

# Cellular Toxicities and Membrane Binding Characteristics of Insecticidal Crystal Proteins from *Bacillus thuringiensis* toward Cultured Insect Cells

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Received August 31, 1992; accepted August 6, 1993

The pattern of *in vitro* toxicity of activated toxins from several classes of entomocidal inclusion genes from *Bacillus thuringiensis* was measured using eight established cell lines from lepidopteran insects. Protoxins representing CryIA(b), CryIA(c), and a mixture of all three CryIA toxins (subtypes a, b, and c; from *B. thuringiensis* subsp. *kurstaki* HD-1) were compared with the protoxin representing CryIC in a bioassay which measured the viability of cultured insect cells upon exposure to entomocidal toxin proteins. The responses of the various cell lines were very specific toward the individual toxin proteins. CryIC activated protoxin was toxic for cells of *Manduca sexta*, *Plodia interpunctella*, and to a lesser extent *Spodoptera frugiperda*. CryIA(b) and CryIA(c) proteins were toxic toward *M. sexta* but relatively non-toxic for *P. interpunctella* or *S. frugiperda*. The toxicity of CryIA(b), CryIA(c), and the composite CryIA activated toxins toward cells of *Choristoneura fumiferana* varied substantially, with the CryIA mixture being slightly more toxic than CryIA(c) alone. CryIC toxin had no effect toward *C. fumiferana* cells. Probit regression analysis of dose-response relationships between insect species and crystal protein composition demonstrated specific patterns of toxicity which may be related to membrane-receptor site binding by specific toxins. Membrane binding analysis of <sup>125</sup>I-labeled CryIA(b), CryIA(c), and CryIC toxins to insect cells from three of the cell lines yielded high specific binding only with *M. sexta* cells toward CryIA(c) toxin. Lower levels of binding were observed with CryIA(b) and CryIC toward cells of *C. fumiferana* and *P. interpunctella*. Although relatively low binding levels for CryIC were observed with *P. interpunctella* cells, toxicity was high for these cells. The results suggest that at least for CryIC, binding may be unrelated to toxicity in cultured insect cells. © 1994 Academic Press, Inc.

**KEY WORDS:** *Bacillus thuringiensis*; insect tissue culture; protoxin; membrane binding; probit analysis.

## INTRODUCTION

The wide diversity of entomocidal activity among *Bacillus thuringiensis* strains toward a variety of insect larvae has been attributed to the multiplicity of protoxin genes harbored by these microorganisms

(Kronstad *et al.*, 1983; Höfte *et al.*, 1988; Jaquet *et al.*, 1987). There are at least 30 recognized subspecies of *B. thuringiensis* based upon flagellar serotype, each containing one or more protoxin genes located either on plasmids or on the chromosome (de Barjac and Fra-chon, 1990). Thirteen of these genes have been classified as to insecticidal spectra and structural complexity (the *cry* genes) (Höfte and Whiteley, 1989). Inclusion bodies oftentimes contain more than one protoxin type which act together to produce a specific pattern of activity toward related insect species. It has been difficult to separate the toxicity characteristics of an individual protoxin protein due to the multiplicity of protoxin types in most inclusions. This has resulted in a type of collective toxicity toward certain larvae due to the contributions from multiple toxins found in inclusion preparations produced by many *B. thuringiensis* subspecies. Consequently, most studies of the histopathology and mode of action have been carried out on larvae with sometimes poorly defined toxin preparations derived from whole *B. thuringiensis* inclusions. Now that clones are available that express individual *cry* genes, however, it is possible to measure the specific activity of a single protoxin (Whiteley *et al.*, 1985; Höfte *et al.*, 1986; Haider and Ellar, 1987).

Cultured insect tissue cells were first shown to be sensitive to *B. thuringiensis* inclusion protein in 1976 (Murphy *et al.*, 1976). The technique proved to be a quick and accurate method for detection and quantitation of entomocidal activity of activated protoxin protein (Johnson, 1981; Thomas and Ellar, 1983). Yet many questions remain, including the contribution of tissue origin, the discrepancy between *in vitro* and *in vivo* tissue CD<sub>50</sub> toxicity, and the lack of strict correlation between tissue cell specificity and the *in vivo* host range of the crystal protein (Lüthy and Ebersold, 1981; Wit *et al.*, 1986). Despite these concerns, the *in vitro* technique remains a valuable alternative to long-term diet studies with intact larvae. The procedure is rapid, quantitative, and surprisingly specific (Johnson and Davidson, 1984; Johnson, 1987a). Not only is spec-

ificity maintained between lepidopteran and dipteran species using appropriate toxin protein from *B. thuringiensis*, but a great deal of interspecies selectivity is maintained as well (Johnson, 1987a,b; 1989). This corresponds for the most part with equivalent species susceptibility among intact larvae for *B. thuringiensis* protoxin activity.

