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Binding of Insecticidal Crystal Proteins of *Bacillus thuringiensis* to the Midgut Brush Border of the Cabbage Looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), and Selection for Resistance to One of the Crystal Proteins

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The susceptibility of *Trichoplusia ni* larvae to several *Bacillus thuringiensis* insecticidal crystal proteins (ICPs) was tested. Neonatal larvae proved to be susceptible to solubilized trypsin-treated CryIA(a), CryIA(b), and CryIA(c) (50% lethal concentrations [LC_{50}s], 570, 480, and 320 ng/cm^2, respectively) but showed little susceptibility to CryIB and CryID (LC_{50}s, 5,640 and 2,530 ng/cm^2, respectively). The toxicity of ICPs was correlated to binding to the epithelial brush border of the midgut, as revealed by immunocytochemical staining with monoclonal antibodies. In vitro binding experiments with iodinated ICPs and brush border membrane vesicles indicated that CryIA(b) and CryIA(c) share the same high-affinity binding site, whereas CryIA(a) binds to a different one. The affinities of CryIA(b) and CryIA(c) for the binding site were similar (K_d = 3.6 and 4.7 nM, respectively), and the mean binding-site concentration was 0.71 pmol/mg of vesicle protein. Selection of a population with increasing concentrations of CryIA(b) produced 31-fold resistance in seven generations. The realized heritability (h^2) was 0.19. The increase of homozygosity (for resistance factors) as selection proceeded was reflected in the increase in the slopes of the dose-mortality curves. Resistance was specific for CryIA(b) and did not extend to CryIA(a) or even to CryIA(c). This result was not predicted by the binding-site model, in which CryIA(b) and CryIA(c) bind to the same high-affinity binding site. This result may suggest a more complicated relationship between in vitro binding of ICPs to specific sites in the epithelial membrane of the midgut and the in vivo toxic effect.

At sporulation, the bacterium *Bacillus thuringiensis* synthesizes a crystal containing one or several proteins. In most cases, these proteins possess insecticidal activity and are therefore called insecticidal crystal proteins (ICPs) or δ-endotoxins (1, 17, 18). *B. thuringiensis* is the most widely used bioinsecticide (7). Because of its specific toxicity spectrum, it does not affect beneficial insects, plants, or animals, including humans. The genes encoding ICPs can be manipulated genetically, allowing their incorporation into plants so that the plants become toxic to the target insect pests (10, 30).

The reason for the specificity of the ICPs is linked to their mode of action (1, 13). The parasporal crystals contain the ICPs in the form of protoxins. Upon ingestion by the insect, the crystal dissolves and the protoxins are activated by means of partial proteolysis, giving rise to the active toxin form of about 60 kDa (17, 18). The toxin crosses the peritrophic membrane and binds to target sites in the epithelial membrane of the midgut. Once it is bound, pores are formed in the cell membrane, probably by the insertion of a hydrophobic domain of the toxin into the lipid bilayer (19), and eventually lead to a colloid-osmotic lysis of the cell. It has been proposed that a key determinant of the high specificity of *B. thuringiensis* ICPs is their binding to specific sites in the insect midgut membrane (16, 31, 32).

An important aspect of *B. thuringiensis* as an insecticide is that, despite its use since 1960, no field resistance was found until very recently. Only one species, *Plutella xylostella* (diamondback moth), has evolved resistance in the field, first in Hawaii and the Philippines (8, 29) and more recently in Florida, Japan, Malaysia, and Thailand (28). In contrast, artificial selection in the laboratory has produced a number of resistant lines in different species: *Plodia interpunctella* (21), *Heliothis virescens* (14, 26), *Leptinotarsa decemlineata* (34), and others (28).

Some advances have been made in the understanding of the biochemical basis of resistance to *B. thuringiensis* ICPs. Modification of the target site was found to be a key factor in the Indianmeal moth and in the diamondback moth (8, 33). However, in *Heliothis virescens*, another type of modification, as yet unknown, seems to be responsible for resistance (14, 20). Also, the genetic basis of resistance is only partially understood (28). It is agreed that the future of this very promising bioinsecticide depends, to a large extent, on choosing the appropriate resistance management strategies. For this, it is desirable to obtain a thorough understanding of the toxicity basis of *B. thuringiensis* ICPs and of the potential of insects to develop resistance to them.

