Human Cell Exposure Assays of Bacillus thuringiensis Commercial Insecticides: Production of Bacillus cereus-Like Cytolytic Effects from Outgrowth of Spores

Azam F. Tayabali and Verner L. Seligy

Mutagenesis Section, Environmental and Occupational Toxicology Division, Bureau of Chemical Hazards, Environmental Health Directorate, Health Protection Branch, Department of Health Canada, Ottawa, Ontario, Canada

Most contemporary bioinsecticides are derived from scaled-up cultures of Bacillus thuringiensis subspecies israelensis (Bti) and kurstaki (Btk), whose particulate fractions contain mostly B. thuringiensis spores (> 10^{12}L) and proteinaceous aggregates, including crystal-like parasporal inclusion bodies (PIB). Based on concerns over relatedness to B. cereus-group pathogens, we conducted extensive testing of B. thuringiensis (BT) products and their subfractions using seven human cell types. The Bti/Btk products generated nonspecific cytotoxicities involving loss in bioreduction, cell rounding, blebbing and detachment, degradation of immunodetectable proteins, and cytolysis. Their threshold dose (Dₜ = 5 × 10^{-16}g BT product/target cell) equaled to a single spore and a target cell half-life (tLD₅₀) of approximately 16 hr. At Dₜ > 10⁴, the tLD₅₀ rapidly shifted to < 4 hr; with antibiotic present, no component, including PIB-related δ-endotoxins, was cytolytic up to an equivalent of approximately 10⁹ Dt. The cytolytic agent(s) within the Bti/Btk-vegetative cell exoprotein (VCP) pool is an early spore outgrowth product identical to that of B. cereus and acting possibly by arresting protein synthesis. No cytolytic effects were seen with VCP from B. subtilis and Escherichia coli. These data, including recent epidemiologic work that suggests containing BT products have an inherent capacity to lyse human cells in vivo and in interactive forms and may also act as immune sensitizers. To critically impact at the whole body level, the exposure outcome would have to be an uncontrolled infection arising from intake of Btk/Bti spores. For humans, such a condition would be rare, arising possibly in equally rare exposure scenarios involving large doses of spores and individuals with weak or impaired microbioclearance capacities and/or immune response systems. Key words: biopesticides, bioreduction, cell death, cytolytic factors, endotoxin, immunodetection, immune sensitization, 35S-methionine, vegetative cells. Environ Health Perspect 108:919–930 (2000). [Online 18 August 2000] http://ehpnet1.niehs.nih.gov/docs/2000/108p919-930tayabali/abstract.html

The major sources of microbe-based biotechnology products (MBPs) released into the environment today are commercial Bacillus thuringiensis (BT) products that are derived from similarly produced, large-scale, sporulation-phase cultures of B. thuringiensis subspecies, mainly, israelensis (Bti) for targeting the larval stage of blood-sucking flies (Diptera) and kurstaki (Btk) for targeting foliage-eating larvae of moths (Lepidoptera) (1,2). The combined production of these BT products exceeds 500 metric tons annually in North America (3). From the point of view of homogeneity, these biopesticides (also known as bioinsecticides, biorational pesticides, or biological control agents) are very complex (2). However, whether in dry (powdered) or liquid states, they are very similar because they are essentially mixtures of culture ingredients that include, in increasing order of their mass, variable amounts of minerals, extracellular nucleic acid, a large spectrum of proteins (mostly sporulation phase-specific), and viable spores, often exceeding 10^{12}L of BT product (2,4,5). The liquid versions of both Bti- and Btk-derived commercial BT products can be easily fractionated by differential centrifugation to yield similar-sized particulate fractions containing > 99.9% of the spores and also the proteinaceous component made up of both regular and irregular amorphous structures (2). This proteinaceous material cannot be quantitatively separated from spores and other culture debris. The regular sized aggregates are predominantly those that are often referred to as crystal toxins (1,6). These structures make up the bulk of the mature parasporal inclusion body (PIB) matrix and contain most of the δ-endotoxins precursors (also any partially processed or degraded forms) encoded by specific cry genes (1,6). The PIBs are known to be coformed during sporulation within the sporangium or spore-mother cell, but exact details concerning molecular and cellular events in their formation and maturation have not been obtained. The PIB components of Bti (spherical) and Btk (bipyramidal) are uniquely different in shape and in protein composition, albeit we note in this study (and also noted by Beegle and Yamamoto (1) and Seligy and Rancourt (5)) that their size and amount in both cases can be highly variable. There is also a considerable amount of poorly defined, amorphous, proteinaceous material that we suggest originated from various stages of the culture process, including trace amounts (usually < 1% of viable spore count) of enzymes such as β-lactamases, proteases, and cuboidal-like crystals, the latter whose composition and cytotoxic effects are unknown (1,2,5). Our previous analyses (2–5,7) suggest that most of these structures are the likely sources of the prominent polypeptide size classes (60–67 kDa and 132–137 kDa) related to different maturation stages and classes of different δ-endotoxins, which essentially define the subspecies in the B. thuringiensis classification system and attribute target organism’s toxicity (1,6).

Through the years, the research and development emphasis on B. thuringiensis-mediated insecticidal activity has focused almost exclusively on the different types of δ-endotoxins, which are uniquely encoded by over 60 cry genes (6). However, only a few of these δ-endotoxins have been actually studied in any critical detail. Also, in comparison, the B. thuringiensis spores and other components that are obviously present in BT products have been greatly understudied either as bona fide components of “active ingredient”...
or as potential hazards (1,2–5,7). In side-by-side tests using insect cell assays similar to those claimed to be effective in the elucidation of B. thuringiensis δ-endotoxins (8–11), we demonstrated that the most toxic constituent of whole BT products is actually spore related (4,7). Depending on dose expressed either in international units or percent of BT per target cell, and temperature (23°C to 37°C), we found that the spore-induced response time, measured as target cell half-life (tLD50), was entirely consistent with the spore outgrowth temperature (23°C to 37°C), we found that the spore-induced response time, measured as target cell half-life (tLD50), was entirely consistent with the spore outgrowth conditions used by the human body defense system (2), these spores have the potential to survive and also to propagate in an in vivo mammalian environment. Our concern over the virulence potential of these organisms focuses on evidence that demonstrates the close genetic similarities between B. thuringiensis or B. cereus and B. anthracis pathogens (12,13) on putative infections arising from various B. thuringiensis subspecies (14–20), and recent epidemiologic evidence of Bernstein et al. (3) showing the occurrence of immune sensitization from use of commercial BT products in the control of lepidopteran pests of agricultural crops. In this agriculture-related exposure study, the immune sensitivities displayed by migrant workers was directed mainly at spore and vegetative cell components, suggesting that the δ-endotoxin components, at least as presented in BT products, were not very reactive, basically masked as compared to other cellular components. Therefore, as a baseline approach to clarify at least some of the concerns raised here, we conducted a detailed comparative study of the exposure effects of contemporary commercial BT products using several biodiagnostic systems with a variety of cells derived from different human and animal tissues.

In the present study, we summarize a large body of these cell and molecular biology experiments and illustrate key findings with data derived mainly from a human cell line (HT29) that has been used to model intestinal epithelial cell differentiation and effects of chemotoxins and microbial pathogens (21–34). Because contemporary Bti/Btk BT products are complex in composition, we carried out a series of experiments using subfractions of BT products (2,3) to determine which ones were the most biologically active and hence might potentially represent the biggest hazard. These experiments included analysis of BT product derivatives that could arise essentially by a form of biotransformation as in the parable of chemical toxicants. In this context, the BT product ingredients are transformed either through proteolysis of the proteinaceous moieties, particularly the δ-endotoxins associated with PIB and amorphic structures, and/or through the production of spore-derived vegetative cells and their exo-products and products made afterward during a second generation of sporeulation-phase activity. In reference to Bti/Btk vegetative cell exoproteins (VCPs) we also investigated their cytotoxic properties using 33S-methionine in experiments to measure effects on human cell biosynthesis and VCPs derived from strains of B. cereus, B. subtilis, and Escherichia coli.

