APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1993, p. 1828–1837
0099-2240/93/061828-10$02.00/0

Vol. 59, No. 6

Two Different *Bacillus thuringiensis* Delta-Endotoxin Receptors in the Midgut Brush Border Membrane of the European Corn Borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae)

P. DENOLF,[1,2]* S. JANSENS,[1] M. PEFEROEN,[1] D. DEGHEELE,[2] AND J. VAN RIE[1]

Plant Genetic Systems, J. Plateaustraat 22,[1] and Laboratorium voor Agrozoölogie,[2] B-9000 Ghent, Belgium

Received 4 December 1992/Accepted 1 April 1993

Binding of three *Bacillus thuringiensis* insecticidal crystal proteins (ICPs) to the midgut epithelium of *Ostrinia nubilalis* larvae was characterized by performing binding experiments with both isolated brush border membrane vesicles and gut tissue sections. Our results demonstrate that two independent ICP receptors are present in the brush border of *O. nubilalis* gut epithelium. From competition binding experiments performed with ^{125}I-labeled and native ICPs it was concluded that CryIA(b) and CryIA(c) are recognized by the same receptor. An 11-fold-higher binding affinity of CryIA(b) for this receptor correlated with a 10-fold-higher toxicity of this ICP compared with CryIA(c). The CryIB toxin did not compete for the binding site of CryIA(b) and CryIA(c). Immunological detection of ingested *B. thuringiensis* ICPs on gut sections of *O. nubilalis* larvae revealed binding only along the epithelial brush border membrane. CryID and CryIE, two ICPs that are not toxic to *O. nubilalis*, were not bound to the apical microvilli of gut epithelial cells. In vitro binding experiments performed with native and biotinylated ICPs on tissue sections confirmed the correlation between ICP binding and toxicity. Moreover, by performing heterologous competition experiments with biotinylated and native ICPs, it was confirmed that the CryIB receptor is different from the receptor for CryIA(b) and CryIA(c). Retention of activated crystal proteins by the peritrophic membrane was not correlated with toxicity. Furthermore, it was demonstrated that CryIA(b), CryIA(c), and CryIB toxins interact in vitro with the epithelial microvilli of Malpighian tubules. In addition, CryIA(c) toxin also adheres to the basement membrane of the midgut epithelium.

Bacillus thuringiensis is a gram-positive bacterium that produces parasporal, crystalline inclusions during sporulation. These inclusions consist of proteins (insecticidal crystal proteins [ICPs] or delta-endotoxins) having molecular masses of 70 to 130 kDa. Most *B. thuringiensis* strains that have been characterized produce ICPs that exhibit highly specific activity against some lepidopteran (caterpillar), dipteran (mosquito and blackfly), or coleopteran (beetle) larvae (9, 23, 27). In addition, some recently discovered strains are reported to be toxic to protozoan pathogens, nematodes, and mites (8, 10).

Activity of ICPs against members of the Lepidoptera first requires solubilization of the ingested crystal proteins in the alkaline, reducing environment of the larval midgut. Then, solubilized ICPs (protoxins) are activated by midgut proteases, which convert the ICPs into smaller toxic proteins. Finally, binding of the toxins to midgut epithelial cell surface receptors induces pore formation, either directly or indirectly (22, 35). As a consequence, the osmotic balance of the midgut epithelial cells is disturbed. This induces cell lysis, gut paralysis, and larval death (6, 26, 29).

Receptor binding has been demonstrated to be a key factor in the specificity of ICPs (19, 36, 37). Different ICP types may bind to distinct receptors in one insect. ICP receptors have also been implicated in insect resistance to ICPs. In the case of *Plodia interpunctella*, CryIA(b) resistance was due to reduced binding affinity for this toxin (38). The CryIA(b) resistance of *Plutella xylostella* was also due to a modified interaction of the toxin with its receptor (11).

* Corresponding author.

B. thuringiensis ICPs are environmentally sound alternatives to chemical insecticides. It is expected that their use in insect control will increase, applied either as a spray of the natural organisms or in genetically modified organisms such as plant-associated bacteria, cyanobacteria, or transgenic plants. Therefore, selection pressure on target insects will probably increase. In order to forestall the development of resistance in natural insect populations, resistance management strategies are needed. The design of such strategies requires a thorough understanding of the mechanism of action of ICPs in agronomically important pests. In this study, we analyzed the presence of ICP receptors in the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae), the major lepidopteran pest of maize in southern Europe and North America (20).

MATERIALS AND METHODS

Purification and activation of *B. thuringiensis* ICPs. The CryIA(a), CryIA(b), CryIB, CryID, and CryIE ICPs were obtained as recombinant proteins expressed in *Escherichia coli*. CryIA(c) is produced as a single crystal protein by *B. thuringiensis* subsp. *kurstaki* (24). Details about gene sources and purification of protoxins and toxins are described in the accompanying paper (7).

Toxicity assays. Toxicity assays were performed with neonate larvae of *O. nubilalis*. Multiwell-24 plates (Costar) were filled with an artificial diet (32). (Pro)toxins were diluted in phosphate-buffered saline (PBS) (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl; pH 7.4) containing 0.1% bovine serum albumin (BSA). Five different concentrations were tested on 24 larvae each. Sample dilutions (50 μl) were

applied uniformly onto the food surface and allowed to dry. Two larvae were placed in each well. Levels of mortality were determined after 5 days. The toxicity data were analyzed by probit analysis (13).

Iodination of ICPs. CryIA(b) and CryIA(c) toxins were iodinated by using the chloramine T method (21). To 50 μg of purified toxin 1 mCi of $Na^{125}I$ and 20 μg of chloramine T were added. After gentle shaking for 30 s, the reaction was stopped with 12 μl of $K_2S_2O_5$. Free iodine and possible degradation products in the reaction mixture were removed by Bio-Gel P-30 (Bio-Rad Laboratories) column chromatography.

From each fraction one sample (10^5 cpm) was separated on a 10% sodium dodecyl sulfate–polyacrylamide gel (25). The dried gel was exposed to Fuji XR safety film to evaluate the integrity of labeled toxins.