As part of an effort to generate resistant insects for biochemical and genetic studies, we selected the cabbage looper, *Trichoplusia ni* (a polyphagous species that prefers cabbage and tomato as host plants), for resistance to one *B. thuringiensis* ICP. For the selection experiments we chose the proteolytically activated toxic form of CryIA(b) (instead of the protoxin). The rationale for this was to simulate the situation that insects will face when exposed to transgenic plants, since generally the toxic fragments of ICPs, and not their respective protoxin forms, are the expression products in transgenic plants (10, 30).

Our results show that the selected colony developed resis-

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tance specific for the ICP used in the selection, CryIA(b). Susceptibility to the structurally related ICPs, such as CryIA(a) and CryIA(c), did not change. In contrast with other cases in which a specific resistance to one ICP could be related to a change in its binding site, the binding-site model obtained with the susceptible *T. ni* colony did not predict such a specificity for CryIA(b) and the lack of cross-resistance to CryIA(c), an ICP that was found to compete with CryIA(b) in binding experiments.

MATERIALS AND METHODS

Insect rearing and origin. Insects were obtained from the laboratories of Novo Nordisk A/S, Bagsvaerd, Denmark, in two batches. The first batch, which was used for selection experiments, was a colony that had been maintained in the laboratory of Novo Nordisk for 3 years after being obtained from a laboratory in Quebec, Canada. This strain, while still in Novo Nordisk, suffered a spontaneous infection by *B. thuringiensis*. After that, the surviving insects were crossed with insects from another strain and the colony was reestablished from a small number of adults. From then on, some subcolonies started to show reduced susceptibility to *B. thuringiensis* subsp. *kurstaki*, and therefore the whole colony was not suitable for quality control of *B. thuringiensis* products. After several attempts to eliminate the less-susceptible subcolonies, the whole colony was discarded and a sample of 200 pupae was sent to our laboratory. This occurred approximately 1 year after the *B. thuringiensis* infection. The second batch had the same origin as the first one (the laboratory in Quebec) and was received in our laboratory 1 year after the first batch and used as the control colony.

Insects were reared on a general-purpose lepidopteran artificial diet at 25°C, with 60% relative humidity and a light-dark photoperiod of 16 and 8 h, respectively. Eggs were sterilized by immersion into a bleach-diluted solution in water (15).

Toxicity assays. The larvicidal activities of CryIA(a), CryIA(b), CryIA(c), CryIB, and CryID were determined by using neonate larvae. Five dilutions of the ICP were prepared 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). Aliquots (50 μl) of the ICP solutions, plus a control containing only PBS, were applied to the surface of the artificial diet in 2-cm^2 wells and allowed to dry. One neonatal larva was placed in each well. Twenty-four insects were used for each ICP concentration, and three replicates per bioassay were performed. Mortality was recorded after 5 days, and the data were analyzed by probit analysis (9) with the POLO-PC computer program (24).

We used a similar method for selection experiments with CryIA(b). Neonatal larvae were placed on artificial media (five insects per well) to which an appropriate amount of CryIA(b) had been applied (50 μl per well). After 5 days, surviving larvae were transferred to fresh standard media [not containing CryIA(b)] to complete their development. Insects that reached maturity were used to produce the next generation of selection.

Source of ICPs and antibodies. ICPs and antibodies directed against them were obtained from Plant Genetic Systems, Ghent, Belgium. CryIA(a), CryIA(b), CryIB, and CryID were recombinant proteins expressed in *Escherichia coli*. CryIA(c) was obtained from *B. thuringiensis* subsp. *kurstaki* HD73, a strain that produces only this ICP. Since ICP preparations often contain a mixture of the full-length protein and the proteolytically activated toxin, only activated ICPs were used for the experiments. Trypsin activation of protoxins was used to generate the toxic fragments. Details about purification of protoxins and toxins and ICP gene sources are given elsewhere (5).

