and gut juice were kept at −20°C until used.

PH was measured by using micro-combination glass pH probes MI-410 (Microelectrodes Inc.) and ion concentrations were determined (courtesy of Mr. T. Dozaki of our Laboratories) using an atomic absorption spectrophotometer NF–IB (Toshiba-Beckman).

RESULTS

Symptoms

Soon after the administration of the lethal dose of the toxin, the Pieris larvae stop feeding. They become gradually sluggish in 2 to 4 days suffering vomiting and diarrhea, and then die curving the body backwards. Strong feeding-inhibition was observed even when the larva received a minor amount of the toxin. Although they do not show a typical general paralysis, their symptoms are very similar to those of
δ-Endotoxin: Changes in Blood pH and Ions

Bombbyx larvae (Nishitsutsuji-Uwo and Endo, 1980a) Tanada (1953) described similar observations in Pieris rapae, although at the time he had no knowledge of the crystal-toxin. Vaňková (1957) also observed similar symptoms in the larvae of Euproctis phaeorrhoea.

Cessation of feeding and occasional vomiting and diarrhea were also observed in Lymantria dispar, but they act normally for 3–5 days until death with an extreme softening of the body. These symptoms bear resemblance to those seen in Ephesia-larvae, which stop feeding and die after shrinkage of the body as noted by Nwanze et al. (1975).

Chemistry of the hemolymph

Generally speaking, pH and ion concentrations in hemolymph vary among species and even in the same species they change from stage to stage. Chemical composition of gut juice also differs with species, food and stages. However, hemolymph and gut juice of lepidopterous insects have some properties common to all. Hemolymph is a weak acid and gut juice is alkaline. In the hemolymph as well as in the gut juice, the K+ concentration is much higher than the Na+ concentration. The K+ level in midgut juice is much higher than that in hemolymph, while the Mg++ and Ca++ levels in gut juice are far less than those in hemolymph.

Pieris: As shown in Table 1, shortly after the forced injection of the pure crystals per os, K+ leven in hemolymph rose sharply as seen in Bombbyx (Nishitsutsuji-Uwo and Endo, 1980a) and within 60 min it was up to 2 times that of normal hemolymph. Thereafter, no rise in K+ was observed. The Mg++ concentration seemed to decrease gradually. Slight fluctuations in the level of Na+ and Ca++ were observed.

According to Heimpel and Angus (1959, 1960), there is no hemolymph pH increase in Type II insect, while the pH of gut juice falls slowly as it does in a starved insect. In our experiment, although the pH in gut juice decreased gradually for 2 hr from 8.3 to 7.5 as Heimpel and Angus found, the hemolymph pH increased from 6.8 to 7.4 within 60 min (followed by no more increase). At the histopathological

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<tr>
<th>Table 1. Changes in Hemolymph and Gut Juice of Fifth Instar Larvae of Pieris rapae Following the Injection Per Os of Crystals (5 μg/larva)</th>
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<td>No. of specimens</td>
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<td>Hemolymph</td>
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<td>toxin 15 min</td>
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* ±: standard error of mean.

b pH and ions of gut juice were measured in the pooled samples of four larvae.
point of view on the columnar cells of midgut epithelium, the 60 min-stage of Pieris corresponded to the stage 3 of Bombyx paralyzed completely (Endo and Nishitsutsuki-Uwo, 1981). When the collapse of the cytoplasmic projections was observed in many goblet cells (120 min), the rise of K+ level in hemolymph seemed to cease.

Lymantria: Although ultrastructural changes observed in the midgut epithelial cells 2 hr after the injection corresponded to those appearing in the midgut of the paralyzed Bombyx, the level of K+ in hemolymph was only up to 1.4 times that of normal blood and the pH and other ion concentrations did not change. A slight rise of K+ level in hemolymph might correlate with a weak condensation of cytoplasm observed in the goblet cells.

Ephestia: The pH and ions including K+ in hemolymph had scarcely changed in living larvae 24 hr after the administration of the toxin diet. It will be worth noting that in Ephestia the electron density of cytoplasm of the goblet cells was not changed.

DISCUSSION

The bulk of lepidopterous species are said to belong to Type II which suffers no blood pH increase and dies in 2 to 4 days without any symptoms of general paralysis (Hempel and Angus, 1959, 1960). In the present studies using three species of so-called Type II insects, Pieris rapae seemed to be allied to Bombyx mori and Ephestia cautella was akin to Galleria mellonella (Nishitsutsuki-Uwo and Endo, 1981) and Lymantria dispar was in between the two, on symptoms and hemolymph physiology after the administration of the toxin. Pieris larvae became sluggish and hemolymph pH rose from 6.8 to 7.4 accompanying a rise in K+ level up to 2 times that of normal hemolymph. In the stage when columnar cells of midgut epithelium showed the obvious histopathological changes corresponding to that of the paralyzed Bombyx, the hemolymph pH of Lymantria and Ephestia did not change and the K+ concentration rose up to 1.4 times in the former and 1.1 times in the latter those of normal hemolymphs. Thus, the degree of rise in the pH and K+ concentration of hemolymph seemed to be associated with symptomatic intensity as follows: Bombyx > Pieris > Lymantria > Galleria > Ephestia.

Fig. 1. shows a temporal sequence of change in K+ concentration of hemolymph on five species following the administration of δ-endotoxin.

The hemolymph of lepidopterous larvae have weak acid levels and the K+ concentrations are 25–40 mM, while the gut juices contain an enormous amount of K+ (150–300 mM). The hemolymph and gut juice are simply separated by the monolayer of midgut epithelial cells. The idea that δ-endotoxin would have damaged the midgut tissue through which gut juice flows into the blood may not be the case, since the degree of pH-increase is not parallel to an increasing (or decreasing) rate of any ion levels in the hemolymph. Moreover, increases of K+ and pH in the hemolymph were not necessarily dependent on K+ concentration and pH in the gut juice. For example, in Lymantria the K+ concentration of gut juice was twice as much and the pH was higher than in Pieris, and yet changes in hemolymph of the former following the impact of the toxin were far less than those of the latter.

A temporal sequence of histopathological changes which appeared in the columnar cells was the same among species, while changes which appeared in the goblet
cells varied in succession from specie to species. The intensity of the condensation of the cytoplasm of the goblet cells would probably reflect the degree of rise in $K^+$ concentration of hemolymph.

There is a sequential difference from species to species on general pathology induced by $B. thuringiensis$ including symptoms, hemolymph physiology, histopathology of midgut epithelium and the relative role of spores. Thus, to clearly classify the insects either to Type I, II or III seems to be difficult.

REFERENCES