Iodination of the CryIB toxin was accomplished by using the iodogen method (14). A 100-μl volume of an iodogen (Pierce Chemical Co.) solution in chloroform (0.1 mg/ml) was transferred with a pipette into a reaction tube and dried under a stream of nitrogen gas. After rinsing with Tris buffer (20 mM Tris, 150 mM NaCl; pH 8.65), 50 μg of toxin and 1 mCi of $Na^{125}I$ were added. The reaction was stopped after 7 min by adding 1 M NaI (one-quarter of the reaction volume). Purity and structural integrity were controlled in the same way that was used for the chloramin-T-labeled toxins.

Determination of specific activity of iodinated toxins. The specific activities of radioiodinated toxins were determined by a sandwich enzyme-linked immunosorbent assay (39) as described by Van Rie et al. (36). Microtiter plates were coated with polyclonal antibodies raised in rabbits against the CryIA(b), CryIA(c), and CryIB toxins. The secondary antibody was monoclonal antibody 4D6 [to detect CryIA(b) and CryIA(c)] or 22A2F1 (to detect CryIB). The specific radioactivities for CryIA(b), CryIA(c), and CryIB were 2.66, 2.33, and 0.46 μCi/μg of toxin, respectively.

Radioligand binding assay. Brush border membrane vesicles from *O. nubilalis* were prepared by the method of Wolfersberger et al. (40), as described in the accompanying paper (7). Binding assays were performed under standard conditions, as described by Van Rie et al. (36). To determine the optimal brush border membrane vesicle concentration for the binding experiments, different concentrations of vesicles (in PBS–0.1% BSA) were incubated for 60 min with ^{125}I-labeled toxins [0.13 nM CryIA(b), 0.14 nM CryIA(c), 0.77 nM CryIB] in the presence or absence of an excess of unlabeled toxin [750 nM CryIA(b), 735 nM CryIA(c), 833 nM CryIB]. In competition experiments, vesicles [20 and 10 μg in experiments performed with CryIA(b) and CryIA(c), respectively] were incubated with ^{125}I-labeled toxins [1 nM CryIA(b) and 2 nM CryIA(c)] and unlabeled toxins in PBS–0.1% BSA (total volume, 100 μl). After 30 min of incubation, bound and free toxins were separated by ultrafiltration through Whatmann GF/F glass fiber filters. The filters were immediately washed with 5 ml of ice-cold PBS–0.1% BSA. The radioactivity retained on the filters was measured with a gamma counter (model 1275 Minigamma; LKB Instruments). The datum points given below correspond to the average values from triplicate tests that differed by less than 10%.

Binding data were analyzed by using the LIGAND computer program (31). This program allows workers to assess on a statistical basis which model gives the best representation of experimental data (e.g., one-site model versus two-site model). A Student *t* test (17) was used to determine whether the values of the binding constants for the different toxins and receptors were significantly different.

TABLE 1. Toxicities of nonactivated *B. thuringiensis* ICPs (protoxins) and activated *B. thuringiensis* ICPs (toxins) to L_1 larvae of *O. nubilalis*[a]

Delta-endotoxin	LC_{50} (ng/cm²)	
	Protoxin	Toxin
CryIA(a)	NT[b]	1,247 (273.3–5,694.0)
CryIA(b)	50.8 (31.5–82.2)	50 (26.3–95.3)
CryIA(c)	NT	531.2 (317.2–889.7)
CryIB	81.2 (52.9–124.8)	105.8 (63.5–176.3)
CryID	NT	>1,350
CryIE	NT	>1,350

[a] LC_{50} values and 95% confidence limits (values in parentheses) were calculated by probit analysis. Doses are expressed in nanograms of (pro)toxin applied per square centimeter of artificial medium.
[b] NT, not tested.

Histopathological effects of ICPs. To visualize binding and the effects of *B. thuringiensis* ICPs, last-instar larvae of *O. nubilalis* were force fed with 10-μg portions (1 μg of ICP per μl of PBS) of the activated CryIA(b), CryIA(c), CryIB, CryID, and CryIE ICPs. Control larvae were fed with the same volume of a 0.1% BSA solution in PBS. Larval midguts were isolated 15 and 30 min and 1, 4, and 12 h after toxin administration.

Fixation, embedding, and sectioning were performed as described in the accompanying paper (7). To visualize histopathological ICP effects, tissue sections were stained by the Heidenhain's Azan method (5). Sections were first deparaffinated and hydrated (twice with 100% xylol for 5 min; three times with 100% ethanol for 3 min; once with distilled water for 1 min). Heidenhain's Azan staining results in a red coloration of epithelial cells and nuclei (dark red), while the brush border membrane is blue.

Immunocytochemical localization of ICPs. For the detection of ICPs in the midguts of intoxicated larvae, four rabbit polyclonal antisera were used; polyclonal antiserum IA recognizes the 60-kDa toxic fragments of CryIA(b) and CryIA(c), while polyclonal antisera IB, ID, and IE react with the toxic fragments of CryIB, CryID, and CryIE, respectively. The specificities and sensitivities of the different polyclonal antibodies were determined by a dot blot assay. The antibodies reacted very specifically with their antigens, and the detection limit for the four toxins was 5 ng.

Sections of intoxicated gut tissue were mounted on 0.1% poly-L-lysine-coated glass slides. After deparaffination and hydration, tissue sections were immersed twice for 2 min each time in lugol (0.5% I_2 and 1% KI in distilled H_2O) to remove $HgCl_2$. To bleach the preparations, the sections were incubated for 2 min in a 5% $Na_2S_2O_5$ solution. Finally, the sections were washed in distilled H_2O for 1 min and immersed in Tris-saline-Triton X-100 buffer (TST buffer) (10 mM Tris, 150 mM NaCl, 0.1 mM thimerosal, 0.1% Triton X-100; pH 7.6) for 5 min.

