Bacillus thuringiensis Enzyme-Digested Delta Endotoxin: Effect on Cultured Insect Cells

Abstract. *Cells from three insect cell lines responded to the enzyme-digested delta endotoxin of* Bacillus thuringiensis *with swelling, lysis, and vesicle formation. Sufficient toxin was taken up in 1 minute to cause half-maximal cell damage. Cytotoxic activity was neutralized by specific antiserum to the endotoxin.*

Microbial insecticides formulated with the spores and delta endotoxin of *Bacillus thuringiensis* are used in agriculture and forestry for the control of lepidopterous pest insects (*1*). Insecticidal activity is primarily due to the delta endotoxin (*2*), which is a bipyramidal proteinaceous crystal produced by the bacillus as a parasporal inclusion (*3*).

The crystal is atoxic until digested by the proteolytic enzymes in the midgut fluids of susceptible lepidopterous larvae (*4*). Ingestion of crystals results in a cessation of feeding and, in some insects, in a general paralysis within a few hours. Within 60 minutes there is extensive disruption and disintegration of the midgut epithelial cells (*5*). Numerous studies have been published on the histopathological and biochemical effects of endotoxin on cells in vivo (*6*) but, to our knowledge, no reports on the effects on cells in vitro. We report here that cultured insect cells respond to the enzyme-digested delta endotoxin (EDD) with swelling, lysis, and vesicle formation.

The delta endotoxin was produced from a culture of *B. thuringiensis* var. *kurstaki* (*7*) grown on half-strength BBL-Trypticase soy broth by using the active culture technique (*8*). After sporulation the crystals were separated from the spores and other debris by density-gradient centrifugation with Renografin-76 (*9*). Endotoxin was digested by treating the crystals with partially purified proteolytic enzymes from fifth-instar larvae of *Trichoplusia ni* prepared from regurgitated gut fluid (*10*). The crystals (< 0.1 percent spore protein) were incubated at 30°C with *T. ni* enzymes (1 mg of crystals per microgram of enzyme protein) in 0.05*M* cyclohexylaminopropane sulfonic acid (CAPS) buffer, *p*H 10.5. After 2.5 hours the suspension was centrifuged and the supernatant containing the EDD was sterilized by filtration and stored at − 20°C. Insecticide activity of EDD was determined by bioassay (*11*) against fourth-instar larvae of *Bombyx mori* (strain, Nichi-102). Forced-feeding of the EDD resulted in an LD_{50} (dose that is lethal to 50 percent of the larvae) of 4.0×10^{-4} mg of protein per gram of larva. Protein was measured by the method of Lowry *et al.* (*12*).

Four insect cell lines were used: (i) Cf-124, started from minced neonate larvae of *Choristoneura fumiferana* (*13*); (ii) Md-108, developed from larval hemocytes of *Malacosoma disstria* (*14*); (iii) Md-66, also derived from *M. disstria* hemocytes but cultured in a medium containing insect hemolymph (*15*); and (iv) TN-368, developed by Hink (*16*) from *T. ni*. The first three cell lines were cultured in Grace's medium (Grand Island Biological) supplemented with *B. mori* hemolymph and fetal bovine serum (Microbiological Associates) according to the requirements of the line. The TN-368 line was cultured in Hink's medium without egg ultrafiltrate (*16*). All stock cultures were grown in 25-cm^2 Falcon flasks (Becton, Dickinson) at 28°C and subcultured at 7-day intervals, except TN-368 which was subcultured every 3 to 4 days. The TN-368 cells were 3 to 4 days old at the time of use, and the others were 5 to 7 days old. The cells to be tested were washed once with buffered saline (BS) (*17*) and then incubated at 28°C in Leighton tubes with EDD diluted in BS (0.2 ml per 2×10^5 cells in each Leighton tube). Growth medium (1.0 ml) was added after 2 hours of incubation, and the cultures were examined by phase microscopy. Normal cells and damaged cells were counted in ten fields (each 6.9×10^{-2} mm^2) at preset intervals along the long axis of the Leighton tube window.

The EDD-induced cell damage, illustrated in Fig. 1 for the Cf-124 line, was similar in all cell lines. Initial cytotoxic response was visible as distinct swelling of the cells, apparent thickening of the cell membrane, change in refractility of the cytoplasm from phase dark to phase bright, and a more prominent nucleus (Fig. 1a). In contrast, the untreated cells were round or spindle-shaped with a dense phase dark cytoplasm without a prominent nucleus (Fig. 1b). Within 3 hours the swollen cells were vacuolated and many had lysed, forming large vesicles with thin membranes (Fig. 1c). Such morphological damage was seen as early as 15 minutes after addition of toxin. Different stages of cellular disintegration (Fig. 1d) were present in EDD-treated cultures 1 to 5 days after treatment. The morphological damage was similar to that reported for midgut epithelial cells in endotoxin-treated lepidopterous larvae (*6*).