Immunocytochemical localization of ICP-binding sites. Binding of ICPs to larval tissues was investigated by using histological sections of midguts of last-instar larvae (7 days old) as described by Bravo et al. (3). Dissected midguts were fixed in Bouin Hollande's 10% sublimate and embedded in Paraplast blocks. Longitudinal sections (5 μm thick) were attached to mounting glasses coated with an ovalbumin solution (5% glycerol, 1% ovalbumin, 77 μM NaN_3). Just before use, tissue sections were deparaffinated and hydrated by successive incubations in xylol, ethanol, and distilled water. Subsequently, tissue sections were equilibrated in TST buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM thimerosal [pH 7.6], 0.1% Triton X-100).

Immunocytochemical detection of ICP-binding sites was performed by incubation of the tissue slides with ICPs [5 μg/ml of TST buffer for CryIA(a), 1 μg/ml of TST buffer for CryIA(b) and CryIA(c)] for 1 h, followed by a wash with TST and then by overnight incubation with monoclonal antibodies diluted at 0.5 μg/ml in TST buffer. The monoclonal antibodies used were $4D_6G_8G_7$ for CryIA(a), CryIA(b), and CryIA(c) and $22A_2F_1$ for CryIB. After a wash with TST buffer, tissues were incubated for 1 h with secondary antibody (alkaline phosphatase-conjugated rabbit anti-mouse antibody; Sigma Chemical Co., St. Louis, Mo.) diluted 1:400 in TST buffer. After a wash with TST buffer, color development was induced by incubation with 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 100 mM Tris–100 mM NaCl–5 mM $MgCl_2$ [pH 9.5]). The reaction was stopped by transferring the samples to TST. Finally, to preserve the results, tissues were dehydrated and mounted with Entellan (Merck, Darmstadt, Germany) onto a coverslip. Staining was not observed when individual steps were omitted.

Iodination of ICPs. CryIA(b) and CryIA(c) were labeled with $Na^{125}I$ by using the chloramine-T method as described elsewhere (32). Low-molecular-weight degradation products resulting from the iodination of CryIA(c) were separated by ultrafiltration in Ultrafree-MC 10K (Millipore Ibérica, Barcelona, Spain) centrifugal units (4,000 × *g* for 30 min at room temperature). The specific activity of the labeled toxins was 6 mCi/mg for CryIA(b) and 0.3 mCi/mg for CryIA(c) (1 μCi = 37 kBq).

Preparation of BBMV. Midguts of last-instar larvae were used to prepare brush border membrane vesicles (BBMV). Midguts were pulled out of the dissected body submersed in ice-cold MET buffer [0.3 M mannitol, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA)–17 mM Tris-HCl (pH 7.5)]. After removal of malpighian tubules and the peritrophic membrane, midguts were rinsed in ice-cold MET buffer, frozen in liquid nitrogen, and stored at −80°C until required. BBMV were prepared by the differential magnesium precipitation method as described by Wolfersberger et al. (36) and stored at −80°C. The yield was around 0.9 mg of vesicle protein per g (wet weight) of midgut. The protein concentration was determined by the method of Bradford (2) with BSA as the standard.

Binding assays with iodinated ICPs. BBMV were thawed immediately before use. The storage buffer was replaced with PBS–0.1% BSA by briefly centrifuging the tube, replacing the supernatant, and homogenizing the mixture by vortexing. The conditions used for the binding assays were essentially the same as previously published (8). The optimum conditions for these assays in *T. ni* were determined to be 15 μg of vesicle protein and 0.35 nM labeled CryIA(b) or 0.018 nM labeled