Prior to ICP detection, tissue sections from intoxicated larvae were treated with a solution containing 1 mg of trypsin (type XI; Sigma) per ml of TST buffer. This was done to unmask ICP epitopes that were hidden by intermolecular bridges formed during fixation (12). Subsequently, the sections were washed with TST buffer for 3 min, and the trypsin was inactivated by addition of a solution containing 0.5 mg of trypsin inhibitor (type II-S; Sigma) per ml of TST buffer. The

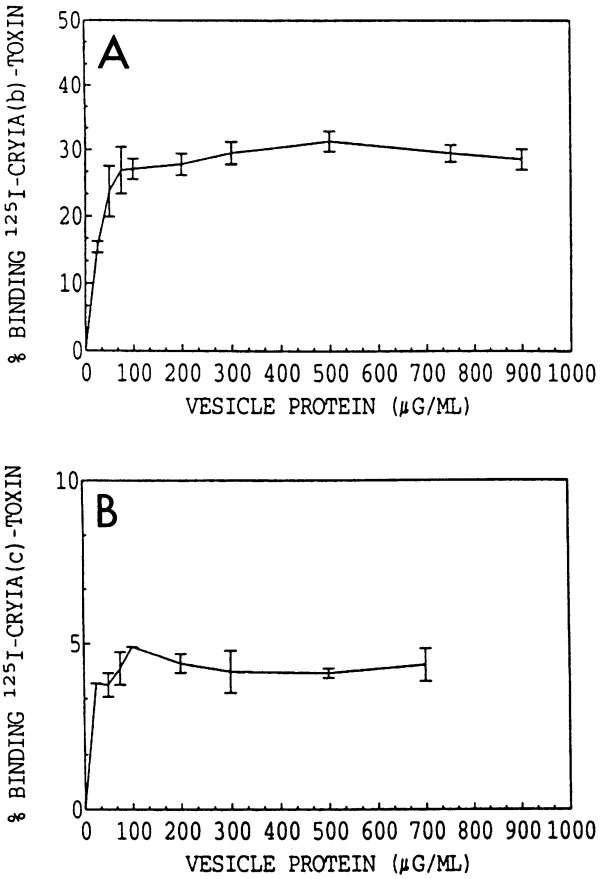

FIG. 1. Specific binding of ^{125}I-labeled CryIA(b) toxin (A) and CryIA(c) toxin (B) as a function of *O. nubilalis* brush border membrane vesicle concentration. ^{125}I-labeled toxins [0.13 nM CryIA(b) and 0.14 nM CryIA(c)] were incubated with vesicles in the presence or absence of unlabeled toxins [750 nM CryIA(b) and 735 nM CryIA(c)]. Samples were incubated for 60 min. Bound and free toxins were separated by using ultrafiltration through Whatmann GF/F filters. Nonspecific binding values were subtracted from total binding values. Each datum point is the mean of values from two independent experiments (two independently prepared batches of vesicles). Error bars were calculated by using the GraphPAD computer program (30).

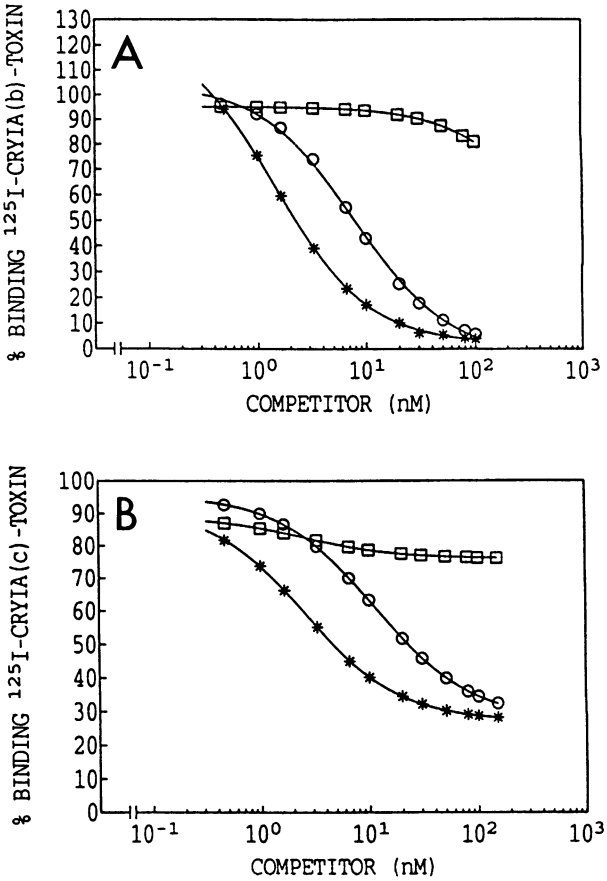

FIG. 2. Binding of ^{125}I-labeled CryIA(b) toxin (A) and CryIA(c) toxin (B) to brush border membrane vesicles of *O. nubilalis*. Vesicles were incubated for 30 min with ^{125}I-labeled toxins in the presence of increasing concentrations of unlabeled CryIA(b) (*), CryIA(c) (○), and CryIB (□). Levels of binding are expressed as percentages of the amount bound after incubation with labeled toxin alone. Nonspecific binding values were not subtracted. The curves are the curves predicted by the LIGAND and GraphPAD (30) computer programs. Each point is the mean of values from three independent experiments (three independently prepared batches of vesicles).

excess trypsin inhibitor was washed off with TST buffer for 3 min.

Immunolocalization of ICPs was performed by incubating tissue sections for 12 h with the appropriate purified polyclonal antiserum (0.5 µg of polyclonal antiserum IA, ID, or IE or 1 µg of polyclonal antiserum IB per ml of TST buffer; 300 µl of solution on each mounting glass) (2). The excess antibody was washed off with TST buffer for 5 min, and slices were incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody (5 µg/ml of TST buffer) for 1 h. Unbound secondary antibody was removed by washing with TST buffer for 5 min, and tissue sections were preincubated for 5 min in $MgCl_2$ buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$; pH 9.5). Finally, an alkaline phosphatase substrate solution (1.75 mg of 5-bromo-4-chloro-3-indolyl phosphate and 2.5 mg of nitroblue tetrazolium in 10 ml of $MgCl_2$ buffer) was added. The signal-to-noise ratio was optimal after 5 to 15 min of staining. The color reaction was inhibited by transfer of the sections to TST buffer.

To preserve stained tissues, the sections were dehydrated (70% ethanol for 15 s; 100% ethanol for 30 s [twice]; 100% xylol for 3 min [twice]) and covered with Entellan mounting medium (Merck).

In vitro binding of ICPs on tissue sections. The conditions used for in vitro binding experiments performed with native and biotinylated ICPs are described in the accompanying paper (7). Biotinylation ratios of 3.4 biotin molecules per ICP molecule were used.

