Immunocytochemical localization of the *Bacillus sphaericus* binary toxin components in *Culex quinquefasciatus* (Diptera: Culicidae) larvae midgut

Maria Helena N.L. Silva-Filha a, * and Christina A. Peixoto b, 1

**a** Departamento de Entomologia, Centro de Pesquisas Aggeu Magalhães-FIOCRUZ, Av. Moraes Rego s/n Cidade Universitária, Recife-PE 50670-420, Brazil

**b** Departamento de Patologia e Biologia Celular, Centro de Pesquisas Aggeu Magalhães-FIOCRUZ, Av. Moraes Rego s/n Cidade Universitária, Recife-PE 50670-420, Brazil

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Abstract

This study describes the immunocytochemical localization of the *Bacillus sphaericus* 2362 binary toxin components, BinA and BinB, in *Culex quinquefasciatus* larvae that had been intoxicated with this entomopathogen. Ultrathin sections of *C. quinquefasciatus* larvae midgut embedded in the hydrophilic resin L.R. White were incubated with the antibodies anti-BinA or anti-BinB and then revealed with goat anti-rabbit IgG coupled to gold particles. Immunocytochemical detection demonstrated the presence of specific labeling in ultra-thin sections that had been incubated with the BinA antiserum. Gold particles were detected on the apical areas of cell membranes and inside the epithelial cell cytoplasm, particularly the mitochondria, of cells from the gastric caeca and posterior stomach in larvae exposed during 2 or 24 h to the entomopathogen. A similar labeling pattern was observed in ultrathin sections from both midgut regions when incubated with BinB antiserum.

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1. Introduction

*Bacillus sphaericus* is a Gram positive, sporulating bacterium with insecticidal properties against Culicidae larvae and has been successfully used in mosquito vector control [1–4]. Its toxicity is attributed mainly to the production of a protein crystal upon sporulation that is toxic when ingested by some culicide larvae. The crystal contains a binary (Bin) toxin made of two polypeptides of 42- and 51-kDa [5,6], named BinA and BinB, respectively. The toxic effect on the epithelial cells from larval midgut is dependent on the synergy of these components, the optimal toxicity
level is achieved when the components are present in equimolar amounts [7–10]. Investigations into the mode of action of the *B. sphaericus* crystal toxin on *Culex pipiens*, a highly susceptible species, have demonstrated that following ingestion, the crystal is solubilized under the alkaline pH in the larval midgut, where the serine-proteases promote the proteolytic activation of the protoxin into toxin [11–13]. After ingestion of the crystal toxin, the ultrastructure of epithelial midgut cells is dramatically modified. The cytopathological alterations reported in *Culex* larvae include cytoplasm vacuoles and mitochondria swelling [14,15], in addition to damage observed in the neural and muscle tissues [16]. The interaction of the Bin toxin with *C. quinquefasciatus* larvae midgut, investigated through qualitative binding assays of the fluorescent-labeled toxin, demonstrated a distinct and regionalized toxin binding localized in the gastric caeca and posterior stomach [17,18]. The toxicity of *B. sphaericus* depends on the specific binding of the Bin toxin to a single class of receptors present in the midgut brush border membranes of mosquito larvae [19–21]. The Bin toxin receptor in the midgut cells of *C. pipiens* was identified and cloned as being an α-glucosidase of 60-kDa attached to the cell membrane by a glycosyl phosphatidyl inositol anchor, named Cpm1 [22,23].

Each component of the Bin toxin has distinct and complementary role on the interaction of the toxin on the *C. pipiens* midgut cell. BinB is responsible for the binding of the Bin toxin to the Cpm1 receptor present in the apical membrane of epithelial cells—its binding affinity is similar to that of the Bin toxin [24]. The component BinA, however does not show saturable and specific binding to those receptors [24]. On the other hand, the BinA component alone, can display in vivo toxicity to *C. pipiens* larvae when administered in high doses, a feature not displayed by BinB [10].