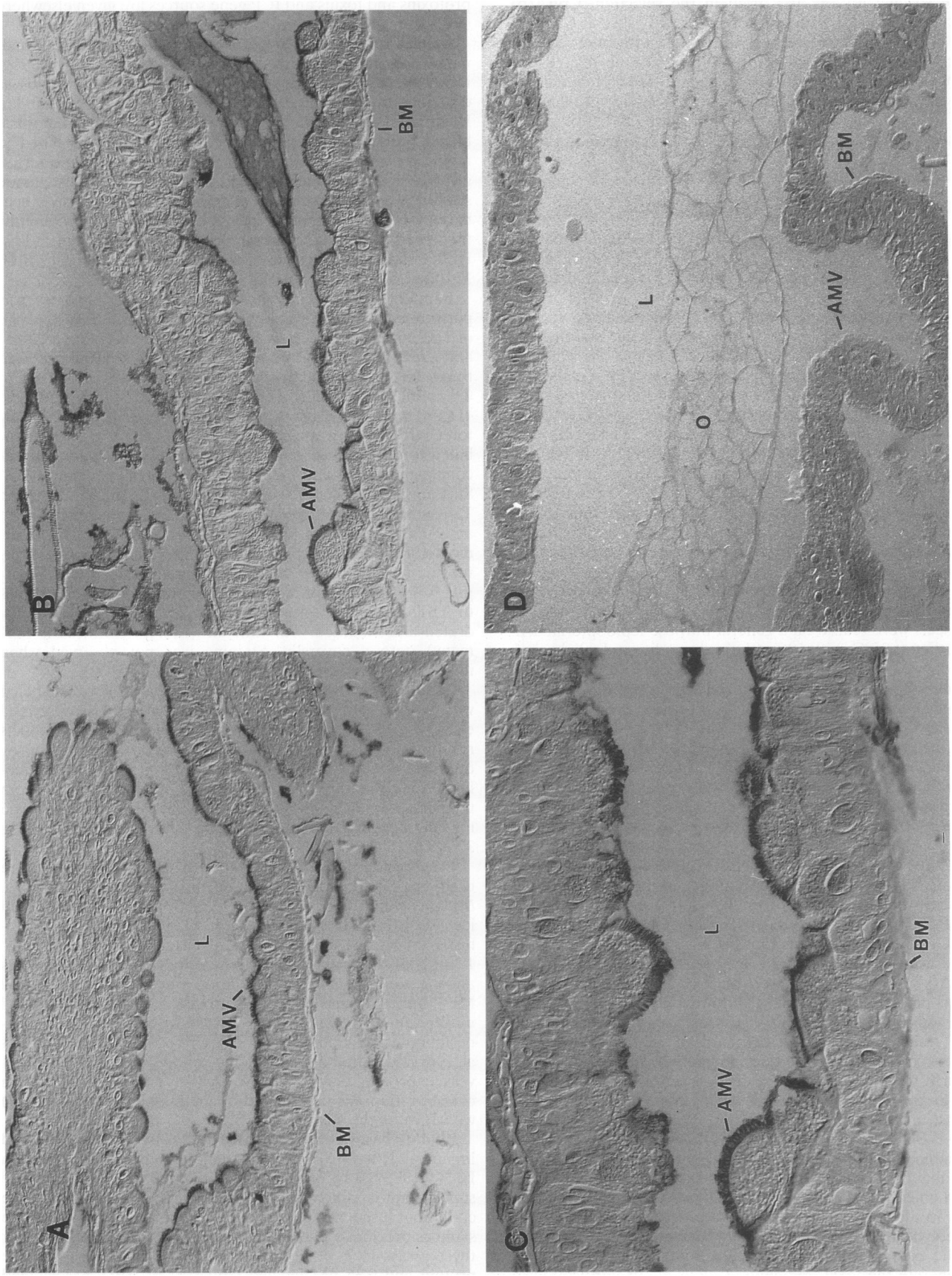

FIG. 1. Immunodetection of in vitro bound ICPs to midgut tissue sections of *T. ni*. (A) CryIA(b). Magnification, ×200. (B) CryIA(c). Magnification, ×200. (C) CryIA(c). Magnification, ×400. (D) Control without toxin. Magnification, ×200. BM, basement membrane; L, lumen; AMV, apical microvilli. Light micrographs obtained with Nomarski differential interference contrast illumination.

TABLE 1. Toxicity of trypsin-activated ICPs to neonatal larvae of *T. ni*

ICP	LC_{50}[a]	FL_{95}[a]	Slope (SE)[b]
CryIA(a)	570	380–770	2.50 (0.36)
CryIA(b)	480	380–600	1.68 (0.10)
CryIA(c)	320	300–340	7.35 (0.76)
CryIB	5,640	3,580–33,900[c]	2.28 (0.68)
CryID	2,530	1,570–9,700	2.26 (0.27)

[a] LC_{50}s and 95% fiducial limits (FL_{95}s) are expressed as nanograms of protein per square centimeter of artificial diet.
[b] SE, standard error.
[c] 90% fiducial limits instead of 95%.