Most of the published accounts using cultured insect tissue for bioassay of *B. thuringiensis* insecticidal crystal protein (ICP) have utilized the same toxin preparation techniques as for whole insect bioassay. Thus, the toxins usually represent mixtures of protoxin species. The advent of cloned toxin genes now permits investigation of specific toxin protein activities toward a variety of cultured insect cell lines. This paper describes the toxicities of two cloned gene proteins [CryIA(b) and CryIC], a single plasmid-mediated protoxin CryIA(c) (*B. thuringiensis* HD-73), and a mixture of protoxins [CryIA(a), CryIA(b), and CryIA(c)] (*B. thuringiensis* subsp. *kurstaki* HD-1) to insect cells in culture. Trypsin-activated preparations of these four protoxin types were analyzed for their activity and specificity toward a variety of lepidopteran cell lines. The membrane-binding characteristics of these toxins to the cultured insect cells were analyzed to provide evidence to support the theory of selective toxicity in this system.

#### MATERIALS AND METHODS

**Protoxins.** Toxin proteins were from a variety of sources. Cloned toxin proteins [CryIA(b)] from *B. thuringiensis* subsp. *berliner* 1715 (*Bt2*) and CryIC from *B. thuringiensis* subsp. *entomocidus* HD-110 (*Bt15*) were kindly provided by J. Van Rie (Plant Genetic Systems, Gent, Belgium) (Höfte *et al.*, 1986; Van Rie *et al.*, 1989). Purified crystal proteins [CryIA(c)] from *B. thuringiensis* subsp. *kurstaki* HD-73 and a mixture of CryIA subtypes (a, b, and c) were prepared from *B. thuringiensis* subsp. *kurstaki* HD-1 in our laboratory by methods already described (Johnson, 1987a).

**Insect cell lines.** The following lepidopteran cell lines were used in this study: spruce budworm (*Choristoneura fumiferana* Clemens) (IPRI-CF-1) (Sohi, 1973); two lines of tobacco hornworm (*Manduca sexta* L.), one derived from embryonic tissue (MRRL-CHE-20) (Eide *et al.*, 1975) and another derived from midgut tissue (BTIPR-MGA) supplied by Dr. R. Granados, Boyce Thompson Institute for Plant Research (Ithaca, NY); four separate lines of the Indianmeal moth (*Plodia interpunctella* Hubner) (IAL-PID2, KSU-PI5.3, KSU-PI5.4, and KSU-PI8.5) (Johnson, 1989; Lynn and Oberlander, 1981); and the fall armyworm (*Spodoptera frugiperda* Smith) (IPLB-SF21AE) (Vaughn *et al.*, 1977). Grace's insect cell culture medium supplemented with 15% fetal calf serum (FCS) and 1.5% tryptose was used to culture the spruce budworm and fall armyworm cells; the tobacco hornworm and Indian-

meal moth cells were cultured in Grace's supplemented with 8% FCS, 0.3% TC yeastolate, and 0.3% lactalbumin hydrolysate (pH 8.5). All culture ingredients were supplied by GIBCO BRL (Grand Island, NY). No antibiotics were used in the culture media. Cell lines were grown in 25-cm<sup>2</sup> tissue culture flasks at 26°C. Cells were counted before transfer and before bioassay with a Model ZF Coulter counter (Coulter Electronics, Hi-aleah, FL) equipped with a 100- $\mu$ m aperture tube.

**Bioassay.** Insecticidal toxicity of trypsinized protoxin digests of each of the four ICPs was measured by a determination of residual ATP content in treated cells by the luciferin-luciferase ATP quantitation method previously reported (Johnson and Davidson, 1984; Johnson, 1987a). This method measures cell toxicity based on a comparison of normal levels of ATP in untreated control versus treated cells. The loss of ATP in treated cells is a measure of the cytotoxicity of the insecticidal crystal protein, and was measured by the firefly luciferase reaction using a Lumac Biocounter Model M2010 (Lumac B. V., Schaesburg, Netherlands). Cell toxicity was converted to mortality by calculating the percentage cytotoxicity in a treated culture. Log-probit estimates of the cytotoxic dose (CD<sub>50</sub>) for each toxin were calculated according to the method of Finney (1971) using a probit analysis program written by G. A. Milliken (Kansas State University, Manhattan, KS).

**Protein iodination.** Activated toxin was iodinated by the Iodo-Beads method (Pierce, Rockford, IL). A 0.5-mCi amount of Na<sup>125</sup>I in NaCO<sub>3</sub>-buffered saline (pH 9.5) was added to 50–80  $\mu$ g of purified toxin. Three Iodo-Beads were added to the solution with gentle shaking for 10 min. The reaction was stopped by removal of the solution from the iodination vessel. Iodinated toxin was removed from free Na<sup>125</sup>I by desalting through an Excellulose GF-5 column (Pierce). The toxin was eluted by using 20 mM Tris-HCl, 150 mM NaCl, 0.1% BSA, pH 7.4. The resulting specific activities were 9.16, 38.65, and 5.28 mCi/mg of input toxin for CryIA(b), CryIA(c), and CryIC, respectively.