Materials and Methods

Human target cells. The American Type Culture Collection (ATCC; Rockville, MD, USA) supplied the colonic epithelial cells (Caco-2, lot F-10803, and HT-29, lot F-12101), liver cells (Chang, lot F-11873, and Hep-G2, F-11225), and human blood derivatives, HL-60 (F-11917) and K-562 (F-11533). Mature erythrocytes were collected as previously described (3). Conditions for short- and long-term cultures are described elsewhere (4,7,29,30). Briefly, cells were cultured using Dulbecco’s Modified Eagles Medium (DMEM) with 25 mM glucose, 2 mM glutamine, 10% (v/v) fetal bovine serum (FBS), and 50 g/mL gentamicin in either T25 or 6- and 12-well culture plates with porous membranes for transfeeding (0.22 µm pore size). Monolayers (2 × 106 cells/cm²) were established 1 day before testing. All cell types were rinsed with DMEM (2 times) to remove antibiotic immediately before treatments. For scanning electron microscopy (SEM) analyses, cell monolayers were established on glass coverslips. Following exposure, cells were fixed with 4% (v/v) glutaraldehyde in 100 mM sodium cacodylate (pH 7.2) at room temperature (RT), post-fixed with 1% (w/v) osmium tetroxide in 100 mM sodium cacodylate, and dehydrated with an ethanol series. Samples were dried in a critical point drier (Autosamdri 814; Toumso Inc., Rockville, MD, USA), sputter-coated with 10 nm gold, and viewed with a JEOI LSM 6400 scanning electron microscope (LEO USA, Peabody, MA, USA) operating at 10 kV.

BT products and subfractions. We used primarily the BT products F48B (Btk strain H D 1) and VB12AS (Bti strain H D 14); however, several others were also investigated and are described in detail elsewhere, along with methods of manipulation and quality control analyses (2,5,7). The concentration of any BT product or derivative subfraction (also vegetative cells) was determined according to recently validated methods (7,13) and expressed as percent of BT product or equivalent, based on the content of viable spores or bacterial cell and/or protein contents relative to contents of undiluted (whole) BT product as the standard. Generally, side-by-side comparisons of BT products required only minor volume adjustments (<5%) to equalize spore contents. BT subfractions were prepared by carefully partitioning each BT product into supernatant (particulate-free) and pellet (particulate-rich) fractions by centrifugation (12,000 × g for 10 min at RT). Particulate-free filtrates (PFFs) were made by filtering (0.45 µm pore size) the supernatants diluted to 10% (v/v) with phosphate-buffered saline (PBS). Alternatively, PFFs were first concentrated 20-fold by reverse osmosis for 6 hr at 4°C. Semipurification of the PIB fractions was performed according to Thomas and Ellar (10) with the following modifications: 500 µL aliquots of each BT product were diluted 2-fold and vigorously vortexed (5 min at RT) with 50 µL sterile crushed glass to disrupt aggregates. After the discontinuous sucrose gradient centrifugation step (80,000 × g for 14 hr at 4°C, using a Beckman SW 50.1; Beckman Instruments, Mississauga, Ontario, Canada), the crystal-rich PIB layer was verified by SEM and protein analysis. Sucrose was removed by dilution with two volumes of ice-cold double distilled H2O and centrifugation. This purification procedure was repeated 3 times. Solubilization of PIB contents and conversion of pro-δ-endotoxin (~132–137 kDa) to activated δ-endotoxin (~60–67kDa) involved incubating either BT products or PIB-enriched fractions in 40 mM sodium carbonate (pH 10) and trypsin (0.1% w/v) at 37°C (10). We used protein electrophoresis to monitor digestion, and we collected residual particulates, including all spores, by centrifugation followed by membrane filtration (0.2 µm pore size). Equivalent dose was based on spore count and total protein of BT product.

Preparation of vegetative cell cultures and exoproteins. Spore outgrowth from BT products as well as from the controls, B. cereus (ATCC 14579; lot 90-075V), B. subtilis (ATCC 6051; lot 91-115V), and E. coli C600 (ATCC), were grown in Luria-Bertani (LB) broth at 37°C for 6–18 hr. Alternatively, they were grown in Grace’s insect cell medium or DMEM with or without human cells. Following enumeration of colony-forming units (cfu) per milliliter (2), each culture was adjusted to a concentration equivalent to...
10% (w/v) BT product (~3 × 10^{10} cfu/mL) with PBS. Cultures were partitioned into cell (pellet) and cell-free (supernatant) fractions by centrifugation (12,000 × g for 10 min at RT). The supernatants, or VCPs, were filter sterilized (0.22 µm pore) before and after concentrating 1,000-fold by reverse osmosis (6 hr at 4°C). We monitored VCP production from washed cells after cells were rapidly resuspended in the same volume of fresh culture medium and harvested at various times thereafter. We conducted various stability tests on VCP before it was tested with human cells. We incubated aliquots of VCP (1 mL) at 0–100°C for intervals from 10 sec to 48 hr. Samples were also subjected to freeze-thaw from –80°C to 37°C in 15 min cycles and treatment with proteases (trypsin at 0.25% w/v or proteinase K at 0.0012% w/v) for various time intervals at 37°C. To control samples, we used fresh insect cell medium or DMEM treated in the same manner as the VCP. We used a series of molecular mass cut-off membranes (Centricon 10–100 kDa; Amicon, Beverly, MA, USA) and gel filtration (Sephadex G-150,100,50; Amersham Pharmacia Biotech, Baie d’Urfe, Quebec, Canada) to approximate the dynamic size and homogeneity of the VCP toxic constituent(s).

**Biological assays.** Bioreduction or cell redox activity, measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Chemical Co., St. Louis, MO, USA) and polypeptide analyses and immunodetection assays were conducted as described for insect cells (4,7). Electrophoretically separated polypeptides were stained with either 0.1% Coomassie Blue or silver nitrate. We obtained anti-actin (5 µg/mL), anti-cyclin (5 µg/mL), and anti-tubulin (2 µg/mL) antibodies from Roche Diagnostics (Laval, Quebec, Canada). Other probes included anti-cry 1Ab and 1Ac antibodies (1:500 dilution; Agdia Inc., Elkhart, IN, USA), anti-glutathione-S-transferase (1:20 dilution; Cedarlane, Hornby, Ontario, Canada), and anti-heat shock proteins 60 (hsp60; 10 µg/mL; M onosan, Am U den, Netherlands) and 70 (hsp70; 11 µg/mL; Sigma). Specificity was based on the polypeptide target size (diagnostic molecular mass) and the absence of cross-reaction with polypeptides from either BT products or vegetative B. thuringiensis cultures. In radiolabeling experiments, we incubated HT29 monolayers with [35S]-methionine (8.5 µCi/mL DMEM, methionine free) in 30 min pulses before or during the dosing regime. At each end point, the supernatants containing any detached cells and cellular debris were transferred to microtubes and frozen at −80°C or centrifuged (1,000 × g for 10 min) first to recover fully intact cells. Radiolabeled polypeptides were assessed by autoradiography (Kodak X-Omat XAR-5; Sigma) after gels were dried and sprayed with radioautographic fluor (Enhance; Dupont, Mississauga, Ontario, Canada). For quantification, X-ray films were exposed for varying durations at −80°C to achieve optimal band resolution before densitometry (7).