RESULTS

Toxicity of *B. thuringiensis* ICPs to *O. nubilalis* larvae. Toxicity assays performed with neonate larvae of *O. nubilalis* showed that this insect was highly susceptible to the CryIA(b) toxin [50% lethal concentration (LC_{50}), 50 ng of CryIA(b) per cm^2] (Table 1). The toxicities of the CryIB, CryIA(c), and CryIA(a) toxins were 2, 10, and 25 times lower, respectively (LC_{50}, 105.8, 531.2, and 1,247 ng/cm^2, respectively). The LC_{50} values of CryID and CryIE ex-

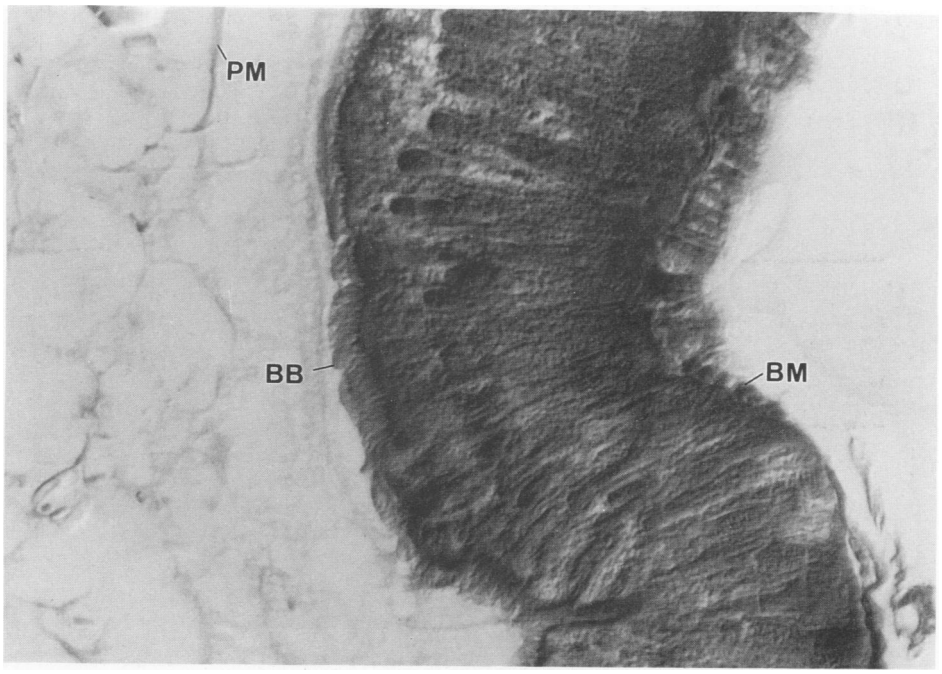

FIG. 3. Heidenhain's Azan-stained midgut epithelium of an L_5 larva of *O. nubilalis*. The epithelium is separated from the hemocoel by a basement membrane. The anterior part of the midgut epithelium is characterized by the presence of columnar cells. Columnar epithelial cells possess apical microvilli which make up the brush border. The brush border is separated from the gut lumen by the peritrophic membrane. BB, brush border membrane; BM, basement membrane; PM, peritrophic membrane. Light micrograph obtained with Nomarski differential interference contrast illumination. Magnification, ×500.

ceeded the highest concentration tested (1,350 ng of protein per cm^2), indicating that these crystal proteins had very low levels of toxicity or were not toxic.

The toxicities of the CryIA(b) and CryIB protoxins were comparable to those of the activated toxins (Table 1), demonstrating that the differences in the toxicities of the ICPs were not due to differential proteolytic activation in the larval midgut.

Binding of ^{125}I-labeled *B. thuringiensis* ICPs on brush border membrane vesicles of *O. nubilalis* larvae. To determine the optimal concentrations of brush border membrane vesicles to be used in competition experiments, ^{125}I-labeled toxins were incubated with increasing concentrations of vesicles. The level of binding in the presence of excess unlabeled ligand was subtracted from the total binding for each datum point (Fig. 1). For the CryIA(b) toxin, binding was maximal (32%) at a vesicle protein concentration of 500 μg/ml but comparable (28%) at a concentration of 200 μg/ml. In binding experiments performed with CryIA(c), 5% of the labeled toxin was bound at an optimum vesicle protein concentration of 100 μg/ml. In contrast to CryIA(b) and CryIA(c) binding, no specific binding of the CryIB ICP was found. Whereas unlabeled CryIB is highly toxic to *O. nubilalis*, toxicity tests with iodinated CryIB revealed only very slight growth reduction after 5 days (data not shown). Incorporation of ^{125}I apparently interferes with the toxicity of CryIB for the European corn borer and probably inhibits its binding to *O. nubilalis* tissue.

Homologous competition experiments (competition between a labeled ligand and its unlabeled analog) were performed with CryIA(b) and CryIA(c) to quantify their brush border membrane vesicle binding characteristics (Fig. 2). Processing of binding data by using the LIGAND computer program provided evidence of a single high-affinity binding site (K_d, 0.66 ± 0.33 nM) on the brush border membrane vesicles for the CryIA(b) toxin. The binding affinity for CryIA(c) to a single binding site was 11 times lower (K_d, 7.38 ± 2.0 nM). The binding site concentrations for the two toxins were not significantly different, as determined by Student's *t* test (17) [4.75 ± 1.7 and 3.3 ± 0.085 pmol/mg of vesicle protein for CryIA(b) and CryIA(c), respectively].

Heterologous competition experiments (competition between a labeled ICP and another, unlabeled ICP) were performed to determine the relationship between binding sites for different ICPs (Fig. 2). Iodinated CryIA(b) was completely displaced by unlabeled CryIA(c). Also, unlabeled CryIA(b) competed for the binding site of CryIA(c). In contrast, CryIB could not displace the labeled CryIA(b) and CryIA(c) toxins. Together with the results of homologous competition experiments, these data indicate that CryIA(b) and CryIA(c) recognize the same receptor on *O. nubilalis* midgut epithelium. The shift to the right of the CryIA(c) competition curve confirms the lower binding affinity of the CryIA(c) toxin for this receptor.