**Binding assay.** The basic procedure of Van Rie *et al.* (1990a) was followed. Washed cell suspensions (Johnson and Davidson, 1984) of IPRI-CF-1, KSU-PI5.3, and MRRL-CHE-20 were incubated in duplicate at room temperature for 60–90 min in combination with various amounts of <sup>125</sup>I-labeled CryIA(b), CryIA(c), and CryIC toxins in a total volume of 0.1 ml of binding assay buffer (20 mM Tris, 150 mM NaCl, 0.1% BSA, pH 7.4). Ultrafiltration through Whatman GF/F glass fiber filters was used to separate bound from free toxin. Each filter was washed with 5 ml of cold (4°C) binding assay buffer. The radioactivity of the filter was measured in a gamma counter (1470 Wizard; Pharmacia LKB Nuclear, Gaithersburg, MD). Nonspe-

cific binding was determined in the presence of 300- to 500-fold excess unlabeled toxin and was subtracted from binding data. Competition binding analysis data were calculated using the LIGAND computer program (Munson and Rodbard, 1980).

**Protein determination.** The protein contents of cultured insect cells and protoxins were measured by the bicinchoninic acid assay at room temperature (Smith *et al.*, 1985).

**Autoradiography.** Labeled toxins were incubated with washed cells from *M. sexta*, *C. fumiferana*, and *P. interpunctella* for 90 min at room temperature. The samples, along with toxin incubated alone, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) (Laemlli, 1970). The dried gel was exposed to Kodak X-Omat AR film for 2 days.

## RESULTS

Toxin stability in the presence of viable insect cells was determined by autoradiography. Each of the cloned or single gene toxin proteins involved in the present work was radiolabeled with  $^{125}\text{I}$  and autoradiography performed on the separated proteins after the normal bioassay sequence. Electrophoretic profiles of  $^{125}\text{I}$  toxins incubated either alone or in the presence of  $1 \times 10^6$  cells/ml (see Materials and Methods) revealed that the toxins were unchanged as a result of exposure to the insect cells (Fig. 1). All three of the toxins [CryIA(b), CryIA(c), and CryIC] exhibited a single band, were similar in molecular weight, ranging from 65 to 68 kDa, and no deviation was observed in molec-

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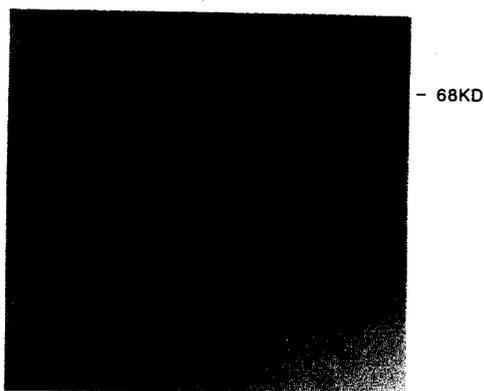


FIG. 1. Stability of *B. thuringiensis* toxins in the presence of cultured insect cells. Autoradiograph of  $^{125}\text{I}$ -labeled CryIA(b) (lanes 1-4), CryIA(c) (lanes 5-8), and CryIC (lanes 9-12) protein after incubation at room temperature for 90 min either alone (lanes 1, 5, 9) or with cells from *C. fumiferana* IPRI-CF-1 (lanes 2, 6, 10), *P. interpunctella* KSU-PI5.3 (lanes 3, 7, 11), and *M. sexta* MRRL-CHE-20 (lanes 4, 8, 12).

TABLE 1  
Toxicity of Insecticidal Crystal Proteins of *Bacillus thuringiensis* toward Cultured Lepidopteran Cell Lines

Cell line	CD <sub>50</sub> <sup>a</sup> Crystal protein type			
	CryIA(b)	CryIA(c)	CryIA(a), CryIA(b), CryIA(c)	CryIC
<i>M. sexta</i>				
MRRL-CHE-20	0.94 (0.07) <sup>b</sup>	0.02 (0.01)	0.31 (0.10)	1.57 (0.06)
BTIPR-MGA	NT <sup>c</sup>	NT	NT	19.21 (0.13)
<i>C. fumiferana</i>				
IPRI-CF-1	131.34 (0.10)	5.29 (0.12)	3.47 (0.08)	123.57 (0.16)
<i>P. interpunctella</i>				
IAL-PID2	NT	NT	NT	0.76 (0.10)
KSU-PI5.3	NT	NT	NT	0.36 (0.06)
KSU-PI5.4	NT	NT	NT	3.91 (0.06)
KSU-PI8.5	NT	NT	NT	1.58 (0.09)
<i>S. frugiperda</i>				
IPLB-SF21AE	68.79 (0.17)	NT	NT	15.82 (0.15)

<sup>a</sup> Cytotoxic dose; micrograms toxin protein/milliliter; approx.  $1-2 \times 10^6$  cells/ml/assay.

<sup>b</sup> Standard error of CD<sub>50</sub>.

<sup>c</sup> No significant toxicity; CD<sub>50</sub> higher (absolute numbers) than 200  $\mu\text{g}$  toxin/ml.

ular weight between the individual ICPs incubated either with or without insect cells.