**Results**

**Cytotoxic effects of whole BT products.** In preliminary studies, several human cell lines, as well as those derived from monkey, mouse, and sheep, were exposed to an array of dilutions of whole BT products or derivative fractions. The results from all of these assays, albeit extensive in detail, were nonetheless nearly identical, including those that compared the apical and basal surfaces of cell monolayers with semipermeable membranes which can separate human cells from actual contact with bacteria and PIB structures of BT products or derivatives. The key difference that distinguished between assays in cell suspension and assays of cells compromised as monolayers was the degree of difficulty in collecting data. The former necessitated additional steps to physically separate BT-related components from both undamaged and damaged monolayers to monitor intermediate changes by microscopic methods. For these reasons, we chose to illustrate most of the salient data with experiments using one human cell monolayer system.

As illustrated in Figure 1A with HT29 cells, quantification of bioreduction activity showed only marginal decreases (<10%) as long as antibiotic was present, even when exposures were extended up to 24 hr. However, without antibiotic, the loss in bioreduction activity was essentially 100% with all doses >5 × 10^{-14}% BT product/target cell. In the dose range of 10^{-16} to 5 × 10^{-14}% BT product/target cell, a threshold response was seen with both Bti and Btk BT products. This threshold dose (DT) response was generated by the presence of one spore per assay. Experiments involving short exposure intervals indicated that the earliest bioreduction changes began between 2 hr to 4 hr at doses ≥10^{-15}% BT product/target cell or ~10^{–2} DT. The results from several dose-time assays, conducted in experiments shown in Figure 1A, were used to derive dose-response times that correspond to target cell LD_{50}, referred to here as tLD_{50}. Using this method, Bti and Btk BT products generated virtually identical tLD_{50} values. The dose-response data (n = 30 separate experiments) using five different human cell systems are summarized in Figure 1B. The timing differences between suspension and monolayer-propagated cell types can be accounted for by the additional steps in the assay procedure required for cells in suspension.

Studies on the changes of human cell morphology and bioreduction loss conducted over various exposure regimes established that loss of bioreduction corresponded directly with numbers of human cells exhibiting visible damage. For example, exposures of 10^{-2}–10^{-1}% BT product/target cell (Figure 2A) resulted in a 50% loss in attached cells by 4.4 ± 0.3 hr and a corresponding loss in bioreduction capacity at 4.4 ± 0.4 hr (Figure 1B). Enumeration of the shed cells based on separate exposure assays (n = 20) revealed that 43 ± 12% of the cells had degraded beyond recognition as long as one spore was present per assay well and its outgrowth was permitted. Companion studies using SEM

![Figure 1](image-url)
confirmed the dramatic changes between control (Figure 2B, C) and treated cells (Figure 2D, E). In addition to numerous vegetative cells adhering to the surfaces of the residual HT29 cells, the microvilli of these cells were absent and none of the cells were actually intact (Figure 2E).

Further investigations were made to establish the fate of cellular proteins by immunodetection. Because the results from the six proteins were very similar, examples of actin and cyclin (an exception) are shown to demonstrate derivation of half-lives of these proteins. As shown in Figure 3A–D using data from 10^{-7} % BT product/target cell, the variation in half-life of cellular proteins was 4.7–5.5 hr. They correspond to the tLD_{50} values derived from enumerating attached cells. The early drop in HT29 cyclin content may be unique, as other cell systems exhibited little or no expression of this isoform. Similar analysis of the shed cell fraction indicated that concentrations of all of the protein markers (including total protein) were underrepresented by at least 80%. This pattern of degradation of cellular protein was virtually identical to that seen in exposure regimes using the Bti-based commercial product.

**BT product particulate-free fractions.**

We assessed the cytotoxic contribution of the liquid PFF of BT products before and after micropore filtration (Figure 4). These fractions were free of spores and aggregates of any kind, as observed by phase-contrast microscopy, SEM, and incubation on LB agar plates. The polypeptide contents of BT products and PFFs (concentrated 10 times) are shown in Figure 4A. The most abundant polypeptides in Btk BT products were those corresponding to pro-δ-endotoxin (132–134 kDa) and activated-δ-endotoxin (60–67 kDa) pools. In the case of the Bti product, there was an additional polypeptide with a molecular mass of the CytA toxin (24–27 kDa) (10).

The PFF was ~ 67% of whole Bti or Btk BT product volume, but it contained ≤ 0.02% total BT protein (or 5 µg/mL). The Btk polypeptides measured 18, 34, 80, and > 200 kDa, whereas those of Bti PFF measured 34 and 95 kDa. When either of these PFF sources was used in exposure assays, the bioreduction activity of human cells decreased, but as shown in Figure 4B, this occurred only with doses equivalent to ≥ 10^{-4} % BT product/target cell. This dose is ~ 1,000-fold over the BT product dose routinely used in other experiments (Figures 2 and 3). Comparisons of phase-contrast micrographs of HT29 cells exposed to PBS or Btk PFF (Figure 4C, D) revealed that treated cells remained attached during the 24-hr exposures and that they had lost tight cell-to-cell associations (junctions) compared to PBS-treated cells. Occasionally treated cells formed cell–cell fusions or syncytia (Figure 4F). In treatments with Bti PFF, entire monolayers detached within a 2-hr exposure interval (Figure 4G). However, these cells still exhibited tight junctions and redox activities (Figure 4H) comparable to PBS-treated cells (Figure 4E). Also, these cells could be replated and grown with a viability > 95%, essentially comparable to that seen with trypsin (2.5 mg/mL) treatment, routinely used in the passage of cell monolayers.

**Cytotoxic effects of parasporal inclusion bodies.**

We used discontinuous sucrose gradient centrifugation to enrich for PIB structures of BT products. The fractionation of
Btk products routinely gave five opaque particulate layers (bands), whereas the Bti product resulted in only four. Polypeptide analysis of these fractions indicated that the bulk of Btk δ-endotoxin proteins (67 kDa and 132 kDa) were found in fractions 3 and 4 (Figure 5A, fractions 3 and 4). However, SEM analysis revealed that fraction 4 contained the most PIBs (Figure 5B), representing a 9-fold increase over the original spore content. In addition, cuboidal crystals were frequently seen (about one per every four Btk spores). For Bti, fraction 3 had the highest content of polypeptide and spherelike PIBs (relative to spore number) (Figure 5C, fraction 3, and 5D). Solubilization of the PIB enrichments was accomplished by treatment with buffer, pH 10, and trypsin. Solubilized Btk fraction 4 (centrifuged and filtered) contained only the 63–67 kDa polypeptides (Figure 5A, lane 7), whereas, in addition to the 67 kDa polypeptide, the spore-rich pellet (lane 7) had the 78 kDa polypeptide but no 22 kDa constituent.

As shown in Figure 6A, results were different when fractions enriched in intact PIBs and solubilized versions were exposed for 4 hr. Intact PIBs from either Bti or Btk sources registered little effect. However, the use of solubilized Btk PIB gave a linear response and, at doses of 100 µg/mL, resulted in 30% loss of bioreduction activity. This dose is about 10 times greater than the highest dose of BT product routinely tested. Identical assays with solubilized Bti PIB initially followed the Btk profile, but then rapidly changed as its dose was increased (Figure 6A). These levels of cytotoxicity changed little (~5%) on retesting at 6 hr. In controls, HT29 cells were treated separately with carbonate buffer and trypsin. All of these treatments resulted in ≤10% loss in bioreduction.

We conducted experiments involving the measurement of cell proteins. Results with intact PIB treatments were the same as untreated controls. As shown in Figure 6B, actin levels and attached cells only gradually decreased as the dose of solubilized Btk-PIB or Bti-PIB was increased up to 100 µg/mL. The loss of these two indicators was 30 ± 10%. However, the corresponding SEM analysis revealed no obvious change in either morphology or volume of attached cells, and shed cells also appeared to be intact (35). Assuming a linear dose response, extrapolation of the curves in Figure 6B would predict an LD_{50} of 130–140 µg/mL.