Immunocytochemical localization and binding of *B. thuringiensis* ICPs on the midgut epithelium of *O. nubilalis* larvae. Most cells of the anterior part of the *O. nubilalis* midgut epithelium are columnar with an apical microvillar border. Toward the posterior midgut half, the number of goblet cells increases. Both columnar and goblet cells stand on a basement membrane which is continuous all round the gut (Fig. 3). Epithelial cells are shielded from crude ingested food particles by the peritrophic membrane. This porous tube basically consists of a network of chitin fibrils set in a protein-carbohydrate matrix (4).

At 15 min after intoxication of *O. nubilalis* larvae with

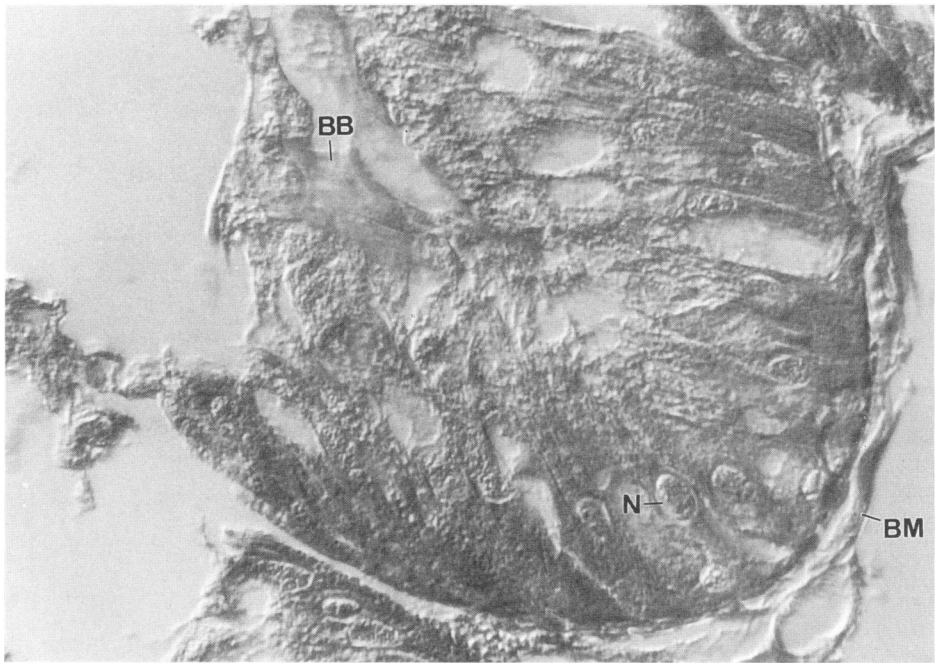

FIG. 4. Midgut epithelium of an L_5 larva of *O. nubilalis* 1 h after intoxication with CryIA(b) toxin. The histopathological effects include hypertrophy and vacuolation of epithelial cells and disruption of the microvillar brush border. The same histopathological changes were observed after intoxication with CryIA(c) and CryIB. BB, brush border membrane; BM, basement membrane; N, nucleus. Light micrograph obtained with Nomarski differential interference contrast illumination. Magnification, ×500.

CryIA(b), CryIA(c), and CryIB, epithelial cells already exhibited hypertrophy. After 1 h, vacuolation of cytoplasm took place, and the microvillar brush border membrane became disrupted (Fig. 4 and 5). Force feeding with nontoxic CryID did not affect regular membrane structure (data not shown).

An immunocytochemical analysis showed that the ingested toxic proteins were localized in the gut lumen and on apical microvilli of columnar cells throughout the gut (Fig. 5). Nontoxic CryID did not bind to the epithelial brush border membrane (data not shown).

In vitro binding of ICPs on tissue sections confirmed the results of the immunolocalization studies. Toxins were bound to the microvillar brush border throughout the midgut (Fig. 6A and 7). Again, nontoxic CryID and CryIE proteins did not bind to epithelial cells (Fig. 6B). Staining of the epithelial brush border was as intense following incubation with poorly toxic CryIA(a) toxin as it was after incubation with CryIA(b), indicating that there was a lack of correlation between toxicity and staining intensity (data not shown).

In vitro binding experiments performed with CryIA(c) and CryIB revealed that these toxins were retained by the peritrophic membrane of *O. nubilalis* larvae (data not shown). Treatment of tissue sections with blocking agents or addition of blocking reagents to the toxin solution gave the same results. CryIA(a), CryIA(b), and nontoxic crystal proteins were not retained.

CryIA(c) toxin also adhered to the basement membrane of the midgut and to microvilli of Malpighian tubules (Fig. 7). CryIA(b) and CryIB were also detected on microvilli of Malpighian tubules.

Biotinylated CryIA(b), CryIA(c), and CryIB produced the same binding pattern on tissue sections of *O. nubilalis* as the unlabeled ICPs (Fig. 8). Furthermore, heterologous competition experiments confirmed the results of the radioligand binding studies. In the presence of a 100-fold excess native CryIA(b), binding of biotinylated CryIA(c) to the microvilli of the midgut was completely inhibited. In contrast, a 100-fold excess of CryIA(c) could not completely inhibit the binding of biotinylated CryIA(b). Incubation of sections with biotinylated CryIB in the presence of a 100-fold excess CryIA(b) resulted in a staining pattern identical to the pattern obtained after incubation with biotinylated CryIB alone. Similar results were obtained from the inverse experiment. Finally, heterologous competition between biotinylated CryIA(c) and CryIA(b) or CryIB did not eliminate biotinylated CryIA(c) interaction with the basement membrane of the midgut.

DISCUSSION

In this study, experiments to determine binding of ICPs to both isolated brush border membrane vesicles and tissue sections were performed to investigate the interaction of *B. thuringiensis* ICPs with the larval gut of *O. nubilalis*.

Bioassays revealed the different toxicities of three crystal proteins, CryIA(b), CryIA(c), and CryIB. Two other lepidopteran-specific ICPs, CryID and CryIE, are not toxic to *O. nubilalis* larvae. The similar patterns of activity of two protoxins and activated toxins indicate that the differences in the toxicities of these ICPs to *O. nubilalis* are not due to differential proteolytic activation.