Despite their similarities, the three toxin clones and the HD-1 toxin mixture provided widely varying toxicities toward certain lepidopteran cultured cell lines (Table 1). A high degree of specificity exists between CryIC and the CryIA toxins toward these cell lines, even though the tissues of origin vary between cell lines (embryonic, neonate, and organismic) (Johnson, 1989). One tobacco hornworm cell line (MRRL-CHE-20) responded to all four toxin proteins. Response (CD<sub>50</sub>) was maximal with CryIA(c) protein (0.02  $\mu\text{g}$  protein/ml) and least with CryIC protein (1.57  $\mu\text{g}$  protein/ml). The midgut-derived cell line of *M. sexta* (BRIPR-MGA) was moderately sensitive only to CryIC protein. Spruce budworm cells (IPRI-CF-1) were sensitive to a significant extent only to CryIA(c) protein and the HD-1 toxin mixture [indicating a possible contribution or synergistic effect from CryIA(a) protein], whereas response to either CryIA(b) or CryIC protein was minimal. The remaining cell lines were also selective toward the four toxins, with only CryIC protein possessing significant toxicity toward cell lines of *P. interpunctella*. All four individual cell lines of *P. interpunctella* were sensitive to CryIC toxin protein (range from 0.4 to 3.9  $\mu\text{g}/\text{ml}$ ), but were insensitive to the other three toxin types. *S. frugiperda* was marginally sensitive to CryIC and CryIA(b) toxins.

Probit analysis of each of the cell types against all four toxin proteins revealed markedly different characteristics in which the slopes and intersects of the probit regression lines yielded several distinct patterns. Cells from *M. sexta* responded in varying degrees to all four crystal protein types, but the slopes of the

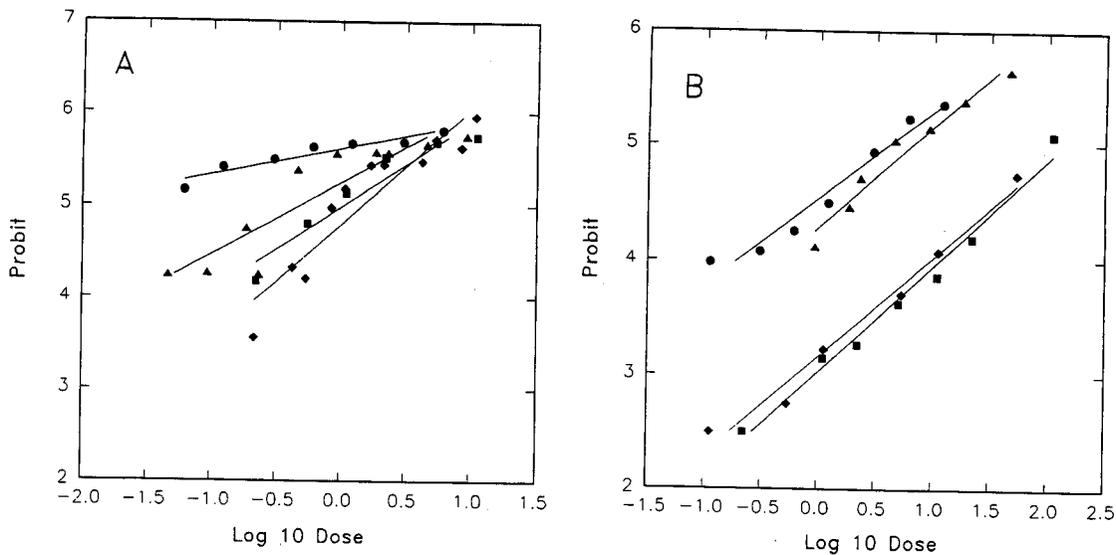


FIG. 2. Probit regression analysis of the toxicities of insecticidal crystal proteins from *B. thuringiensis* toward cultured insect cells from (A) *M. sexta* (MRRL-CHE-20) and (B) *C. fumiferana* (IPRL-CF-1). ICPs: CryIA(b) (■); CryIA(c) (●); CryIC (◆); and HD-1 (▲). Individual data points represent mean values of experiments performed in triplicate.

probit regression plots were significantly different (Fig. 2A). All four plots intersected at nearly the same coordinates but were clearly not parallel. In comparison, a probit regression plot of the response to one toxin (CryIC) by four separate *P. interpunctella* cell lines revealed essentially parallel lines, as one might expect from the responses of similar host tissues to a uniform toxin (Fig. 3). Conversely, *C. fumiferana* cells proved to be highly selective in their response to the four different toxin protein types that were examined (Fig. 2B). In this case, the regression plots, although parallel, appeared to represent two separate levels of toxin re-