Experiments with a commercially available antibody probe considered specific for cry1Ab and 1Ac δ-endotoxins (see “Materials and Methods”) indicated that cultures of Btk spores produced detectable amounts of these proteins.

**Figure 3.** Changes in specific proteins during HT29 exposure to BT products. Example immunoblot assays for actin (A) and cyclin (B) after exposure to 10^{−7}–10^{−6} BT product/target cell (see “Materials and Methods”). Lane 65 shows only trace levels of actin and cyclin in shed cells at 6 hr. (C and D) Predicted TI_{50} values determined from the quantification of proteins of attached cells. Abbreviations: ACT, actin; GST, glutathione-S-transferase; hsp, heat shock protein; TUB, tubulin.

**Figure 4.** Protein content and toxicity of BT product particulate-free supernatant fractions. (A) Coomassie-stained SDS-PAGE gels of undiluted Btk and Bti BT products (4% final) and corresponding (PFFs concentrated to 10X 10 µL/lane). (B) Bioreduction assay of HT29 cells exposed 24 hr to PBS or a series of PFF dilutions. Antibiotic had no effect. The dashed line represents the artifactual loss of bioreduction due to premature shedding of the entire monolayer induced by Bti PFF trypsin-like activity. Data are from three determinations ±SD. (C–H) Phase-contrast micrographs of HT29 cells exposed 24 hr to PBS (C,D,E) or PFFs of Btk (D,F) or Bti (G,H) products at a dose equivalent to 10^{−5} % BT product/target cell. Comparison of 1X (D,G) and 15X (E,H) image magnifications of monolayers in assay wells directly after MTT-formazan production shows the release of the entire monolayer (or portions of it).
polypeptides, but only at about 18 hr and up to at least 48 hr of culture (35). This expression profile corresponded favorably with the initial buildup of spores, but not with the cytotoxic activity produced by exposing human cells to BT product for only 4–8 hr in the absence of antibiotic. These data added to the certainty that the cytotoxicity observed from BT products was not attributable to either PIBs present within BT products or "second generation" PIBs generated from sporulated cultures.

**Cytotoxicity of vegetative cells and their exoproducts** Exposures to intact or solubilized PIBs and the liquid, particulate-free fractions of BT products (Figures 4 and 6) caused little human cell damage as compared to that seen whenever spores were present and their outgrowth was not prevented by antibiotic. Further investigations revealed that there was a dependence on the presence of human cells for efficient vegetative cell and VCP production to occur from spores. Also, peak destruction of human cells occurred early during the buildup of vegetative cells, and this cytotoxicity (per microgram of total VCP) produced by either Btk or Bti cells exhibited a relative decrease as these cells entered sporulation phase and other proteins accumulated after approximately 18 hr. Experiments with a commercially available antibody probe considered specific for cry1Ab and 1Ac δ-endotoxins (see "Materials and Methods") indicated that cultures of Btk spores produced detectable amounts of these polypeptides but only at about 18 hr and up to at least 48 hr of culture (35). This expression profile corresponded favorably with the initial buildup of spores (also seen for Bti), but not with the cytotoxic activity produced by exposing human cells to BT product for only 4–8 hr in the absence of antibiotic. These data add to the certainty that the cytotoxicity observed from BT products was not attributable to either PIBs present within BT products or "second generation" PIBs generated from sporulated cultures.

In the absence of human cells, spore outgrowth was found to be ≤4% in either fresh DMEM or in medium conditioned for 8 or 24 hr with any of the human cell monolayers. These observations are in contrast to those using Graces' insect cell medium, which alone supports spore outgrowth at levels comparable to or better than in LB broth (2). However, in all types of media, where at least some spore outgrowth actually occurred, the VCP cytotoxicity levels were comparable if they were made relative to number of vegetative cells. To simplify our investigations, 8-hr outgrowths were mass-produced from BT products incubated in Graces' insect cell medium without FBS. The removal of FBS eliminates its masking of cell proteins during analysis. Tests showed that the absence of FBS shifted the Bti/Btk growth curves by ~2 hr, but otherwise had little or no effect on the level of VCP exocytolytic activity produced by either Btk or Bti sources. In addition, we removed fine particulate matter (spores, vegetative cells, cell wall debris, and potentially PIB) from culture supernatants by filter sterilization before concentrating and retesting supernatants. These steps also had little effect (<5%) on the severity of the cytolytic response, but improved the storage qualities of VCP stockpiled at various stages of cultures harvest from 4 hr to 48 hr.

In 2-hr exposure regimes with Btk VCP from 8-hr cultures, we observed considerable cell damage as compared to control cells (Figure 7), but the overall damage was no different than that seen in exposures with BT product (Figure 2). In assays using VCP dilutions, there was a saturation effect at 0.5X to 1X VCP, as both doses generated similar LD50 values of 6–8 min (Figure 7E). Based on the number of vegetative cells present at 8

![Figure 5](image-url) Properties of PIB enrichments and their solubilized derivatives. SDS-PAGE analysis of PIB enrichments obtained by sucrose gradient centrifugation using (A) Btk and resultant fractions (lanes F1–F5) and (C) Btk BT products and resultant fractions (lanes F1–F4). Fractions were trypsin solubilized (SOL) and separated into supernatant filtrate (FILT) and a spore-containing pellet (PEL). (B) SEM of Btk fraction 4 (shown in (A)), the most enriched for Btk bipyramidal PIB crystallike structures. (D) SEM of Bti fraction 3 BT product (shown in (C)), the most enriched in spherical PIB structures and its trypsin-solubilized derivative.

![Figure 6](image-url) Cytotoxicity of PIBs and solubilized derivatives. (A) Changes in HT29 cell bioreduction activity after exposures to dilutions of unsolubilized fractions (F) and their trypsin-solubilized (SOL) filtrates. In all cases, the exposures were for 4 hr at 37°C with antibiotic added as a precaution to control possible spore contamination. (B) Corresponding changes in cell number and actin levels of attached HT29 cells after exposure to trypsin-solubilized PIB of Btk or Bti. All data points are the means of four separate experiments ± SD.
hr, 0.5X VCP was estimated to be equivalent to approximately $1.4 \times 10^{-7}$% BT product (spores)/target cell. In parallel assays with 0.5X VCP, the average LD$_{50}$ value predicted by loss of specific proteins and cell adhesion was 42 min for hsp 60 and hsp 73, 60 min for actin, and 90 min for loss of cell adhesion (Figure 7F). Corresponding analysis of the shed cell population indicated total cytolyis and an inconsistent loss of protein indicators (Figure 7F). In all of these assays, Btk VCP gave results similar to those of Btk VCP, if based on an equivalent number of Btk vegetative cells or the total Btk protein content.

Further exposure tests using washed vegetative cells determined that an LD$_{50}$ required at least 1.5 hr of preincubation or at least one vegetative cell doubling to take effect. The addition of gentamicin also decreased the regenerated cytolytic activity by > 90%. Also, based on total protein content per bacterial cell, VCP cytolytic activity harvested at later stages of culture decreased rapidly (> 90% by 18–20 hr). These results show that the production of cytolytic factor(s) is likely dependent on the early growth phase and de novo synthesis rather than on the simple release of presynthesized product(s).