Whereas ^{125}I-labeled CryIA(b) exhibited a level of binding to brush border membrane vesicles of 32%, only 5% of the CryIA(c) [which is 10 times less toxic than CryIA(b)] was bound. Since the two ICPs had comparable specific radioactivities, these preliminary binding study results indicate

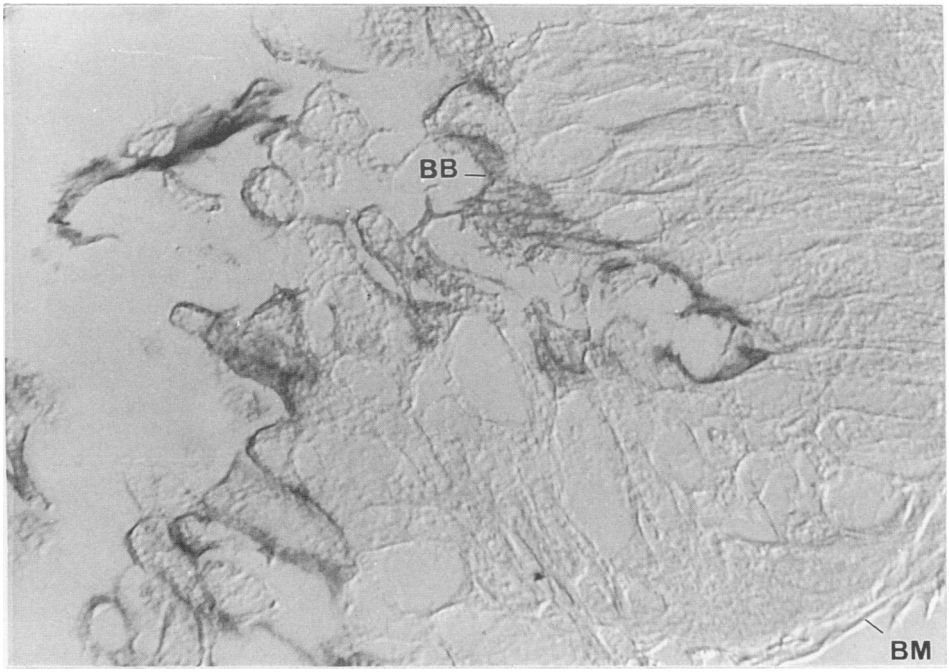

FIG. 5. Immunolocalization of force-fed CryIA(b) toxin in the midgut of an *O. nubilalis* L_5 larva. Midgut tissue was fixed 1 h after toxin administration. CryIA(b) is specifically localized on the disrupted brush border of gut epithelial cells. BB, brush border membrane; BM, basement membrane. Apparent increased shading of the basement membrane occurred because of uneven illumination across the micrograph. Light micrograph obtained with Nomarski differential interference contrast illumination. Magnification, ×500.

that there is a qualitative correlation between ICP potency and binding.

The results of homologous and heterologous competition experiments strongly suggest that CryIA(b) and CryIA(c) bind to the same receptor in the gut epithelium of *O. nubilalis*. Although ICP binding characteristics are not the only factors determining ICP potency (15), it seems likely that the 10-fold-lower toxicity of CryIA(c) compared with CryIA(b) can be attributed mainly to the lower affinity of CryIA(c) for the binding site.

No specific binding of radiolabeled CryIB toxin could be shown. Bioassays performed with iodinated CryIB indicated that there was a loss of toxicity of the labeled crystal protein. Incorporation of ^{125}I into tyrosine residues and the importance of these residues for toxicity of some ICPs (41) probably explain the observed reduction in toxicity. Problems with binding of radiolabeled CryIB were also reported by Ferré et al. (11). The use of radioligand binding experiments allowed us to demonstrate a lack of competition of unlabeled CryIB for bound, ^{125}I-labeled CryIA(b) and CryIA(c) toxins. The binding of CryIB could be demonstrated by performing in vitro binding experiments on tissue sections with native CryIB and biotinylated CryIB. Also, by performing immunolocalization studies we could visualize the binding of CryIB to the brush border membranes of intoxicated larvae.

Taken together, the results of radioligand binding studies and binding experiments performed with gut tissue sections suggest that two *B. thuringiensis* delta-endotoxin receptors are present in the *O. nubilalis* midgut brush border membrane. CryIA(b) and CryIA(c), two highly related crystal proteins, are bound by the same receptor, although with different affinities. In contrast, CryIB, which is another lepidopteran-specific crystal protein, is bound by a second type of receptor. This is consistent with the findings of Hofmann et al. (19) and Van Rie et al. (36, 37) that there is a family of distinct insect membrane receptors to which different toxins may bind.

In addition to CryIB binding, the immunocytochemical analysis also revealed binding and toxic effects of CryIA(b) and CryIA(c). For each ingested or in vitro bound toxin, binding to the microvillar brush border was shown. Nontoxic CryID and CryIE are not bound to the brush border membrane. The ICP binding pattern along the total length of the midgut indicates that there are uniformly distributed, membrane-associated molecules responsible for ICP binding. Bravo et al. (2, 3) also demonstrated that activated ICPs bind to the apical microvilli of epithelial cells along the larval midguts of *Manduca sexta* and *Plutella xylostella*. Visualization of ICP binding on insect tissue by immunocytochemical analysis allows a qualitative interpretation of ICP binding, providing data in addition to the quantitative analysis data provided by radioligand binding assays.