Cytotoxic response was at its maximum within 1 hour, but after that the percentage of damaged cells decreased. This decrease may be accounted for by the disintegration of the damaged cells and the multiplication of the surviving cells. Thus, treated cultures should be examined within 2 to 3 hours after the addition of EDD.

The quantitative response of the cell

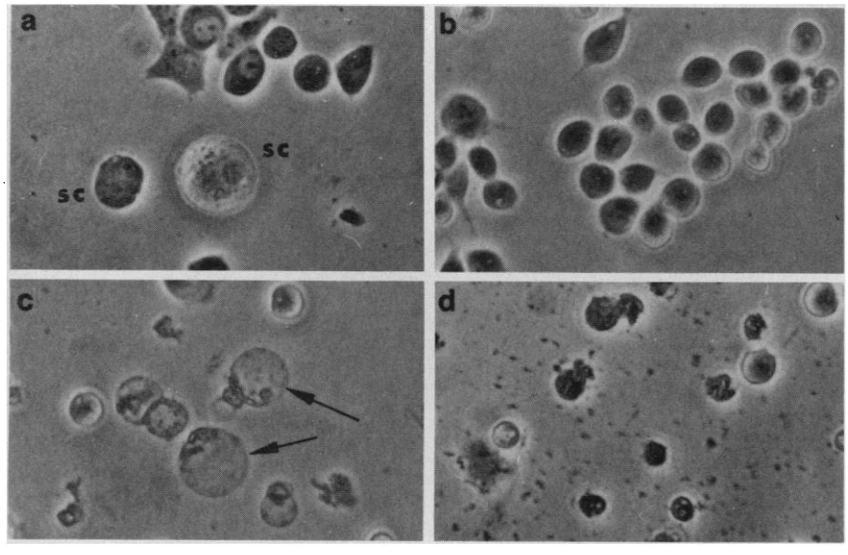


Fig. 1. Cells of line Cf-124 in the 89th passage in vitro. (a) EDD-treated culture showing swollen cells (*SC*) and prominent nuclei at 60 minutes. (b) Untreated culture in which cells are normal. (c) EDD-treated culture demonstrating vesicles (arrows) at 3 hours. (d) EDD-treated culture showing cellular disintegration at 2 days. The EDD concentration was 0.68 mg/ml (phase contrast; × 400).

lines to EDD at 0.68 mg/ml is shown in Table 1. Significant cytotoxic response occurred in three of the cell lines, the Cf-124 line showing the greatest response (45 percent), followed by Md-108 (32 percent) and TN-368 (25 percent). There was some cell damage in the Md-66 line; however, it was not statistically significant when compared with the controls. The cells and insect larvae showed no significant response to either the CAPS buffer or the heat-treated EDD or the *T. ni* enzyme system at the concentrations used. Thus cell damage in the EDD-treated cultures was not due to nonspecific causes. The Cf-124 line was selected for further work since it gave the greatest cytotoxic response.

When EDD was reacted with dilutions of specific antiserum (*18*) and then bioassayed, cytotoxicity and insecticidal activity were neutralized at dilutions of antiserum lower than 1 : 64 (Fig. 2). Enzyme-digested delta endotoxin plus normal rabbit serum and EDD alone resulted in 95 to 100 percent insect mortality and 35 to 40 percent damaged cells. The maximal response of the Cf-124 cells ranged from 35 to 70 percent.

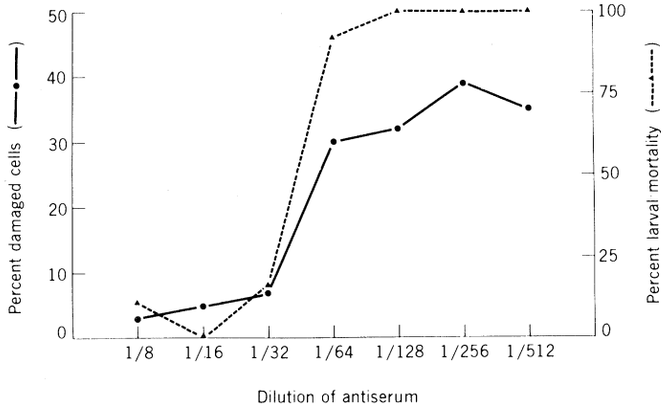
Table 1. Response of insect cells to EDD. The cultures were prepared as described in the text. Heated EDD was prepared by incubating toxin at 121°C for 30 minutes.