**VCP effects on human cell protein biosynthesis.** We investigated possible effects of VCP on human cell protein synthesis by examining changes in the polypeptide composition of each human cell type used in time-course experiments of 0–24 hr exposure. These experiments involved protein staining (silver or Coomassie blue) and demonstrated that loss was not specific to any protein species (35). To detail early events, HT29 cells were pulsed with $^{35}$S-methionine at 30 min intervals over a 6-hr exposure period, using either PBS or 8-hr VCP at a dose equivalent to $1.4 \times 10^{-7}$% BT product/target cell. Resultant autoradiographs of $^{35}$S-labeled polypeptides (Figure 8B, C) permitted calibration of the changes in protein synthesis of attached cells. In control experiments, we detected > 150 putative polypeptide products using various autoradiographic exposures. With VCP treatment, polypeptide synthesis was rapidly reduced without apparent bias for any abundant polypeptide species. The corresponding LD$_{50}$ estimate for loss of $^{35}$S-methionine incorporation was ≤ 15 min or approximately one-fourth to one-third the rate of loss of specific protein indicators (see “Cytotoxicity of vegetative cells and their exoproducts”). At the same time, the $^{35}$S label associated with cell shedding (Figure 8C) was < 7% that of control cells and soon reduced to background levels thereafter. This indicates a rapid shutdown of protein synthesis culminating in a less rapid, nonspecific degradation of various cellular proteins.

**VCP properties.** Polypeptide analysis of VCP produced at various stages of spore outgrowth indicated considerable size heterogeneity, ranging from 5 kDa to > 200 kDa. Tests conducted before and after crude fractionation of the nondenatured VCP, using selective membrane (pore size) filtration as well as gel and bead matrices (see “Materials and Methods”), indicated that the cytolytic constituent(s) was between 50 and 100 kDa in size. In terms of total protein, this activity was approximately 20 times higher in VCP harvested at 6–8 hr than at 18–24 hr when spores and cry products accumulate (see “VCP effects on human cell protein biosynthesis”).

Further studies (34) demonstrated that loss was not specific to any protein species (see “Cytotoxicity of vegetative cells and their exoproducts”). The arrow shows characteristic initial overstimulation effect. (F) Changes in bioreduction activity during the time-course exposure to PBS or VCP diluted to 0.1X, 0.5X, or 1.0X of the stock dose. These data were adjusted (subtracted) to take into account contributions from $Eri$-related exorhoductase activity that otherwise would give an underestimation of toxic effects. The arrow shows characteristic initial overstimulation effect. (F) Changes in attached cell number and corresponding levels of actin and hsp 73 after exposure to 0.5X VCP. The dashed line indicates the profile of detached cells. The inset shows a sample immunoblot using total protein from lysates of attached cells separated by SDS-PAGE and probed with the actin antibody.
nicotinamide adenine dinucleotide (NAD; ≤ 0.1 mM), and adenosine triphosphate (≤ 0.1 mM), which are suggested to inhibit or modify activities of B. cereus (18). In all cases, control tests with these agents (no VCP) resulted in losses of bioirradiation of ≤ 5%.

Exposures to VCPs from other bacterial species. Stocks of B. cereus, B. subtilis and E. coli cells and their VCPs were prepared in the same manner as those derived from Btk and Bti spore outgrowths. The tests with B. cereus (spores or vegetative cells) showed that both biomass and VCP cytotoxic effects were comparable to those of Bti and Btk cells (Figure 9A–C). In contrast, B. subtilis and E. coli produced only approximately 1% of the B. thuringiensis biomass (Figure 9A). The strong binding to human cells exhibited by B. subtilis and E. coli influences human cell MTT substrate utilization by either increasing or decreasing bioreduction activity in relation to untreated control cells (Figure 9B). In all cases, these negative changes could be blocked by gentamicin to arrest vegetative or decreasing bioreduction activity in in vitro testing of Btk VCP stock made equivalent by serial dilutions indicated that the international unit (IU) and the percentage of “active ingredient”.

The IU is essentially an arbitrary unit, reflecting a relative measure of insect larval death attributed to ingestion of BT product (1,36–38). In terms of abundance, the most consistent and dominant components of BT products, the “active ingredient” has been considered synonymous with δ-endotoxins, encoded by various cry genes, whose presence or absence in plasmid form justifies the rationale for subspecies classification (6). However, the assumption that the sole toxic ingredient is equivalent to that reported from using laboratory-processed and purified δ-endotoxins merits debate because in most cases the importance of controlling spore contamination and related effects was not recognized.

Given the complexity of current BT products, it is difficult to rationalize what either the IU or percent of “active ingredient” directly measures in the context of environmental applications (36–38). Our previous in vitro toxicology tests with insect cells showed that spores played a major role as a toxic ingredient, requiring about the same time interval for septicemia to take place as effects predicted from the in vivo breakdown of PIBs (4,7). In those experiments, the dose was expressed in terms of IU per target cell, where it was determined that 1 IU was equivalent to approximately 2,400 spores (2). For the baseline study of human cell effects presented here, all the data were expressed as percent of BT product per target cell. This format allows us to assess (or reassess at a later date) any subcomponent of these BT products as long as the concentration is known in relation to other known components (e.g., spores). This approach would also be useful for testing plant biotechnology products as fresh or dried powders. Further, by supplying appropriate parameters about the target cell or tissue/organoid assay system (e.g., number and type of cells in suspension per milliliter of culture medium or per square centimeter of surface), any manner of exposure unit can be derived. For our purposes, because of the abundance, hardness, and likelihood of potential risk (13,37), we used the spore to estimate relative enrichment and dose equivalence of PIBs and also vegetative cells and their exoproducts. Thus, a typical application potentially delivering approximately 3 × 10^10 IU/hectare or approximately 300 IU/cm^2 (~ 7.2 × 10^5 spores/cm^2) (2,13,37) would equate to a dose of approximately 2.4 × 10^5–5% BT product/cm^2 or 10^10–1% BT product/target cell as tested in this study. Also, in relation to target insects, such a dose would be intermediate to those contained within the 52–84 μm diameter BT droplets that gave maximum (optimal) larval mortality over a 1-week exposure interval (38). From this dose optimization and an approximation of the surface area of an insect larval midgut (< 0.013 cm^2), the in vivo cell surface dose would be ≥ 1.5 × 10^12% BT product/target cell, which falls within the range for human cell assays.

Discussion

Complexity of biopesticides and dose estimations. As compared to chemical pesticides, the details provided in material safety data sheets for biopesticides are unusually sparse and vague, considering the extent to which they are used and promoted for community and even household use. Recently we addressed some of these data gaps (2–5,7) by introducing sampling strategies and physical, biochemical, immunologic, and molecular genetic methods that should be applicable for assessing quality control and quantification of health effects and efficacy of these and other biotechnology products, including transgenic plant derivatives, intended for release into the environment. The data derived from the analysis of several BT products demonstrate that these products are inherently heterogeneous and impure with respect to a defined active ingredient when compared to chemical counterparts.

The most common descriptors used by the industry for Btk- and Bti-based products in parallel calibrations are the international unit (IU) and the percentage of “active ingredient”. The IU is essentially an arbitrary unit, reflecting a relative measure of insect larval death attributed to ingestion of BT product (1,36–38). In terms of abundance, the two most consistent and dominant components of BT products that we have found are the spores, which in most cases actually exceed 10^15/L, and the PIB crystalloids, whose concentrations are equal to or often 25–50% of the spore content (5,36). In the promotion of BT products, the “active ingredient” has been considered synonymous with δ-endotoxins, encoded by various cry genes, whose presence or absence in plasmid form justifies the rationale for subspecies classification (6). However, the assumption that the sole toxic ingredient is equivalent to that reported from using laboratory-processed and purified δ-endotoxins merits debate because in most cases the importance of controlling spore contamination and related effects was not recognized.