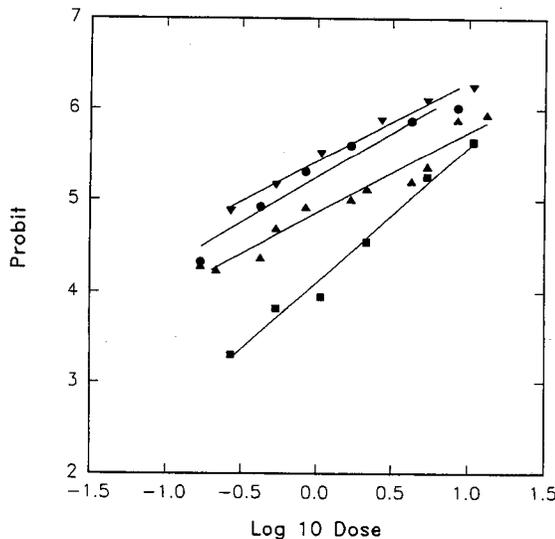


FIG. 3. Probit regression analysis of the toxicity of *B. thuringiensis* CryIC protein for cultured insect cells of four separate cell lines of *P. interpunctella*. IAL-PID2 (●); KSU-PI5.3 (▲); KSU-PI5.4 (■); KSU-PI8.5 (▼). Individual data points represent mean values of experiments performed in triplicate.

sponse toward spruce budworm tissue. A similar case was obtained with *S. frugiperda* cells, in which two different toxin protein types yielded parallel probit regression lines (equivalent slopes), but exhibited quite different sensitivity to the toxins in question [CryIA(b) and CryIC] (data not shown). In all instances, slopes of the regression lines ranged from 1.5 to 0.5, which is generally low when compared with regression analysis using whole larvae.

Membrane binding studies were performed with each of the iodinated toxins using washed insect cell preparations from each species.  $^{125}\text{I}$ -labeled toxins (concentrations ranging from 1.5 to 3.0 nM) were incubated with increasing cellular protein concentrations of 20 to 1200  $\mu\text{g/ml}$ . Nonspecific binding in the presence of excess toxin (800–1000 nM) was subtracted from total binding for each sample point. With the exception of *M. sexta*, which bound tightly with CryIA(c) protein, cells from this and the other species tested failed to bind tightly to crystal proteins from *B. thuringiensis* (Fig. 4). However, the relative affinities between cell species and toxin protein types were very specific. *M. sexta* MRRL-CHE-20 cells bound approximately 10% of the iodinated toxin and exhibited the greatest affinity for CryIA(c) protein, with less but approximately equal affinity for CryIA(b) and CryIC proteins (Fig. 4A). Homologous competition studies with CryIA(c) protein were performed on cultured cells of *M. sexta* MRRL-CHE-20 (Fig. 5). These cells bound with relatively high affinity ( $K_d = 1.17$  nM) to CryIA(c) protein with a calculated binding-site concentration of 2.10 pmol/mg of cellular protein. However, cells of *P. interpunctella* bound only about 1% of the protein from CryIC, with no detectable binding of CryIA type proteins (Fig. 4B). Spruce budworm cells showed low but discernable affinity for all three toxin protein types at an equivalent