Even though many aspects concerning toxin binding to epithelial cells have been elucidated, events after binding and leading to the development of the toxic process in the cells have not been completely clarified. Previous in vivo localization studies of the fluorescent-labeled toxin on *C. quinquefasciatus* midgut, showed apparently internalization of the Bin toxin [17,18]. On the other hand, in vitro binding assays between Bin toxin and *C. pipiens* midgut brush border membranes, showed that bound toxin displays fast and almost complete dissociation, suggesting that toxin would not be able to insert in the plasma membranes [21]. Consequently, it has not been completely elucidated whether the Bin toxin is able to insert in *Culex* midgut cell membranes and it remains as a major question concerning the *B. sphaericus* mode of action. Recently an in vitro study has demonstrated that the Bin toxin can permeabilize artificial lipid membranes and that the BinA component was mainly responsible for pore formation [25].

This study is the first to localize antigenic sites to the components BinA and BinB of the *B. sphaericus* toxin in ultrathin sections of *C. quinquefasciatus* larvae midgut, providing insights into the events that may occur following the Bin toxin binding to cell receptors.

2. Materials and methods

2.1. Insects

The *Culex quinquefasciatus* 4th instar larvae used in this work were obtained from a 10-year-old colony from the insectarium of the Departament of Entomology, in the Centro de Pesquisas Aggeu Magalhães-FIOCRUZ. The insects were reared at 27°C, 70% relative humidity and with a 12:12 (L:D) h photoperiod. Larvae were reared in tap water and fed on cat biscuits. The adults were maintained on a 10% sugar solution and females were fed on *Gallus* sp.

2.2. Larvae intoxication

The *B. sphaericus* 2362 lyophilized reference powder (SPH88) from the Pasteur Institute was used in this work. A bacterial solution of 5000 mg/L was prepared and serially diluted. Groups of 20 early 4th instar larvae were placed in disposable cups each containing 100 ml of distilled water. Larvae were fed with a small amount of food and treated with the respective aliquot of the bacterial
suspension in order to provide a lethal concentration to kill 90% of larvae in a 48 h exposure (LC$_{90}$ = 0.074 mg/L), that was previously determined for that colony. Control groups, consisting of non-treated larvae, were kept in the same conditions as treated groups.

2.3. Dissection

Larvae treated during 2 or 24 h with *B. sphaericus* 2362, and non-treated larvae were recovered and dissected for transmission electron microscopy (EM) procedures. Ten larvae from each group, that were exhibiting normal behavior, were chilled on ice and the midguts were excised under a binocular microscope. After removing the peritrophic membranes and malpighian tubules, the gastric caeca and posterior stomach, which had been previously identified as the main target areas of the Bin toxin in the midgut epithelium of *Culex* [17], were processed for EM analysis.

2.4. Transmission electron microscopy

Specimens were fixed for 2 h at room temperature, or overnight at 4°C, in 2.5% glutaraldehyde and in 0.1 M cacodylate buffer, pH 7.2. After fixation, the samples were washed twice in the same buffer, and then they were post-fixed in a solution containing 1% osmium tetroxide, 2 mM CaCl$_2$, and 0.8% potassium ferriyanide in 0.1 M cacodylate buffer, pH 7.2. They were then dehydrated in acetone and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate.

2.5. Immunocytochemistry

The immunolocalization samples were washed twice with phosphate-buffered saline (PBS) and fixed for 60 min in a solution containing 0.1% glutaraldehyde and 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M cacodylate buffer, pH 7.2. After fixation, the specimens were washed twice with PBS, dehydrated in acetone, and embedded in L.R. White resin (Sigma, St. Louis, MO). The hydrophilic nature of this resin is highly suitable for labeling thin sections with colloidal gold probes. Polymerization was performed at 37°C for 5 days. Ultrathin sections from gastric caeca and posterior stomach were collected on 300-mesh nickel grids and incubated for 60 min at room temperature (RT) in PBS, pH 8.0, containing 0.1% bovine serum albumine and 0.1% Tween (PBS/BSA/T). The sections were then incubated for 1 h at RT with rabbit polyclonal antibodies anti-BinB or anti-BinA, diluted 1:250 in blocking solution (PBS/BSA/T). The antigens were obtained by separating *B. sphaericus* crystals on SDS–PAGE electrophoresis with subsequent protein transfer to a nitrocellulose membrane. The individual bands on the membrane, corresponding to BinA and BinB, were visualized with Ponceau's solution, ground into a powder and used as antigens to raise the rabbit polyclonal antibodies against each component. After incubation with anti-BinA or anti-BinB antibodies, the sections were then washed with PBS and incubated for 1 h at RT with goat anti-rabbit IgGs coupled with colloidal gold 10 nm (Sigma, St. Louis, MO). One control group consisted of thin sections of treated larvae midgut incubated only in the presence of the gold-labeled antibody. Another control group consisted of ultrathin sections of non-treated larvae midgut processed for immunocytochemistry and incubated with both first and second antibodies. After incubation, the sections were washed with PBS, followed by distilled water, and were counterstained with uranyl acetate and lead citrate. Sections were examined with a ZEISS EM 109 transmission electron microscope.