Given the complexity of current BT products, it is difficult to rationalize what either the IU or percent of “active ingredient” directly measures in the context of environmental applications (36–38). Our previous in vitro toxicology tests with insect cells showed that spores played a major role as a toxic ingredient, requiring about the same time interval for septicemia to take place as effects predicted from the in vivo breakdown of PIBs (4,7). In those experiments, the dose was expressed in terms of IU per target cell, where it was determined that 1 IU was equivalent to approximately 2,400 spores (2). For the baseline study of human cell effects presented here, all the data were expressed as percent of BT product per target cell. This format allows us to assess (or reassess at a later date) any subcomponent of these BT products as long as the concentration is known in relation to other known components (e.g., spores). This approach would also be useful for testing plant biotechnology products as fresh or dried powders. Further, by supplying appropriate parameters about the target cell or tissue/organoid assay system (e.g., number and type of cells in suspension per milliliter of culture medium or per square centimeter of surface), any manner of exposure unit can be derived. For our purposes, because of the abundance, hardness, and likelihood of potential risk (13,37), we used the spore to estimate relative enrichment and dose equivalence of PIBs and also vegetative cells and their exoproducts. Thus, a typical application potentially delivering approximately 3 × 10^10 IU/hectare or approximately 300 IU/cm^2 (~ 7.2 × 10^5 spores/cm^2) (2,13,37) would equate to a dose of approximately 2.4 × 10^5–5% BT product/cm^2 or 10^10–1% BT product/target cell as tested in this study. Also, in relation to target insects, such a dose would be intermediate to those contained within the 52–84 μm diameter BT droplets that gave maximum (optimal) larval mortality over a 1-week exposure interval (38). From this dose optimization and an approximation of the surface area of an insect larval midgut (< 0.013 cm^2), the in vivo cell surface dose would be ≥ 1.5 × 10^12% BT product/target cell, which falls within the range for human cell assays.

Toxicity of BT products and subcomponents in the absence and presence of antibiotic. In the absence of antibiotic, all doses from approximately 5 × 10^14% to 10^5% BT product/target cell were cytotoxic to all types of human cells tested. Detailed tests with serial dilutions of BT products indicated that the threshold dose would be approximately 5 × 10^14% BT product/target cell. At this dose, the tLD 50 was approximately 16 hr, a time point corresponding to when a single spore (per assay well) has reached midexponential growth. Similarly, differences in spore contents of the various subfractions tested were found to account for the observed differences in lag time before dose-induced cell changes were observed. Thus, the no-observed-adverse-effect level (NOAEL) of BT products and their subfractions would correspond to doses in which there were either no spores present or there was an inhibitor (antibiotic) present to block spore-associated activity. In the latter case, concerning whole BT products and their particulate-rich (pellet) and particulate-free (supernatant) fractions, the dose defining Dt (NOAEL) of BT products and their subfractions would correspond to doses in which the rationale for subspecies classification (6).

Hence, the assumption that the sole toxic ingredient is equivalent to that reported from using laboratory-processed and purified δ-endotoxins merits debate because in most cases the importance of controlling spore contamination and related effects was not recognized.
Materials and methods

Toxicity of activated δ-endotoxin(s). The supernatant fraction (with antibiotic) and its filtered version were shown to be essentially nontoxic unless used at near full BT product strength. Aside from its coloration (usually brownish yellow), the supernatant (1X) fraction of either Bti or Btk BT product sources contained < 0.02% of the total protein content and only a few discernable polypeptides, which did not resemble constituents detected in PIB enrichments. Further, the Btk fraction failed to react with commercially prepared pCA1Ab or 1Ac antibody probes (35), indicating that little or no “activated” δ-endotoxin material may be present. After the BT product was concentrated to an equivalent T of ≥ 1010, the Btk and Bti supernatants caused multicell fusions and shedding, similar to that seen after treatment with a virus or polyethylene glycol (35,40). This shedding may be caused by trypsin-like protease activity, and the presence of such factors in BT products suggests a carryover of fermentation products as noted for β-lactamase (5,13).

Aside from the liquid and spore fractions, the only other major constituent of BT products is the rather heterogeneous collection of protease-sensitive amorphous and crystalline PIB particulates. The PIB structures are usually less abundant than spores (2,5), but in theory should be equal in amount (1.36). SEM and light microscope analyses with protein stains indicated that both types of particulates have protein as a major part of their composition. Because similar amorphic structures were also seen in Btk and Bti vegetative cell phase cultures (35), we suggest that the amorphic particulates are aggregates of exopolypeptides and possibly other culture residues. After treatment with a broad-substrate protease and detergent, > 90% of the spores in BT products can be recovered free of protein, mainly those related to the δ-endotoxins and their cleavage products (2). However, in our experience using BT products and various purification methods for δ-endotoxins (10,39,41–46), definitive, quantitative separation of amorphous and crystalline PIB particulates from each other, and from spores and other cellular debris, is not very realistic. When tested at concentrations up to 100 µg/mL (~1.6 × 10⁻⁵% BT product/target cell), exposures using either Bti or Btk PIB enrichments estimated to be 9-fold relative to spore content showed only transient changes (Figure 6) as long as antibiotic was present to control spore contamination (35). There were no distinct changes in cell morphology, and cell viability was ≥ 95% with subsequent changes of medium and cell passage. The lack of cytolyis with Bti PIB, which contains CytA protein (~10% of the total protein), indicates that it may be unavailable to interact with target cells as reported earlier (8).

Following PIB solubilization with trypsin treatment, we found that residual aggregates and spores could be effectively removed by microfiltration, as shown by the absence of bacterial colonies after culturing filtrates for 48 hr. With the Btk BT product, this approach resulted in the disappearance of 200 kDa and 132–134 kDa proteins and a marked increase in 63–67 kDa protein (Figure 5A), consistent with a conversion of pro-δ-endotoxin to “activated” δ-endotoxin (1.6,10). The trypsin treatment of Bti BT product resulted in a similar pattern of polypeptide solubilization; its unique polypeptide profile may account for the differences in bioreduction response, which was not seen when either cell or actin content was assayed (Figure 6A, B). The dose-dependent changes using these two bioindicators for Bti and all three bioindicators for Btk indicate a crude linear response beginning at a dose of approximately 5µg/mL (~10⁻⁶% BT product/target cell) with an extrapolated T of approximately 130–140 µg/mL. This dose corresponds to approximately 2 × 10⁻⁵% BT product/target cell or ≥ 4 × 10⁸

Figure 9. Toxic effects of B. subtilis, E. coli, and B. cereus and their VCPs. (A) Growth of B. cereus (Bc), B. subtilis (Bs), E. coli (Ec), and B. thuringiensis (Btk or Bti) incubated at 37°C in DM EM without phenol red and antibiotic and in the presence (+) or absence (-) of HT29 cells. Changes in optical density (OD; 450nm) caused by vegetative cell production (biomass) were automatically recorded starting 15 min. (B) Changes in HT29 bioreduction activity after 24 hr exposure to Bc, Bs, or Ec at 37°C in the absence or presence of antibiotic. (C) Changes in bioreduction activity of HT29 cells on exposure to VCP. The overall concentration of VCP was made equivalent to BtVCP at a dose of 3.5 × 10⁻⁷% BT product/target cell, based on total protein concentration.

Environmental Health Perspectives • VOLUME 108 | NUMBER 10 | October 2000 927
Dt, and suggests that Bti or Btk BT product-induced human cell toxicity is not caused by their PIB-related moieties. In support of this conclusion, VCP harvested from late Btk cultures (>18 hr) reacted to cry1Ab and 1Ac antibodies, but was 8–10 times less cytotoxic than VCP harvested from 6- or 8-hr cultures (35). Therefore it is doubtful that even a second generation of δ-endotoxin production contributes much to the BT product toxicity so far observed.