CryIA(c) in PBS–0.1% BSA (final volume, 0.1 ml). Incubation was performed at room temperature for 60 min. Radioactivity retained by the BBMV was separated from unbound labeled ICP by filtration (Whatman GF/F fiberglass filters) and measured in a 1282 Compugamma CS gamma counter (LKB).

Quantitative data were obtained from competition experiments, which were performed by incubating a fixed amount of labeled ICP with increasing concentrations of unlabeled competitor. Data were analyzed by the LIGAND computer program (23).

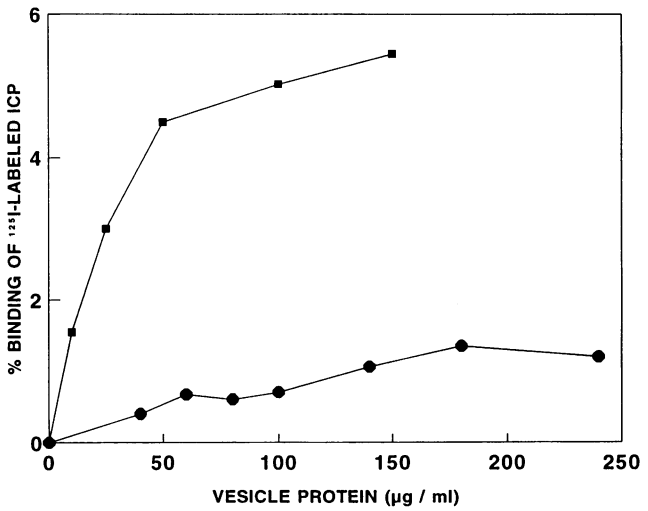

FIG. 2. Specific binding of ^{125}I-labeled CryIA(b) (●) and CryIA(c) (■) as a function of *T. ni* BBMV concentration. Nonspecific-binding values (obtained with a 100-fold excess of unlabeled ICP) were subtracted from each datum point.

RESULTS

Toxicity assays. The toxicity of several solubilized trypsin-activated ICPs from *B. thuringiensis* was tested by using neonatal larvae of the control colony of *T. ni* (Table 1). The results show that ICPs of the subclass CryIA were about 10 times more toxic than CryIB and CryID. CryIB was the least toxic ICP tested against this species. Among the CryIA subclass, toxicity differed by less than twofold, with CryIA(c) being the most toxic followed by CryIA(b) and CryIA(a).

Binding of ICPs to the midgut epithelium of *T. ni* larvae as revealed by immunocytochemical detection. The mode of action of *B. thuringiensis* ICPs was investigated by immunocytochemical detection of toxin binding to slides containing tissue of larval midguts. CryIA toxins bound to the microvillar brush border of the epithelial cells throughout the midgut. Figure 1 shows the results for CryIA(b) and CryIA(c). Although not shown, essentially the same result was found when using CryIA(a). Under the same experimental conditions, CryIB gave poor staining of the brush border (results not shown): either an almost nondetectable color or a very faint one against a conspicuous background at higher concentrations. CryID was not tested.

Binding of ^{125}I-labeled ICPs to BBMV. Saturable binding of toxic ICPs to the epithelial brush border was demonstrated by using ^{125}I-labeled ICPs. Figure 2 shows specific binding of iodine-labeled CryIA(b) and CryIA(c) as a function of vesicle concentration (nonspecific binding was subtracted from each datum point). Nonspecific binding was relatively high, since maximal total binding was 2.5% for CryIA(b) and 16% for CryIA(c).

Results of competition experiments are shown in Fig. 3, where binding of labeled CryIA(b) and CryIA(c) is measured at increasing concentrations of unlabeled ICPs. Data from Fig. 2 and 3 indicate that there is saturable binding, i.e., that the brush border membrane contains specific target sites for the ICPs. However, the high level of nonspecific binding in CryIA(c) (Fig. 3B) suggests that this toxin may also display a nonsaturable type of binding.

In heterologous competition experiments (Fig. 3) CryIA(b) and CryIA(c) behaved as only one ICP, competing effectively with one another for the specific binding sites. By contrast, CryIA(a) did not compete with labeled CryIA(b) or CryIA(c).