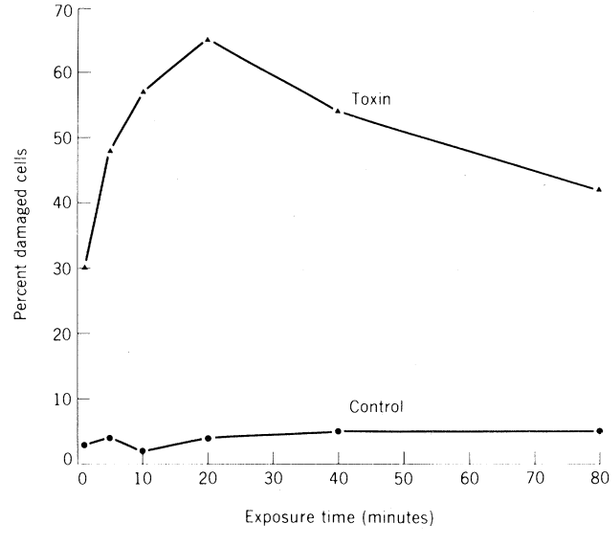
Cell line	Percentage of damaged cells*		
	5 m*M* CAPS buffer (*p*H 10.5)	Heated EDD (0.68 mg/ml)	EDD (0.68 mg/ml)
Md-66	6a (1596)	3a (2463)	10a (1950)
Md-108	11a (1830)	15a (1845)	32b (1575)
Cf-124	4a (2133)	3a (2202)	45b (2202)
TN-368	16a (3330)	15a (1906)	25b (1182)

*The values within each row having similar letters were not significantly different at $P = .05$, Duncan's multiple range test. Numbers in parentheses represent the total number of cells counted per treatment, based on at least three experiments for each cell line.

When a saturating level of specific antiserum was added at different time intervals to EDD-treated cells, a 1-minute exposure was sufficient to cause half-maximal cell damage (Fig. 3). Maximal response required only a 20-minute exposure. Therefore, sufficient EDD must be bound to the cell and inaccessible to antibody, or taken in by the cell, within 1 minute to initiate the metabolic events leading to visible cell damage. In *B. mori* larvae the endotoxin causes significant stimulation of glucose uptake in the midgut within 1 minute, a general metabolic breakdown of the gut between 10 and 20 minutes (*19*), and visible morphological damage to gut epithelial cells within 30 minutes (*20*). Thus the time scales for in vivo and in vitro cytotoxic effects are similar.

The average maximal response to EDD was 45 percent damaged cells. A direct relation between the titer of EDD and the percentage of damaged cells existed at titers giving less than 45 percent response (Fig. 4, curve "Original"), suggesting that only 45 percent of the cells were susceptible. If the dose-response data is normalized so that 45 percent = 100 percent (Fig. 4, curve "Nor-





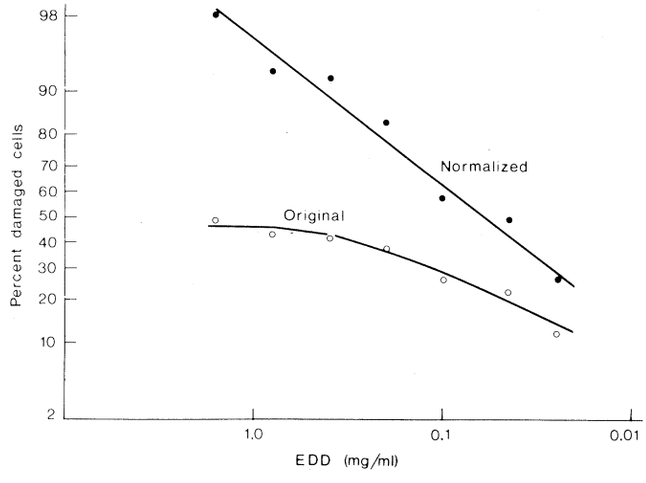


Fig. 2 (top left). Neutralization of EDD by specific antiserum. The EDD at 0.68 mg/ml was mixed with an equal volume of diluted antiserum, incubated for 30 minutes at 23°C, and centrifuged. Toxic activity in the supernatant was determined with Cf-124 cells and fourth-instar larvae of *B. mori*, as described in the text. Fig. 3 (top right). Exposure time and cell response to EDD. The Cf-124 cells were prepared as described in the text and incubated at 28°C with EDD at a concentration of 0.34 mg/ml. At the indicated times specific antiserum plus growth medium was added, and the cultures were examined after 3 hours. Control cells were incubated with 2.5 m*M* CAPS buffer (*p*H 10.5) plus specific antiserum in BS. Fig. 4 (bottom right). Dose-response of Cf-124 cells to EDD. The cells were incubated with seven twofold serial dilutions of EDD, and 400 to 600 cells were examined at each dilution. The assay was replicated three times. Control cells incubated in CAPS buffer diluted with BS had < 5 percent damaged cells. The observed maximum cell damage was considered equal to 100 percent and the data were normalized accordingly and plotted on log-probit paper.

malized") and probit analysis is applied (*21*), the EDD titer giving 50 percent effect was 0.059 mg of protein per milliliter, with 95 percent confidence limits of 0.058 and 0.060 mg of protein per milliliter and a slope of 1.93.