On examining previous studies that used δ-endotoxin preparations derived from a variety of mechanical extraction methods, we observed that no precautions were taken (reported) to eliminate possible effects from spores or VCP carryover in δ-endotoxin preparations before they were tested in various systems (10,39,41–43). Furthermore, these δ-endotoxin preparations were not derived from commercial BT products. This is especially important because of the serious potential for VCP carryover in batch cultures that would exaggerate the effects claimed for δ-endotoxins. At present, Bti PIB cytotoxic effects have been reported for human erythrocytes, canine kidney fibroblasts (MDCK cells), mouse fibroblasts (L929 cells), primary pig lymphocytes, and mouse epithelial carcinoma cells (EC2, EC5 and EC6 cells) (8,10,44,45). Also, PIBs from noninsecticidal strains of B. thuringiensis have been shown to be lethal to leukemic T cells but not to normal T cells (46). These noncommercial sources were deemed toxic according to trypan blue uptake by mammalian cells (LD₅₀ = 10–80 µg/mL in 30 min) and human erythrocytes (LD₅₀ = 1–2 µg/mL in 60 min), but only if they were pre-treated at pH 10–10.5 with trypsin (46). In either case, the morphologic changes (e.g., cell rounding, swelling, membrane blebbing, and lysis) were about the same. In our investigations, an LD₅₀ of approximately 130–140µg/mL was predicated by assays with PIB δ-endotoxin-related proteins from Bti and Btk BT product sources. However, these same effects are caused by VCP from Bti, Btk, and B. cereus vegetative cells, suggesting that VCP components may have been included in the culture residues used to produce PIBs for earlier work.

VCP effects of Btk, Bti, B. cereus, and related bacteria. Experiments with vegetative cells resulting from spore outgrowth demonstrated that the most toxic substance(s) was released into the surrounding medium soon after spores germinated and began proliferating. Use of VCP instead of BT products, but at an equivalent dose, reduced the exposure time seen with BT products by 30-fold or more. Further study of VCP production is in order, but we know that it can be generated in bacterial culture broth as well as in insect and human cell media, using a range of temperatures from approximately 15°C and up to 45°C (35). The marked reduction of B. thuringiensis proliferation in fresh human cell medium (DMEM) or in medium conditioned by preincubation with human cells indicates that a direct interaction with human cells is probably necessary for stimulation of B. thuringiensis growth and possibly VCP production. Also, low VCP concentrations (Figure 7E) induced an initial stimulation in target cell bioreduction activity. Because a very similar stimulation was observed in human and insect cell exposures to ionophore A23187 (35), the transient increase in bioreduction could have resulted from cell membrane alterations allowing an increased uptake of tetrazolium (MTT) substrate into cells and/or a stimulation of membrane-coupled electron transport at different intracellular sites (7,47).

We used strains of spore-forming B. cereus and B. subtilis and a gram-negative, nonpathogenic strain of E. coli to investigate the possibility that the cytotoxic effects produced by Btk or Bti vegetative cells could also be produced by other bacteria. The B. cereus strain is used in standard antibiotic testing (7). Polymerase chain reaction-DNA hybridization assays using six different genes indicated that Bti and Btk spores and this B. cereus strain share common sequences (13), whereas B. subtilis is distinctly related. The damage generated from B. cereus in the absence of antibiotic was remarkably the same as that seen with Btk and Bti. However, B. subtilis and E. coli had little or no effect on target cells in terms of morphology and capacity for passage without appreciable cell loss. Also, no toxic effects were observed with their VCPs (Figure 9C), even after their protein contents were concentrated to be roughly equivalent to that of VCP from Btk, Btk, and B. cereus. Unlike B. cereus (and Bti and Btk), neither B. subtilis nor E. coli grew well in human culture medium (DMEM), with or without human cells present. The B. subtilis results are similar to the minimal effects seen when Vero cells (from kidney of African green monkey) were treated 2 hr with culture supernatants from B. subtilis isolated from Lancashire cotton mills (48). Aside from target cell binding, the in vitro results with nonpathogenic E. coli are in contrast to those from E. coli strains classed as enterohemorrhagic (e.g., serogroup O157:H7), enteroinvasive (029:NM), or enterotoxigenic (C845), which showed microvillar destruction (effacement), erythrocyte agglutination, and actin rearrangements or depolymerization (27,49).

Similar effects were observed when we used VCP from Btk, Bti, and B. cereus sources and also when others used 20-fold concentrated VCP (culture filtrate) of Bacteroides fragilis produced in brain-heart infusion medium for 48 hr at 37°C in 1-hr assays with HT29 (C1 clone line) (33). Also, screening assays for B. cereus diarrheal enterotoxin with culture filtrates (VCP) from over 30 isolates revealed gross morphology changes such as monolayer disruption and cell shrinkage in assays with M cCo y cells (unknown mouse tissue) and Vero cells, progressing over a period of 24 hr (19,50). In a more recent assay involving Chinese hamster ovary cells to assess toxicity of 18-hr culture filtrates from several different Bacillus species, including a putative B. thuringiensis strain (isolated from cheese and raw milk), Beattie and Williams (51) showed a 90% loss in bioreduction activity by 72 hr. Compared to these earlier assays that used considerably longer exposure times (overnight to days), the VCPs from Btk and Bti and also from the B. cereus strain that we tested were apparently very toxic. Further side-by-side experiments are needed to survey the toxic constituent(s) of various B. cereus strains in relation to various B. thuringiensis subspecies and the classical view of B. cereus enterotoxins. In related work, we detected enterotoxin gene sequences in the DNA from spores of all BT products, and showed that VCP contains an immunologically related component which uses two different commercially available Bacillus enterotoxin test kits (13,35).

Possible candidates for the B. thuringiensis VCP cytotoxin. Compared to δ-endotoxin, much less is known about other toxic substances that may contribute to B. thuringiensis toxicity, specifically components from the fermentation stage of the BT production process. Our experiments demonstrate that the most toxic substance(s) is a proteinaceous, thermolabile product common to Bti, Btk, and B. cereus cells. Early toxic effects included rapid loss in reductive capacity and protein synthesis. These effects were reversible only by rapidly replacing the VCP with fresh medium within the first 5 min of exposure; by 10 min of exposure, the toxic effects resulted in cell detachment, lysis, and internal protein degradation (35). Possible candidates considered so far are ADP-ribosylating toxins, B. cereus-like enterotoxins, phosphatidylinositol-specific phospholipase C (PI-PLC), and vegetative insecticidal proteins (52). The 35S-methionine experiments clearly show that an early step in toxicification is the cessation of protein synthesis, which is similar to that seen with other human (and animal) cells with ADP-ribosylating toxins of Corynebacterium diptheria and Vibrio cholera, exotoxin A of Pseudomonas aeruginosa, Shiga toxin of Shigella flexneri, and the Shiga-like toxin of E. coli (53). These ribosylating toxins transfer the ADP-ribose moiety to eukaryotic host target proteins, such as elongation factor 2 α,
to render them inactive (54). Our most recent studies indicate that a 45 kDa constituent of the VCP can be covalently labeled using 32P-NAD as substrate (35), a characteristic of some ADP-ribosylating toxins. In other studies we tested B. thuringiensis PL-PC and concluded that the lytic effects are likely the result of another lipase (52).