Data from the homologous competition curves were analyzed by the LIGAND computer program, and a single high-affinity binding-site model was obtained for both CryIA(b) and CryIA(c). Similar binding affinities [K_d = 3.6 ± 0.4 nM for CryIA(b) and 4.7 ± 1.0 nM for CryIA(c)] and binding-site concentrations [R_t, 0.64 ± 0.12 pmol/mg of vesicle protein for CryIA(b) and 0.79 ± 0.12 pmol/mg of vesicle protein for CryIA(c)] were calculated for both toxins.

Selection of *T. ni* for resistance to CryIA(b). A population sample of *T. ni* was subjected to laboratory selection with CryIA(b) for seven generations. Sample sizes, doses of toxin used, and selection pressures applied (as percent mortality) to each generation are summarized in Table 2. The selection pressure was adjusted in each generation to give a mortality above 75%. Except for the first generation of selection, the mortalities achieved ranged between 83 and 98%.

Changes of susceptibility of the selected population were monitored by running bioassays every two or three generations (Table 3). The 50% lethal concentration (LC_{50}) increased 4-fold in the first two generations of selection, 2.6-fold in the following two, and 3-fold in the last three. The overall increase was 31-fold in seven generations. The slope of the regression line was significantly greater in the A_4 and A_7 generations than in A_0 (before selection started), indicating loss of variability as selection proceeded.

Realized heritability (h^2) was calculated as described by Tabashnik (28). This parameter estimates the fraction of phenotypic variation in the population that is attributable to additive genetic variation. In other words, h^2 estimates the potential of a population to develop resistance. The h^2 for the overall experiment of selection was 0.19.

Cross-resistance assessment. The susceptibility of the selected line to ICPs of the CryIA subclass is shown in Table 4. Compared with the LC_{50}s of the control population (Table 1), the results indicate that the selected line developed resistance only to the ICP for which it was selected, i.e., CryIA(b), and not to CryIA(a) or CryIA(c). The apparently higher suscepti-

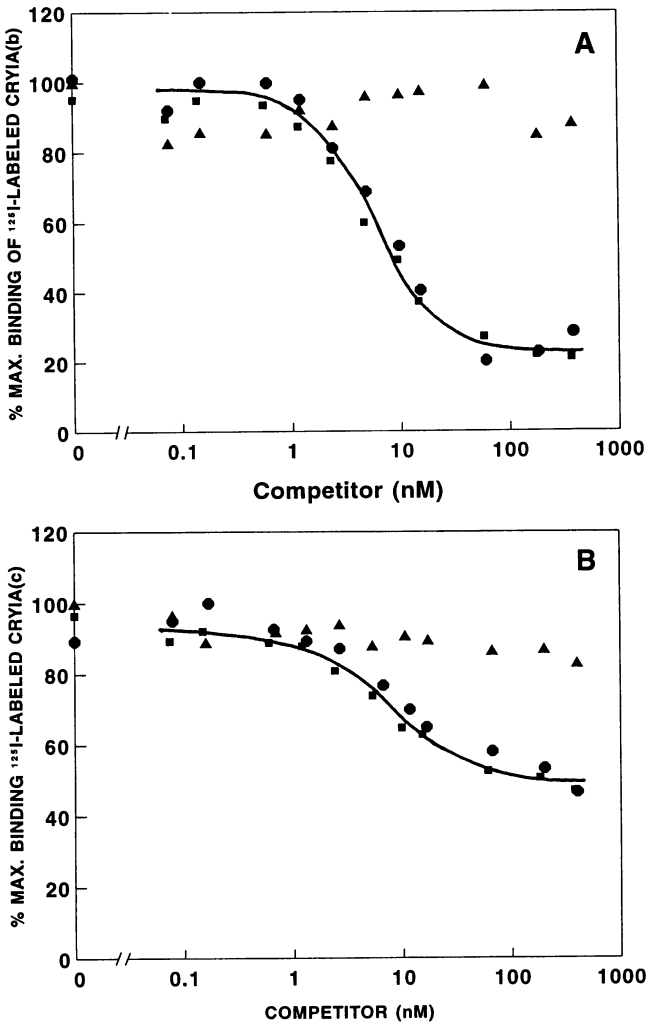

FIG. 3. Binding of ^{125}I-labeled CryIA(b) (A) and CryIA(c) (B) to BBMV of *T. ni* at increasing concentrations of nonlabeled competitor. Nonspecific binding was not subtracted. Each point represents the mean of three independent experiments. ▲, CryIA(a); ●, CryIA(b); ■, CryIA(c).

bility of the selected line for CryIA(c) cannot be considered significant, especially if we take into account that the control line did not derive from the same population as the one from which the selection line derived.