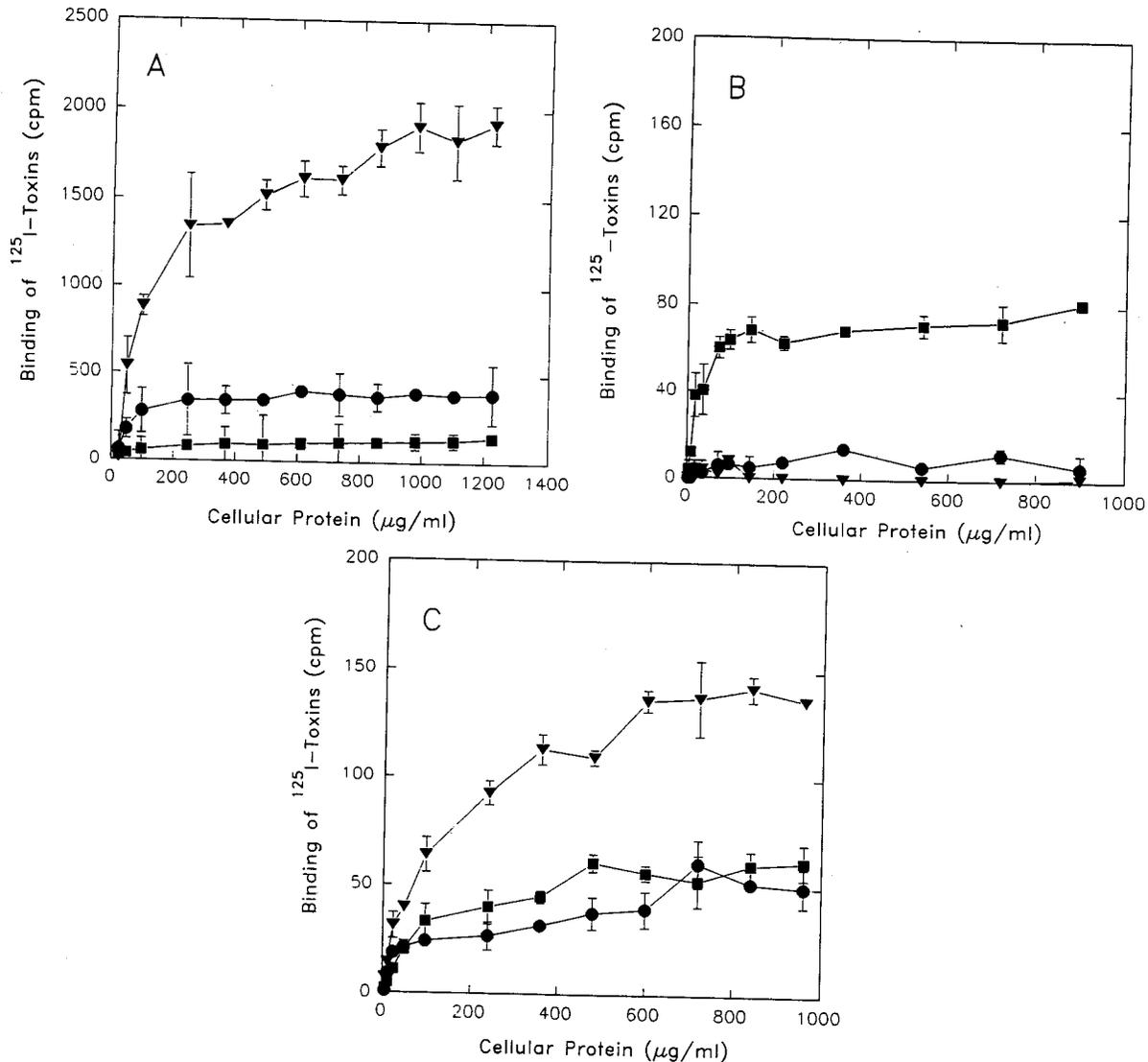


FIG. 4. Specific binding of <sup>125</sup>I-labeled CryIA(b) (●), CryIA(c) (▼), and CryIC (■) as a function of the protein concentration of cultured insect cells of *M. sexta* MRRL-CHE-20 (A), *P. interpunctella* KSU-PI5.3 (B), or *C. fumiferana* IPRI-CF-1 (C). <sup>125</sup>I-labeled toxin [CryIA(b), 3.0 nM; CryIA(c), 1.5 nM; CryIC, 2.35 nM] was incubated with washed insect cells in the presence or absence of an excess unlabeled toxin [CryIA(b), 1000 nM; CryIA(c), 790 nM; CryIC, 850 nM]. Samples were incubated for 60 [CryIA(b) and CryIA(c) toxins] or 90 (CryIC toxin) min. Bound toxin was separated from free toxin by using ultrafiltration through Whatman GF/F filters. Nonspecific binding was subtracted from total binding. Error bars represent standard error about the sample mean.

level of binding (Fig. 4C). Competition studies were not performed with cells from *P. interpunctella* and *C. fumiferana* due to the low level of saturation binding demonstrated with each of the toxins. Likewise, saturation-binding experiments performed with *S. frugiperda* cells resulted in only minimal binding of CryIA(b), while binding of CryIA(c) and CryIC toxins could not be detected (data not shown). Maximum binding for all three toxins with each of the cell lines occurred with approximately 400 μg/ml of cellular protein.

#### DISCUSSION

Despite the remarkable similarity of the CryIA proteins in terms of molecular weight and amino acid ho-

mology, they possess very distinct insect toxicity profiles (Höfte and Whiteley, 1989). Similarly, CryIC protoxin is very similar in size to those of the CryIA family; however, amino acid sequence homology between CryIA and CryIC protoxins range only from 58 to 67%. Consequently, the diverse activities of these protein toxins toward certain lepidopteran larvae appear to be based on finite variations in amino acid sequence and protein conformation. Recent reports on specific binding of different ICPs to brush border membrane vesicles from a variety of insect species suggest that differences in midgut ICP receptors are a major determinant of differences in the insecticidal spectrum of the lepidopteran-specific ICP family (Van Rie *et al.*, 1989; Van Rie *et al.*, 1990a,b).

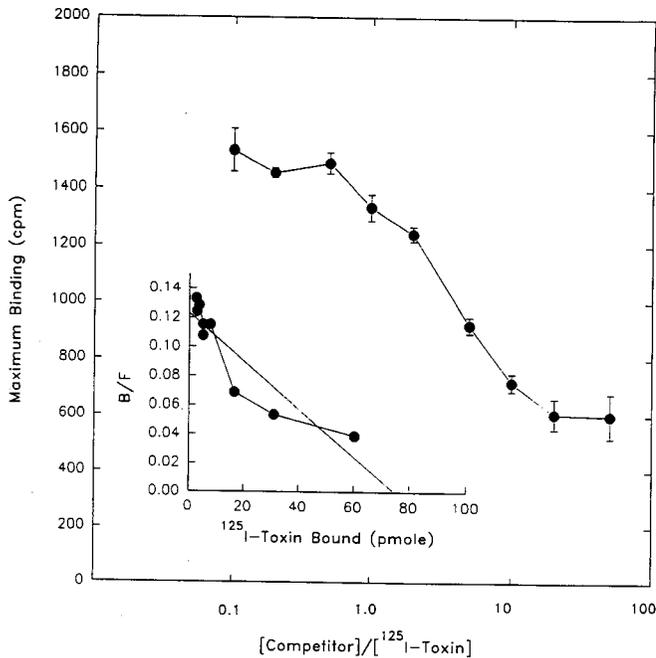


FIG. 5. Homologous competition binding study of  $^{125}\text{I}$ -CryIA(c) toxic protein for cultured insect cells of *M. sexta* MRRL-CHE-20. Inset: Scatchard plot of the data, as computed using the LIGAND program. Error bars represent standard error about the sample mean.