Implications for Human Health

Before our studies, a handful of case reports described skin irritation and infections after spray applications (55–57). The literature also indicates complications in immunologically impaired individuals linked to exposure to B. thuringiensis' organisms (15,17,20,55–57). Further, B. cereus-type ailments can be confused with B. thuringiensis induced poisoning because B. thuringiensis is routinely harvested from common foods such as milk, pasta, and bread (14,20,57). More recently there has been a well-documented case report of B. thuringiensis mediated soft tissue infection and necrosis, along with experimental evidence of pathogenicity, in both immune compromised and, more importantly, normal mice (17,18). The results presented here show for the first time that, at the human cell level, both Bti and Btk BT products can generate potent B. cereus-like toxic effects. To go beyond the scale seen in BT product immunologic sensitization reactions of field workers (3), a sustained infection would be needed to generate sufficient amounts of vegetative cells and their cytolytic exoproducts. What is lacking is a critical understanding of conditions that might concern high-risk groups, those unable to manage microbe invasions through impaired immune responses and other physical–chemical clearance mechanisms manifested during development (the very young, the elderly) and in specific genetic mechanisms manifested during development (the very young, the elderly) and in specific genetic

References and Notes

1. Beegle CC, Yamamoto T. Invitation paper (C.P. Alexander fund): history of Bacillus thuringiensis Berliner Research and Development. Can Entomol 124:587–612 (1992).
2. Seligy VL, Beggs RW, Rancourt J, Yao, T. Bacillus subtilis: a review. J Microbiol Methods 3:69–76 (1984).
3. Bernstein L, Bernstein J, Miller M, Terzieva S, Bernstein DM, Lunnas Z, Seligy VL, Impulse reactions in farm workers after exposure to Bacillus thuringiensis pesticides. Environ Health Perspect 107:357–362 (1999).
4. Tayabali AF, Seligy VL, Semiautomated quantification of cytotoxicity and protein-damage inducing in cultured insect cells exposed to commercial Bacillus thuringiensis biopesticides. J Appl Toxicol 15:365–373 (1995).
5. Seligy VL, Rancourt J, Antibiotic Micro BCM analysis of Bacillus-based commercial insecticides: use of bioreduction and DNA-based assays. J Ind Microbiol Biotechnol 22:565–574 (1999).
6. Cannon R, Bacillus thuringiensis use in agriculture: a molecular perspective. J Environ Saf 33:155–162 (1997).
7. Tayabali AF, Seligy VL, Cell integrity markers for in vitro evaluation of cytotoxic responses to bacteria-containing commercial insecticides. Environ Toxicol 36:109–113 (1999).
8. Gill SS, Hornung M, Cytoytic activity of Bacillus thuringiensis proteins to insect and mammalian cell lines. J Invertebr Pathol 50:16–25 (1987).
9. Smith GP, Merrick JD, Bone EJ, Ellar DJ. Mosquitocidal activity of Cry1C endotoxin from Bacillus thuringiensis ssp. azleai. Appl Environ Microbiol 62:680–684 (1996).
10. Thomas WE, Ellar DJ. Bacillus thuringiensis var. israelensis crystal endotoxin: effects on insect and mammalian cells in vitro and in vivo. J Cell Sci (60):181–189 (1983).
11. Vacch V, Paradis J, Marsolais M, Schwartz J-L, Laprade R. Ionic permeabilities induced by Bacillus thuringiensis in Sf9 cells. J Membr Biol 148:37–63 (1995).
12. Huang H, Edqvist E, Dikshit DA, J ohan H, A, Fouet A, M och, H, Legna J, KoltA. Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis— one of species on the basis of genetic evidence. Appl Environ Microbiol 66:2671–2675 (2000).
13. Seligy VL, Douglas GR, Rancourt J, Tayabali AF, Otvos I, van Frankenhuyzen K, Dugaj J, Rousseau G, Szabo AG. Comparative performance of conventional and molecular dosimetry methods in environmental bio-monitoring assessment using Bacillus-based commercial biopesticides as models. In: Rapid Methods for the Analysis of Biological Materials in the Environment (Stopa P), Bacteroida: Bacterial Adhesion, Mar S, Ed. Dordrecht, Netherlands: Kluwer Academic Publishers, 2000:279–297.
14. Dampaard PH, Larsen HD, Hansen BW, Bresciani J, Jenngers K. Enterotoxir-producing strains of Bacillus thuringiensis isolated from food. Lett Appl Microbiol 23:146–150 (1996).
15. Dampaard PH, Granum PE, Bresciani J, Torregrossa MV, Joergensen K. Enterotoxin-producing strains of Bacillus thuringiensis isolated from food. Lett Appl Microbiol 23:146–150 (1996).
16. Hernandez E, Ramisse F, Cruel T, Cavallo JD. Bacillus thuringiensis subsp. konkukian (serotype H34 isolated from milk, pasta, and bread (14,20,57). More recently there has been a well-documented case report of B. thuringiensis mediated soft tissue infection and necrosis, along with experimental evidence of pathogenicity, in both immune compromised and, more importantly, normal mice (17,18). The results presented here show for the first time that, at the human cell level, both Bti and Btk BT products can generate potent B. cereus-like toxic effects. To go beyond the scale seen in BT product immunologic sensitization reactions of field workers (3), a sustained infection would be needed to generate sufficient amounts of vegetative cells and their cytolytic exoproducts. What is lacking is a critical understanding of conditions that might concern high-risk groups, those unable to manage microbe invasions through impaired immune responses and other physical–chemical clearance mechanisms manifested during development (the very young, the elderly) and in specific genetic disorders (e.g., cystic fibrosis). To justify urban usage of spore-containing BT products, earlier claims of no health effects need to be addressed in terms of current medical views and practices (3,13). This includes testing health effects of vegetative cell exoproducts such as CryW (58) and Vip3A (59), which are proposed for use as novel insecticides.
killing action on human cancer cells. J Appl Microbiol 86:477–486 (1999).
47. Berridge MV, Tan AS, McCoy KD, Wang R. The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. Biochemica 4:14–19 (1996).
48. Hoult B, Tuxford AF. Toxic production by Bacillus pumilus. J Clin Pathol 44:445–458 (1991).
49. Steiner TS, Lima AA, Nataro J P, Guerrant RL. Enteric-aggregative Escherichia coli produce intestinal inflammation and growth impairment and cause interleukin-8 release from intestinal epithelial cells. J Infect Dis 177:88–96 (1998).
50. Hostacka A, Kosiarova A, Majtan V, Kohutova S. Toxic properties of Bacillus cereus strains isolated from different foodstuffs. Zbl Bakt Int Med M 276:303–312 (1992).
51. Beattie SH, Williams AG. Detection of toxigenic strains of Bacillus cereus and other Bacillus spp. with an improved cytotoxicity assay. Lett Appl Microbiol 28:221–225 (1999).
52. Tayabali AF, Beggs RW, Rancourt J M, Seligy VL. PI-PLC-like activity in cultures of human and insect cells exposed to commercial Bacillus thuringiensis-based products. Cell Mol Biol 42:563 (1996).
53. Menestrina G. Electrophysiological methods for the study of toxin-membrane interaction. In: Sourcebook of Bacterial Protein Toxins (Alouf JE, Freer JH, eds). London: Academic Press Ltd., 1991;215–276.
54. Krueger KM, Barbieri J T. The family of bacterial ADP-ribosylating exotoxins. Clin Microbiol Rev 8:34–47 (1995).
55. Bender C, Peck S. Health symptoms reported during BTK spraying spring 1994 in the capital regional district. Environ Health Rev 40:42–44 (1996).
56. Noble MA, Riben PD, Cook GJ. Microbiological and Epidemiological Surveillance Programme to Monitor the Health Effects of Foray 48B BTK Spray. Victoria, British Columbia, Canada: Ministry of Forests, Province of British Columbia, 1992.
57. Samples J R, Buettner H. Corneal ulcer caused by a biological insecticide (Bacillus thuringiensis). Am J Ophthalmol 95:258–260 (1983).
58. Estruch JJ, Warren GW, Mullins MA, Nye GJ, Craig JA, Koziel MG. Vip3A, a novel Bacillus thuringiensis vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc Natl Acad Sci USA 93:5389–5394 (1996).
59. Kostichka K, Warren GW, Mullins M, Mullins AD, Craig JA, Koziel MG, Estruch JJ. Cloning of a cryV-type insecticidal protein gene from Bacillus thuringiensis: the cryV-encoded protein is expressed early in stationary phase. J Bacteriol 178:2141–2144 (1996).