In vitro binding experiments showed that CryIA(c) and CryIB were retained by the peritrophic membrane of *O. nubilalis* larvae. CryIA(a), CryIA(b), and nontoxic CryID and CryIE were not retained. This finding supports the finding of Bravo et al. (2) that there is no correlation between toxicity and toxin retention by the peritrophic membranes of lepidopteran insects. Interaction of CryIA(c) and CryIB with the peritrophic membrane was not inhibited by the addition of protein blocking agents. In the presence of a 100-fold excess of native CryIA(b) and CryIB, biotinylated CryIA(c) and CryIB were not retained. These data indicate that aspecific protein-protein interactions are not responsible for the retention. Furthermore, the fact that CryIA(b) did not adhere to the peritrophic membrane indicates that retention by the peritrophic membrane is not due to an interaction

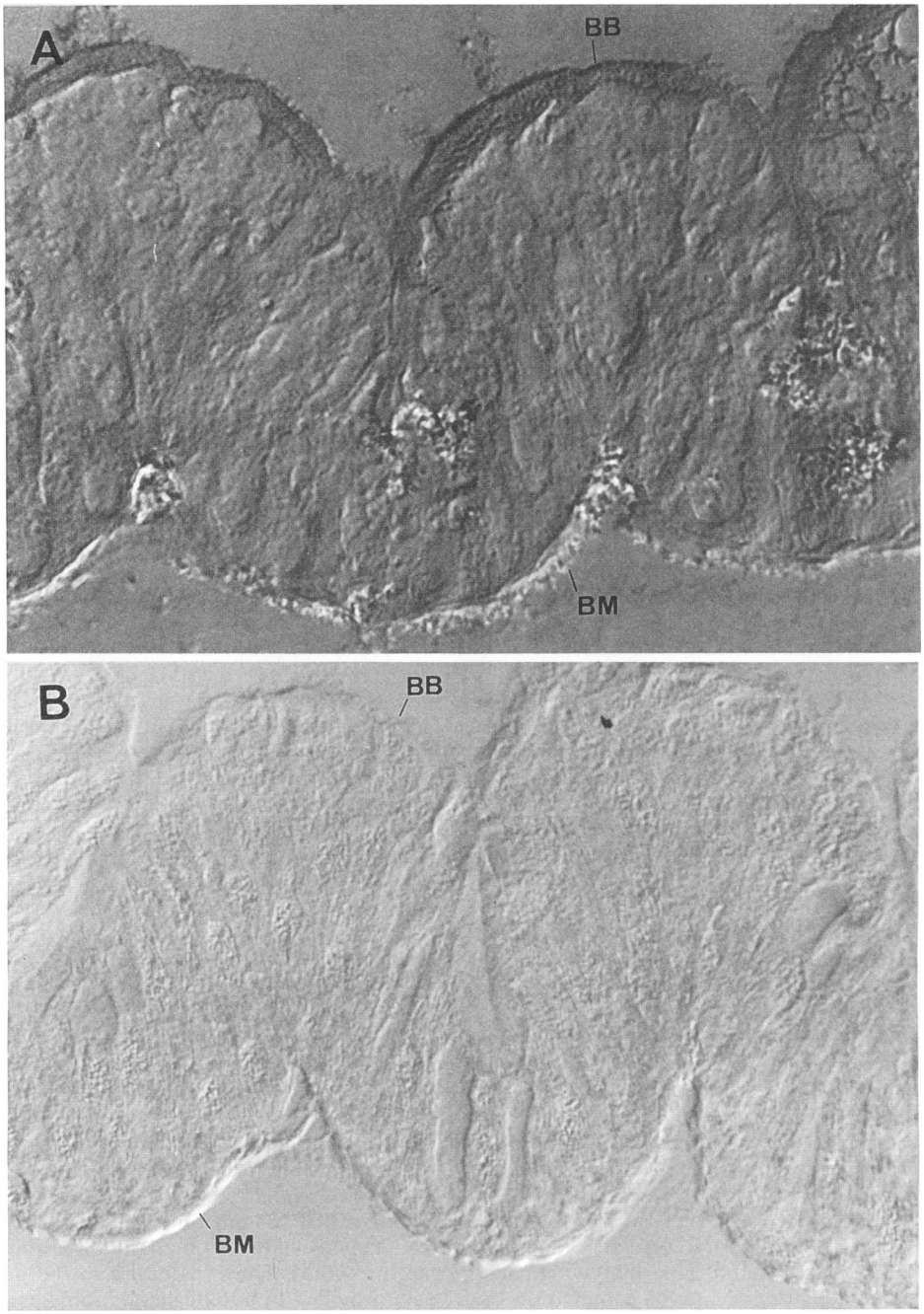

FIG. 6. Immunodetection of in vitro bound ICPs on *O. nubilalis* gut tissue. Midgut sections were incubated with toxic CryIA(b) (A) and nontoxic CryID(B). The CryIA(b) toxin was specifically bound to the microvillar brush border. Nontoxic CryID did not bind to brush border membrane. BB, brush border membrane; BM, basement membrane. Light micrographs obtained with Nomarski differential interference contrast illumination. Magnification, ×500.

with a molecule identical to the brush border membrane receptor. Quantitative binding experiments will be required to determine whether ICP interaction with the peritrophic membrane is saturable and has a high affinity.

In vitro adherence of CryIA(b), CryIA(c), and CryIB to the microvillar membrane of *O. nubilalis* Malpighian tubules seems to be consistent with the results of a previous report that demonstrated CryIA(c)-induced cytolysis of isolated Malpighian tubule epithelia of *Calpodes ethlius* (33). On the basis of their findings, Reisner et al. suggested that CryIA(c) can affect fluid-transporting epithelium other than midgut, at least in vitro. In vivo interaction of *B. thuringiensis* ICPs with Malpighian tubule microvilli of *O. nubilalis* could not be demonstrated 12 h after toxin administration, whereas midgut epithelial cells were affected 15 min after intoxication. Although further investigation will be required, it seems