TABLE 2. Selection procedure for obtaining a *T. ni* population resistant to CryIA(b)

Generation	Dose of CryIA(b) (μg/cm^2)	No. of neonates exposed	No. of adults obtained	% Mortality
A_1	0.28	250	60	76.0
A_2	0.76	600	100	83.3
A_3	2.29	700	43	93.9
A_4	4.59	600	30	95.0
A_5	7.65	820	19	97.7
A_6	11.47	1,000	132	86.8
A_7	19.12	1,600	89	94.4

TABLE 3. Responses to CryIA(b) of progressive generations of the selected line of *T. ni*

Generation	LC_{50} (FL_{95})[a]	Slope (SE)[b]
A_0	260 (180–360)	1.16 (0.19)
A_2	1,040 (710–2,170)	1.37 (0.28)
A_4	2,670 (2,260–3,120)	2.23 (0.24)
A_7	8,060 (6,050–10,400)	2.00 (0.37)

[a] LC_{50}s and 95% fiducial limits (FL_{95}s) are expressed as nanograms of protein per square centimeter of artificial diet.
[b] SE, standard error.

DISCUSSION

Neonatal *T. ni* larvae were more susceptible to CryIA(a), CryIA(b), and CryIA(c) than to CryIB and CryID. Our LC_{50}s for the CryIA toxins differ somewhat from those reported by other authors, especially for CryIA(a), for which a toxicity of 1/10 that of CryIA(c) was reported (11, 12, 22). This is most probably due to differences in the source of ICPs and, to a lesser extent, to differences in bioassay or insect strain. However, in all studies, including the one in this report, CryIA(a) was the least toxic ICP of the three and CryIA(c) was the most toxic.

The toxicity of *B. thuringiensis* ICPs to *T. ni* larvae correlated with binding to the brush border membrane of the midgut epithelial cells, as revealed by immunocytological detection. Under the same experimental conditions as CryIA ICPs, binding of CryIB was difficult to observe. This result suggests that the very limited toxicity of CryIB is probably due to low binding affinity to target sites in the epithelium or to the scarcity of such sites. No differences in the intensity and distribution of immunological staining were found between ICPs of the CryIA subclass.

In vitro binding experiments with iodinated ICPs and BBMV indicate that CryIA(b) and CryIA(c) share the same high-affinity binding sites. The fact that similar binding affinities and binding-site concentrations were found for the two toxins is in agreement with the data from heterologous competition experiments and strengthens the idea that CryIA(b) and CryIA(c) recognize the same specific binding sites. However, CryIA(a) binds to a different binding site. These three ICPs have a high degree of homology (82 to 90% amino acid identity) (17). Despite this similarity, variable results have been found in studies in which the relationship between ICPs of the CryIA subclass and their binding sites was assessed. Thus, similarly to our results, CryIA(b) and CryIA(c) share the same binding site in *Lymantria dispar* (35), *Ostrinia nubilalis* (4), and *Manduca sexta* (16, 31). However, in the last case, CryIA(a) also binds to the same binding site. In *Heliothis virescens* the situation is more complicated: CryIA(a), CryIA(b), and CryIA(c) share one receptor, but CryIA(b) and CryIA(c) bind to a second one, and only CryIA(c) binds to a third one (31).

TABLE 4. Toxicity of trypsin-activated ICPs to neonatal larvae of the selected line of *T. ni* at the sixth [CryIA(a) and CryIA(c)] and seventh [CryIA(b)] generations of selection

ICP	LC_{50}[a]	FL_{95}[a]	Slope (SE)[b]
CryIA(a)	640	480–830	2.78 (0.29)
CryIA(b)	8,060	6,050–10,400	2.00 (0.37)
CryIA(c)	160	60–270	2.46 (0.24)

[a] LC_{50}s and 95% fiducial limits (FL_{95}s) are expressed as nanograms of protein per square centimeter of artificial diet.
[b] SE, standard error.