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Photoreceptor Mutant of *Drosophila*: Is Protein Involved in Intermediate Steps of Phototransduction?

Abstract. *In* norpA *mutants of* Drosophila melanogaster *the phototransduction process is either partially or completely blocked. By using a temperature-sensitive allele, we have found that the* norpA *mutation has little or no effect on either the rhodopsin-metarhodopsin transition or the machinery of quantum bump production. Thus, the* norpA *lesion appears to be localized in the intermediate process of phototransduction. Because a temperature-sensitive allele of* norpA *has been isolated, the* norpA *gene probably encodes a protein involved in the process.*

One of the main functions of the photoreceptor is to convert light signals to physiological responses of the photoreceptor membrane—the phototransduction process. Although much is known about the physiology of the photoreceptor, the mechanism of transduction itself has proved to be elusive. We thought that the use of mutants might be of considerable value in the study of the photoreceptor, because the technique would allow manipulation of any given physiological process at the molecular level. Thus, we set out to mutagenize fruit flies of the species *Drosophila melanogaster* and to isolate mutants with abnormal receptor function (*1*, *2*).

We reported previously that the *norpA* cistron, mapping at about 6.5 units from the tip of the X chromosome, appears to be involved in phototransduction (*2–4*). There are now over 40 independently derived *norpA* mutants. The homozygous phenotypic expression of these alleles varies from a complete absence of the electroretinogram to only a slight change in time course or amplitude of the potential. Alawi *et al.* (*3*, *5*) applied intracellular recording techniques to the photoreceptors of *norpA* mutants to show directly that the mutation indeed affects the receptor potential. They further showed that the photoreceptors of even the most extreme alleles of *norpA* have apparently normal membrane potential and resistance in the dark, but, unlike the wild-type photoreceptors, the potential and resistance do not change when a light stimulus is applied (*3*, *5*). Furthermore, the results of histological and spectrophotometric studies have suggested that the absence of the receptor potential in these mutants cannot be attributed to either morphological abnormality or the lack of the visual pigment (*3*, *6*).

One of the *norpA* alleles, $norpA^{11\,52}$, has been found to be reversibly temperature sensitive (*7*). At 17°C, the electroretinogram and the intracellularly recorded receptor potential of this mutant are very nearly normal. Above 29°C, however, these responses deteriorate, and no response is obtained above 34°C. These temperature-induced changes occur very rapidly and are reversible except when the mutants are exposed to more extreme treatments in temperature, duration, or both. These results have been interpreted to suggest that the *norpA* gene product (presumably a protein) is somehow involved in the generation of the photoreceptor response and that in $norpA^{11\,52}$ the conformation of the *norpA* gene product has become highly sensitive to temperature (*7*). In the present work (*8*), the temperature-sensitive allele, $norpA^{11\,52}$, has been used to localize more precisely the stage of phototransduction affected by the *norpA* gene.

The transduction events in the *Drosophila* photoreceptor may be summarized as follows. The dominant class of visual pigment rhodopsin (*9*) in the fly compound eye absorbs maximally (λ_{max}) at about 485 nm and is photoconverted to a stable photoproduct with a λ_{max} of about 580 nm (*6*, *10*). By still unknown mechanisms, photoexcitation of rhodopsin molecules leads to a change in permeability of the photoreceptor membrane, giving rise to a depolarizing receptor potential. Studies on other invertebrate photoreceptors have shown that most of the receptor current is carried by Na^+ ions (*11*). Moreover, several lines of evidence suggest that the receptor potentials of many invertebrates, including *Drosophila*, arise from the summation of a large number of small membrane voltage fluctuations, commonly referred to as quantum bumps (*12*, *13*). The nature of the intermediate process linking the excitation of rhodopsin and the production of bumps is not known.

At what stage of transduction does the *norpA* gene exert its effect? One possibility is that the visual pigment rhodopsin fails to make its normal transition to metarhodopsin when photoactivated. This possibility was tested by performing spectrophotometry on the temperature-sensitive mutant $norpA^{11\,52}$ at a temperature of 35°C. At a temperature as high as this, no trace of receptor potential can be detected in this mutant (*7*).