3. Results

Ultrathin sections of gastric caeca and posterior stomach from larvae, treated for 2 or 24 h with *B. sphaericus* 2362 (SPH88 powder), showed cytopathological alterations when compared to those groups that were not fed with the bacteria. The major alterations observed in cells from both midgut regions included the appearance of large cytoplasm vacuoles and the disruption of the rough endoplasmic reticulum producing numerous small vesicles (Figs. 1A and B). Other important lesions observed were epithelial swelling (not
shown) and the disruption of microvilli (Fig. 1B). In some cells the mitochondria presented a condensed matrix (Fig. 1A), while others showed a swollen appearance (Fig. 1B). Cell lysis and epithelial destruction were observed in gastric caeca and posterior stomach, following 24h of toxin exposure (not shown).

In this study, the incubation of thin sections of L.R. White-embedded gastric caeca and posterior stomach from larvae exposed for 2 or 24h in the presence of rabbit anti-BinA antibodies, demonstrated labeling of epithelial cells in both regions. In gastric caeca cells some gold particles were observed on apical membranes and also inside the cytoplasm, mainly within mitochondria (Figs. 2A and C). Labeled particles were also found in the microvilli and in their surrounding areas (Figs. 2A and B). A similar labeling pattern was obtained with thin sections of posterior stomach incubated in the presence of rabbit anti-BinA antibodies (Figs. 2B and D).

Specific immunolocalization of the BinB component in thin sections from gastric caeca and posterior stomach from treated larvae demonstrated a similar labeling pattern. In thin sections of gastric caeca from a 2-h-treated larvae, the gold particles were detected bordering the cell membrane, they were also observed inside the cells and some particles were directly related to the mitochondria (Fig. 3A). A homogenous labeling pattern was observed in the thin section of gastric caeca from 24-h-treated larvae, where gold particles were noted within the cytoplasm, inside large vacuoles produced by B. sphaericus toxic action (Fig. 3C). The presence of BinB was also detected in the cytoplasm of posterior stomach epithelial cells and neighboring other cell organelles (Figs. 3B and D). No reaction was observed when thin sections of untreated larvae were incubated in the presence of rabbit anti-BinA and anti-BinB antibodies followed by the gold-labeled antibody (data not shown), nor when the thin sections of treated larvae were incubated only in the presence of the gold-labeled antibody (Figs. 4A and B).

4. Discussion

Larvae treated with B. sphaericus showed cytological alterations in the cells from gastric caeca and posterior stomach, after 2 or 24h exposure. Those data ensured that the immunodetection of Bin toxin components was performed in an appropriated moment, since Bin toxic action was showed to be established in the larval midgut. The

Fig. 1. Ultrathin sections of C. quinquefasciatus: (A) Gastric caeca from larva treated for 2h with B. sphaericus. Note several cytoplasmatic vacuoles (thin arrows), condensed mitochondriae (arrowheads), and disruption of microvilli (short arrow). (B) Posterior stomach from larvae treated for 24h with B. sphaericus. Observe the disruption of the rough endoplasmic reticula producing inumerous small vesicles (thin arrows), condensed (arrowheads) and swollen mitochondriae (short arrow), and also disruption of microvilli (arrows). Bar = 1.0µm.
major cytopathological alterations observed in this work were large cytoplasm vacuoles, disruption of the rough endoplasmic reticula, epithelial swelling, and microvilli destruction, findings consistent with previous works on the toxic action of *B. sphaericus* on *Culex* larvae [15,16,26]. In the present study however, cell lysis and epithelial destruction were observed in gastric caeca and posterior stomach, from larvae exposed to *B. sphaericus* during 24 h. This feature was most commonly reported as a result of larvae intoxication with *B. thuringiensis* serovar. *israelensis*, a pathogen that displays a distinct mode of action on Culicidae larvae [27].