Binding to the midgut epithelium is a necessary step for ICP toxicity. Although in many cases a positive correlation between binding parameters (K_d and concentration of binding sites) and toxicity was described (4, 16, 31), some authors have reported exceptions in which this correlation is not direct. In *Lymantria dispar* (35) and *Plutella xylostella* (8), the correlation is even reversed, and in *Phthorimaea operculella*, CryIA(b) and CryIC bind to BBMV with similar affinity whereas CryIA(b) seems to be about five times more toxic than CryIC (6). The results reported in this paper indicate that in *T. ni* there is a good correlation between binding affinity to BBMV and in vivo toxicity of CryIA(b) and CryIA(c).

Selection of a population sample with increasing concentrations of CryIA(b) produced 31-fold resistance in seven generations. The resistance factor is lower (17-fold) if we compare the selected line with the control population in Table 1. This discrepancy was because of the different degree of susceptibility of the control population (LC_{50}, 0.48 μg/cm^2) and the selected population before selection started (LC_{50}, 0.26 μg/cm^2 [Table 3]). A twofold difference in the LC_{50}s between different strains is not uncommon.

The rapid response to selection probably reflects the fact that the population had been previously selected and therefore possessed resistance genes at a detectable frequency. The h^2 was 0.19, in good agreement with values reported for other members of the Lepidoptera that responded to selection with *B. thuringiensis* (27). The increase of homozygosity (for resistance factors) as selection proceeded was reflected in the increase of the slopes of the dose-mortality lines.

Comparing the selected line with the control population, we can see an important difference in susceptibility only to CryIA(b) (about 17-fold). Therefore, selection with CryIA(b) increased resistance specifically to this ICP and not to the closely related CryIA(a) or CryIA(c). Such specificity should be linked to a specific step in the mode of action of CryIA(b). The best-characterized specific step in the mode of action of ICPs is the binding to membrane binding sites. However, in the *T. ni* model, CryIA(b) binds to the same binding sites as CryIA(c) and a change in the binding site would be expected to affect binding of both CryIA(b) and CryIA(c). This is the case for specific binding; however, CryIA(c) also showed a high level of nonspecific binding. There have been reports that this type of binding seems to be important for toxicity of ICPs (25). Therefore, it is possible that the selected colony adapted to CryIA(b) by eliminating its specific binding sites but still retaining the nonspecific sites where CryIA(c) binds. Another alternative explanation is that although CryIA(b) and CryIA(c) share the same binding molecule, it does not necessarily mean that both toxins recognize an identical epitope on this molecule. It is possible that the toxins recognize different but overlapping epitopes and that the molecular change associated with resistance affects only the CryIA(b) epitope.

Alternatively, resistance may have been due to other factors such as a change in proteolytic activity, interference with pore formation, and change in midgut pH value, specifically affecting CryIA(b) but not CryIA(c) or CryIA(a). Unfortunately, because of contamination of the insect colonies with *Nosema trichoplusiae*, biochemical analyses with the selected insects could not be carried out and so the above hypotheses could not be tested.

This is the first time that resistance to a *B. thuringiensis* ICP has been demonstrated in *T. ni*. Therefore, the potential for developing resistance to *B. thuringiensis* in the field exists, and appropriate measures should be taken to prevent this from happening. Several papers have highlighted the importance of determining the relationship among ICPs in binding to high-affinity sites for developing strategies for managing resistance to *B. thuringiensis* insecticides (4, 6, 8, 33). In this work, we present evidence that CryIA(b) and CryIA(c) seem to share the same high-affinity binding site and therefore that their combination in mixtures is discouraged. The case of specific resistance to CryIA(b) without cross-resistance to CryIA(c) in *T. ni* deserves further investigation. However, this result, along with the above-mentioned cases of quantitative discrepancy between binding parameters and in vivo toxicity, seems to point out a more complicated relationship between in vitro ICP binding to specific sites of the epithelial membrane and the in vivo toxic effect.

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