It may be possible to demonstrate these properties in cultured insect cells as well as in whole larvae. No specific information is available confirming the presence of similar receptors on insect cell surfaces grown *in vitro*, although Knowles and Ellar (1986) described a glycoprotein membrane receptor on the surface of *C. fumiferana* CF1 cells that was sensitive to activated *B. thuringiensis* HD-1 toxin. In our saturation-binding assays, the extent of binding differed appreciably between toxins and insect species, yet toxin specificity was apparent even when specific-binding affinity was low (Fig. 4).

Based upon current knowledge of specific *B. thuringiensis* toxin protein types (Höfte and Whiteley, 1989), it is tempting to relate the differences in mortality to the multiplicity of toxin protein types. Probit analysis is a statistical technique designed to measure the susceptibility of a population to a toxin/pathogen and the variation in their response to that stimulus (Burges and Thomson, 1971). Thus, a test organism with little population variation in terms of viability, stamina, etc., should respond to a singular toxin in uniform fashion. The nonparallel nature of the four treatments toward cells of *M. sexta* shown in Fig. 2A apparently resulted from differences in host tissue response to the individual toxins. CryIC exhibited the steepest slope (1.117), while the CryIA toxins elicited a more shallow response curve. The resulting regression lines may represent significantly different receptor-binding affinities and therefore appear to be dissimilar toxins in

standard probit regression tests. This reasoning is reinforced by the response (both probit regression and  $\text{CD}_{50}$  values) of *C. fumiferana* cells to the same toxins. These cells also respond at different levels to all four toxin preparations. Again, the variations in the probit regression lines could be the result of specific receptor-toxin interactions in each toxin/tissue cell system. In this case, however, where the regression lines segregated into two paired plots, similar receptors may be involved but which possess quite different affinities for the four toxin types and therefore tend to segregate into two response classes. This was not the case with four *P. interpunctella* cell lines, which responded similarly (and with parallel regression lines) to a single (CryIC) toxic protein. In this case, one would expect little difference in the slopes of the regression lines, but only in the lethality of the specific preparations for the tissue cells.

Receptor-binding studies intended to support the above observations were not completely successful. The membrane-binding data obtained with *C. fumiferana* and *P. interpunctella* cells were not clear due to low-binding affinities. The binding results from *M. sexta* (MRRL-CHE-20) cells were consistent with the probit mortality data for the three toxins. CryIA(c) bound with relatively high affinity to the cells, which correlated well with cellular mortality with this toxin. The other two toxins, CryIA(b) and CryIC, bound poorly to *M. sexta* cells, despite being relatively toxic [though less than CryIA(c)] for these cells. With *C. fumiferana* (IPRI-CF-1), the binding efficiency was very low and it was difficult to relate any significance between binding data and cellular mortality. Also, with *P. interpunctella* cells, the lack of association between toxicity and CryIC binding activity (the only toxin with any activity toward this cell line) suggests that the relationship between toxicity and binding may not be a reliable one.

To date, there are no existing studies that describe the binding capacities of cultured insect cells for *B. thuringiensis* toxins. The relationship between receptor binding and Cry protein toxicity in these cells is even less defined. The receptor-binding capacity of cultured insect cells can be shown in select instances; however, not all of the cell lines tested bound crystal protein toxins in a manner equivalent to observed cellular toxicity. Clearly, further work is needed in this area.

#### REFERENCES

- Burges, H. D., and Thomson, E. M. 1971. Standardization and assay of microbial insecticides. In "Microbial Control of Insects and Mites" (H. D. Burges and N. W. Hussey, Eds.), pp. 591-622. Academic Press, New York.
- de Barjac, H., and Frachon, E. 1990. Classification of *Bacillus thuringiensis* strains. *Entomophaga* 35, 233-240.
- Eide, P. E., Caldwell, J. M., and Marks, E. P. 1975. Establishment of