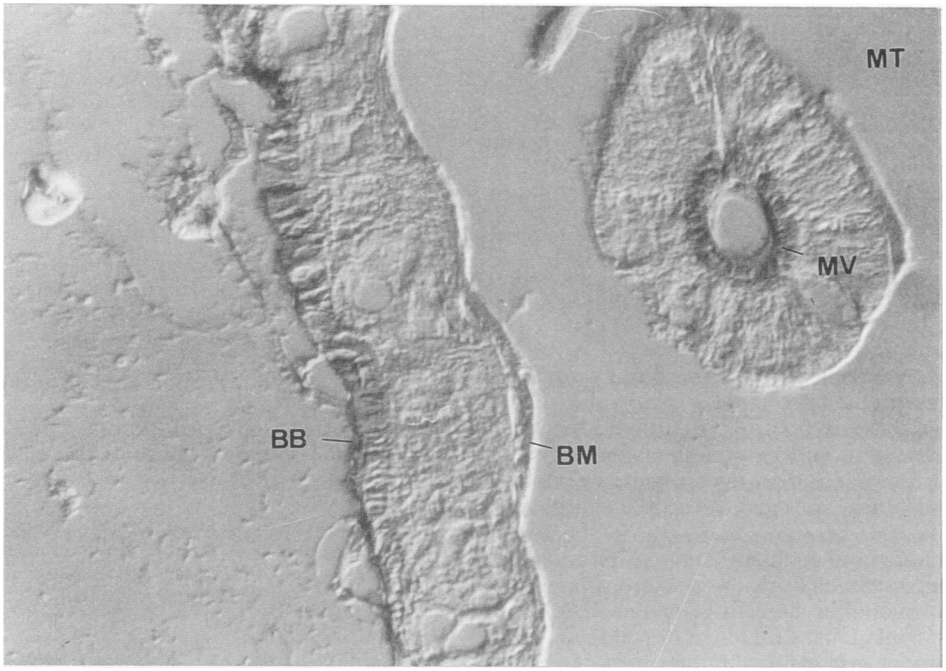

FIG. 7. Immunodetection of in vitro bound CryIA(c) toxin on *O. nubilalis* gut tissue. CryIA(c) not only binds to the epithelial brush border but also interacts with the basement membrane. Furthermore, CryIA(c) is also retained by the Malpighian tubule microvilli. BB, brush border membrane; BM, basement membrane; MT, Malpighian tubule; MV, microvilli. Light micrograph obtained with Nomarski differential interference contrast illumination. Magnification, ×250.

likely that ICP adherence to Malpighian tubules is not relevant for in vivo toxicity.

Immunocytochemical analysis of in vitro ICP binding also showed that CryIA(c) adheres to the basement membrane of the midgut epithelium of *O. nubilalis*. Reisner et al. previously investigated the effects of serosal (basal) CryIA(c) exposure on isolated *C. ethlius* Malpighian tubules (33). Studying fluid secretion and cell disruption of Malpighian tubules, these authors demonstrated that in vitro, CryIA(c) affects plasma membranes not only mucosally (apically) but

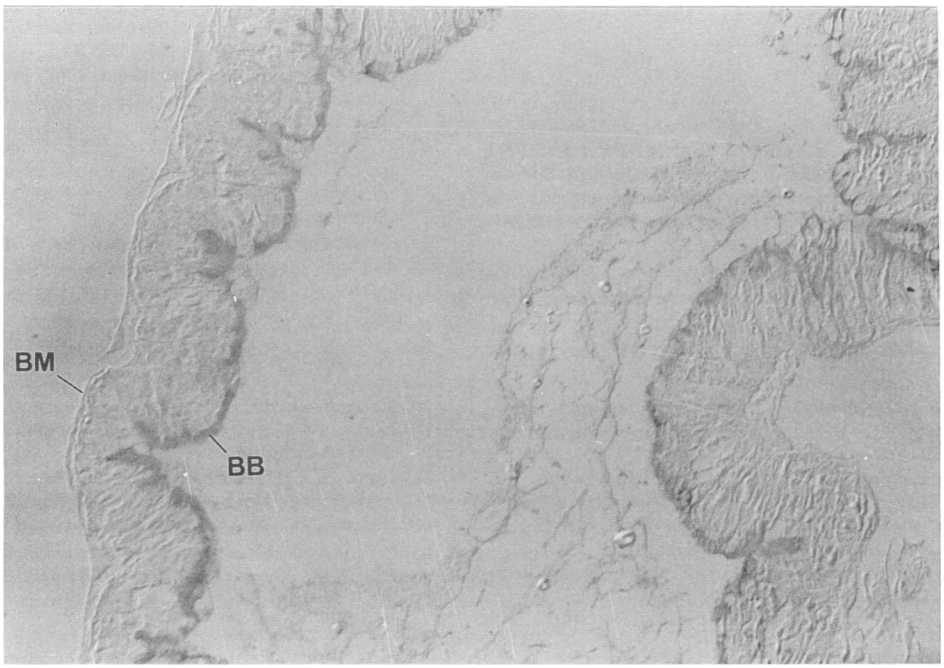

FIG. 8. Binding of biotinylated CryIA(b) to the brush border membrane of *O. nubilalis*. The biotinylated CryIA(b) binds to the microvillar brush border throughout the midgut, even when a 100-fold excess of native CryIB is present. BB, brush border membrane; BM, basement membrane. Light micrograph obtained with Nomarski differential interference contrast illumination. Magnification, ×125.

also serosally (basally). Furthermore, Ryerse and Beck (34) visualized basement membrane adherence of CryIA(c) on *Heliothis virescens* midguts. Taken together, these data indicate that mucosal exposure and serosal exposure of plasma membranes to the CryIA(c) toxin give rise to CryIA(c) adherence and cytopathological effects. However, the results of our in vivo binding experiments indicate that the basal plasma membrane is a physiologically nonrelevant target for *B. thuringiensis* ICPs.

The relationship between different receptor sites, as inferred from the results of radioligand competition experiments, was confirmed by the results of competition binding studies performed with biotinylated and nonbiotinylated CryIA(b), CryIA(c), and CryIB. The inability of a 100-fold excess of CryIA(c) to displace all of the biotinylated CryIA(b) corresponds with the 11-fold-lower affinity of CryIA(c) for its binding site compared with CryIA(b). Furthermore, the results of competition between biotin-labeled CryIA(b) and native CryIB, as well as the inverse experiment, confirmed that the two toxins are recognized by distinct receptors.

Insight into the receptor specificity of the *B. thuringiensis* ICPs should permit a rational decision concerning how these microbial proteins should be applied to minimize selection pressure on pest populations. Combined or rotational application of two or more insecticides interfering with distinct target sites may be useful in delaying the development of resistance (16, 18, 28). Since *O. nubilalis* has at least two different ICP receptors, it may be possible to decrease the resistance potential in this pest by using a mixture of ICPs that bind to these distinct receptors.

ACKNOWLEDGMENTS

We thank Bernadette Saey and Linda Buysse for insect rearing and toxicity assays. P.D. was supported by a grant from the Instituut tot Aanmoediging van Wetenschappelijk Onderzoek in Nijverheid en Landbouw and by the Algemene Spaar- en Lijfrentekas.

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