The results presented here demonstrate the immunological localization of the Bin toxin components, BinA and BinB, in thin sections of midgut cells of *C. quinquefasciatus* larvae intoxicated with *B. sphaericus* 2362. Both components of the binary toxin were detected in close interaction with the cell membrane and inside the cells of gastric caeca and posterior stomach from *C. quinquefasciatus* larvae. Previous works have shown that BinB is responsible for the binding of the toxin to the specific receptor present on the apical membrane of epithelial cells of midgut [19,24], allowing the component BinA to play a role on the toxic action. The post-binding events on the mode of action are
poorly understood and previous investigations, using in vitro systems, have suggested that Bin toxin might be able to form pores or channels in cell membranes. Data previously obtained suggested apparently internalization of fluorescent-labeled binary toxin in a C. quinquefasciatus midgut cells [17,18]. Investigation on the effects of the BinA and BinB on the membrane using cultured C. quinquefasciatus cells, with a patch clamp assay, and showed that the toxin can form pores or channels in the membrane [28]. Recently, in vitro assays proved, for the first time, that the Bin toxin could permeabilize receptor-free large unilamellar phospholipid vesicles and planar lipid membranes, by a mechanism of pore formation [25]. This work showed that BinA is more efficient than BinB to provoke pore formation. Although the BinB component forms pores to a lesser extent, it is able to improve the ability of BinA to permeabilize the large unilamellar vesicles. Nevertheless data from that study demonstrated that BinA is the component that displays the best pore-forming ability and this is probably its major role in the mode of action of the Bin toxin.

The present data on the immunolocalization of the BinA and BinB in midgut cells shows that the Bin toxin not only interacts with the cell apical membrane by specifically binding to the receptors

Fig. 3. Immunolocalization of the Bin-B component of the B. sphaericus toxin. Ultrathin sections of C. quinquefasciatus gastric caeca from larvae treated with B. sphaericus during 2 h (A) or 24 h (C). (A) Observe the gold particles lining the apical membrane (short arrow), and particles are also detected inside mitochondria (arrowhead). (C) Note gold particles inside large vacuoles produced by B. sphaericus toxic action (arrow). m, mitochondria. Ultrathin sections of C. quinquefasciatus posterior stomach from larvae previously treated during 2 h (B) or 24 h (D) with B. sphaericus. (B) Note labeling inside the cytoplasm (arrowheads). (D) The presence of Bin-B component is detected on apical membrane (arrows), and inside the cytoplasm (arrowheads). m, mitochondriae. Bar = 0.5 μm.
[19,22] and forming pores [25], as previously demonstrated, but it also acts directly inside the cells. This is the first report of the immunological detection of the Bin toxin, or its components, in the midgut cells from intoxicated larvae, at the ultra structural level. It is probable that after BinB binding, BinA promotes pore formation allowing the entry of both components into the cell. However, the intracellular role of BinB is not yet clear since in vivo and in vitro studies have shown that BinB alone could not exert toxic effects on *Culex* sp. cells. Its major role is due mainly to its ability to bind specifically to the membrane receptors and to mediate BinA action on pore formation [8,10,24,25].

Despite evidence demonstrating the presence of binary toxin in the midgut cells from intoxicated larvae, very little is known about the physiological effects of the toxin. Data on the action of the Bin toxin from strain 1593 on the mitochondria isolated from larvae treated with *B. sphaericus* showed that mitochondrial oxidative activity was reduced, in addition to a reduced production of the acetylcholine enzyme in the presence of the toxin [29]. These effects may explain the typical cytopathological swelling of mitochondria and the paralysis displayed by intoxicated larvae, respectively. Our immunocytochemistry assay demonstrated that the presence of the Bin components in midgut cells were often associated with mitochondria and, to a lesser degree, to the endoplasmic reticula indicating that the binding of the Bin toxin to a specific receptor might be the first step of a set of more complex interactions that occurs at the intracellular level. These results, in association with previous in vitro evidence demonstrating the pore-forming ability of the Bin toxin, contribute to the elucidation of the post-binding events. Further studies should be conducted to improve understanding of the mode of action of the Bin toxin on susceptible species and furthermore, to identify the key steps in this process that may result in the selection of resistant individuals.

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