- two cell lines from embryonic tissue of the tobacco hornworm, *Manduca sexta* (L.). *In Vitro* 11, 395-399.
- Finney, D. J. 1971. "Probit Analysis," 3rd ed. Cambridge Univ. Press, London.
- Haider, M. Z., and Ellar, D. J. 1987. Characterization of the toxicity and cytopathic specificity of a cloned *Bacillus thuringiensis* crystal protein using insect cell culture. *Mol. Microbiol.* 1, 59-66.
- Höfte, H., de Greve, H., Seurinck, J., Jansens, S., Mahillon, J., Ampe, C., Vandekerckhove, J., Vanderbruggen, H., van Montagu, M., Zabeau, M., and Vaeck, M. 1986. Structural and functional analysis of a cloned delta endotoxin. *Eur. J. Biochem.* 161, 273-280.
- Höfte, H., Van Rie, J., Jansens, S., Van Houtven, A., Vanderbruggen, H., and Vaeck, M. 1988. Monoclonal antibody analysis and insecticidal spectrum of three types of lepidopteran-specific insecticidal crystal proteins of *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* 54, 2010-2017.
- Höfte, H., and Whiteley, H. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* 53, 242-255.
- Jaquet, F., Hutter, R., and Lüthy, P. 1987. Specificity of *Bacillus thuringiensis*  $\delta$ -endotoxin. *Appl. Environ. Microbiol.* 53, 500-504.
- Johnson, D. E. 1981. Toxicity of *Bacillus thuringiensis* entomocidal protein toward cultured insect tissue. *J. Invertebr. Pathol.* 38, 94-101.
- Johnson, D. E., and Davidson, L. I. 1984. Specificity of cultured insect tissue cells for bioassay of entomocidal protein from *Bacillus thuringiensis*. *In Vitro* 20, 66-70.
- Johnson, D. E. 1987a. Incidence of insect cell cytolytic activity among *Bacillus thuringiensis* serotypes. *FEMS Microbiol. Lett.* 43, 121-125.
- Johnson, D. E. 1987b. Entomocidal activity of crystal proteins from *Bacillus thuringiensis* toward cultured insect cells. In "Biotechnology in Invertebrate Pathology and Cell Culture" (K. Maramorosch, Ed.), pp. 45-62. Academic Press, New York.
- Johnson, D. E. 1989. Specificity of cultured insect tissue cells for the bioassay of entomocidal protein of *Bacillus thuringiensis*. In "Invertebrate Cell Systems Applications" (J. Mitsuhashi, Ed.), Vol. II, pp. 85-88. CRC Press, Boca Raton, FL.
- Knowles, B. H., and Ellar, D. J. 1986. Characterization and partial purification of a plasma membrane receptor for *Bacillus thuringiensis* var. *kurstaki* lepidopteran-specific  $\delta$ -endotoxin. *J. Cell Sci.* 83, 89-101.
- Kronstad, J. W., Schnepf, H. E., and Whiteley, H. R. 1983. Diversity of locations for *Bacillus thuringiensis* crystal protein genes. *J. Bacteriol.* 154, 419-428.
- Laemmli, K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature* 227, 680-685.
- Lüthy, P., and Ebersold, H. R. 1981. The entomocidal toxins of *Bacillus thuringiensis*. *Pharmacol. Ther.* 13, 257-283.
- Lynn, D. E., and Oberlander, H. 1981. Development of cell lines from imaginal wing discs of lepidoptera. *In Vitro* 17, 208.
- Munson, P. J., and Rodbard, D. 1980. LIGAND: A versatile computerized approach for the characterization of ligand binding systems. *Anal. Biochem.* 107, 220-239.
- Murphy, D. W., Sohi, S. S., and Fast, P. G. 1976. *Bacillus thuringiensis* enzyme-digested  $\delta$ -endotoxin: Effect on cultured insect cells. *Science* 194, 954-956.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76-85.
- Sohi, S. S. 1973. *In vitro* cultivation of larval tissues of *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae). In "Proceedings of the 3rd International Colloquium on Invertebrate Tissue Culture" (J. Rehacek, D. Blaskovic, and W. F. Hink, Eds.), pp. 75-92. Publishing House of the Slovak Academy of Sciences, Bratislava, Czechoslovakia.
- Thomas, W. E., and Ellar, D. J. 1983. *Bacillus thuringiensis* var. *israelensis* crystal  $\delta$ -endotoxin: Effects of insect and mammalian cells *in vitro* and *in vivo*. *J. Cell Sci.* 60, 181-197.
- Van Rie, J., Jansens, S., Höfte, H., Degheele, D., and Van Mellaert, H. 1989. Specificity of *Bacillus thuringiensis*  $\delta$ -endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target insects. *Eur. J. Biochem.* 186, 239-247.
- Van Rie, J., McGaughey, W. H., Johnson, D. E., Barnett, B. D., and Van Mellaert, H. 1990a. Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. *Science* 247, 72-74.
- Van Rie, J., Jansens, S., Höfte, H., Degheele, D., and Van Mellaert, H. 1990b. Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis*  $\delta$ -endotoxins. *Appl. Environ. Microbiol.* 56, 1378-1385.
- Vaughn, J. L., Goodwin, R. H., Tompkins, G. J., and McCawley, P. 1977. The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *In Vitro* 13, 213-217.
- Whiteley, H., Kronstad, J. W., and Schnepf, H. E. 1985. Structure and expression of cloned *Bacillus thuringiensis* toxin genes. In "Molecular Biology of Microbial Differentiation" (J. A. Hoch, and P. Setlow, Eds.), pp. 225-229. American Society for Microbiology, Washington, DC.
- Wit, D. P., Carlson, H., and Hodgdon, J. C. 1986. Cytotoxicity of *Bacillus thuringiensis*  $\delta$ -endotoxin to cultured CF-1 cells does not correlate with *in vivo* activity towards spruce budworm larvae. In "Fundamental and Applied Aspects of Invertebrate Pathology" (R. A. Samson, J. M. Vlcek, and P. Peters, Eds.), pp. 3-6. Society for Invertebrate Pathology, San Diego, CA.