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

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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^{-14}% BT product/target cell) equated to a single spore and a target cell half-life (tLD_{50}) of approximately 16 hr. At Dts > 10^4, the tLD_{50} rapidly shifted to < 4 hr; with antibiotic present, no component, including PIB-related δ-endotoxins, was cytolytic up to an equivalent of approximately 10^9 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 indicate that spore-containing BT products have an inherent capacity to lyse human cells in free and 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 microbe-clearance capacities and/or immune response systems. *Key words*: biopesticides, bioreduction, cell death, cytolytic factors, endotoxin, immunodetection, immune sensitization, ^{35}S-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 (Dipterans) 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, biorationale 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 amorphic structures (*2*). This proteinaceous material cannot be quantitatively separated from spores and other culture debris. The regular variably 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, amorphic, 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-lactamase, 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 underinvestigated either as bona fide components of "active ingredient"

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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 (tLD_{50}), was entirely consistent with estimates of pest mortality derived in field and laboratory tests with larvae (*7*). Because the fastest response time, measured in terms of tLD_{50}, was < 4 hr at 37 ± 3°C (the optimum *Btk* or *Bti* spore outgrowth temperature) and because 5–20% of these spores remained viable, even under the harshest pH and temperature 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* organisms and *B.cereus* and *B.anthracis* pathogens (*12,13*) reports 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 sensitivity 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 bioindicator 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 parlance of chemical toxicants. In this context, the BT product ingredients are transformed either through proteolysis of the proteinaceous moieties, particularly the pro-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 sporulation-phase activity. In reference to *Bti/Btk* vegetative cell exoproteins (VCPs) we also investigated their cytotoxic properties using ^{35}S-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 T80 flasks (Life Technologies, Burlington, Ontario, Canada). Treatments with BT products and their subfractions were conducted in 6-, 48-, and 96-well plates at 37°C. Effects of dose, head volume over monolayers, and apical–basal surface exposure were assessed using 6- and 12-well culture plates with porous membranes for transfeeding (0.22 µm pore size; Costar, Cambridge, MA). Monolayers (2×10^6 cells/cm^2) 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; Tousimis Inc., Rockville, MD, USA), sputter-coated with 10 nm gold, and viewed with a JEOL JSM6400 scanning electron microscope (JEOL USA, Peabody, MA, USA) operating at 10 kV.

BT products and subfractions. We used primarily the BT products F48B (*Btk* strain HD1) and VB12AS (*Bti* strain HD14); 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 ($12,000 \times g$ for 10 min at RT). Particulate-free filtrates (PFF) 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 ($80,000 \times g$ for 14 hr at 4°C, using a Beckman SW50.1; 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 H_2O 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 90-07SV), *B. subtilis* (ATCC 6051; lot 91-11SV), 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% (v/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 inclubated 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 0.0001% w/v) for various time intervals at 37°C. For control samples we used Grace's insect cell medium or DMEM treated in the same manner as the VCP. We used a series of molecular mass cutoff membranes (Centricon 10–100 kDa; Amicon, Beverly, MA, USA) and gel filtration (Sephadex G-150,100,50; Amersham Pharmacia Biotech, Baie d'Urfè, Quebec, Canada) to approximate the dynamic size and homogeneity of the VCP toxic constituent(s).

Bioindicator 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; Monosan, Am Uden, 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 ^{35}S-methionine (8.5 µCi/L 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 radiosensitive fluor (EnHance; Dupont, Mississagua, 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 assaying cells in suspension culture and assaying cells configured 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 cells and 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^{-14} 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^{-7}% BT product/target cell or ~ 10^7 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^{-7}% 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

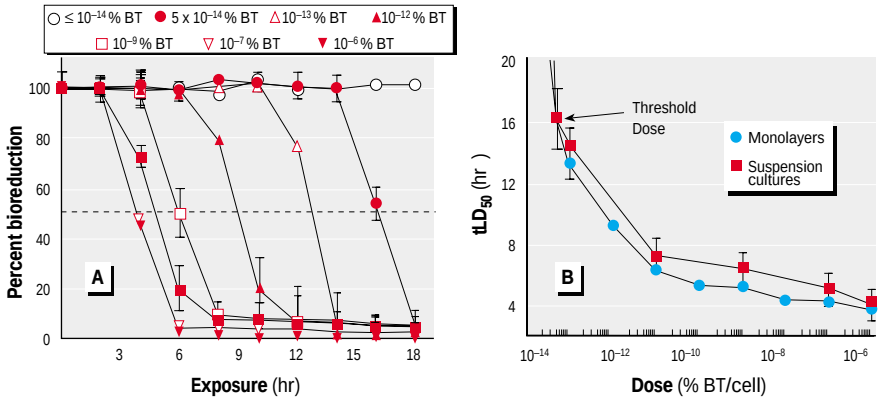

Figure 1. Changes in human cell bioreduction activity after exposure to commercial BT products. (*A*) Time course of HT29 cell exposure to various concentrations of BT product per target cell. Bioreduction was assayed as described in "Materials and Methods." The dashed line indicates a 50% loss of the cell population's bioreduction activity. Data for 10^{-11}, 10^{-10}, and 10^{-8}% BT product doses are not shown. Data points for *Bti* and *Btk* -BT products completely overlapped and are the means of at least three separate determinations ± SD. In the presence of antibiotic (gentamicin), all BT doses gave results equivalent to 10^{-14}% BT product/target cell. (*B*) Average dose response (tLD_{50}) values computed from 120 exposures of *Bti* and *Btk* BT products using various human cell types propagated as monolayers or suspension cultures (see "Materials and Methods" for details).

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 *Cyt*A 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

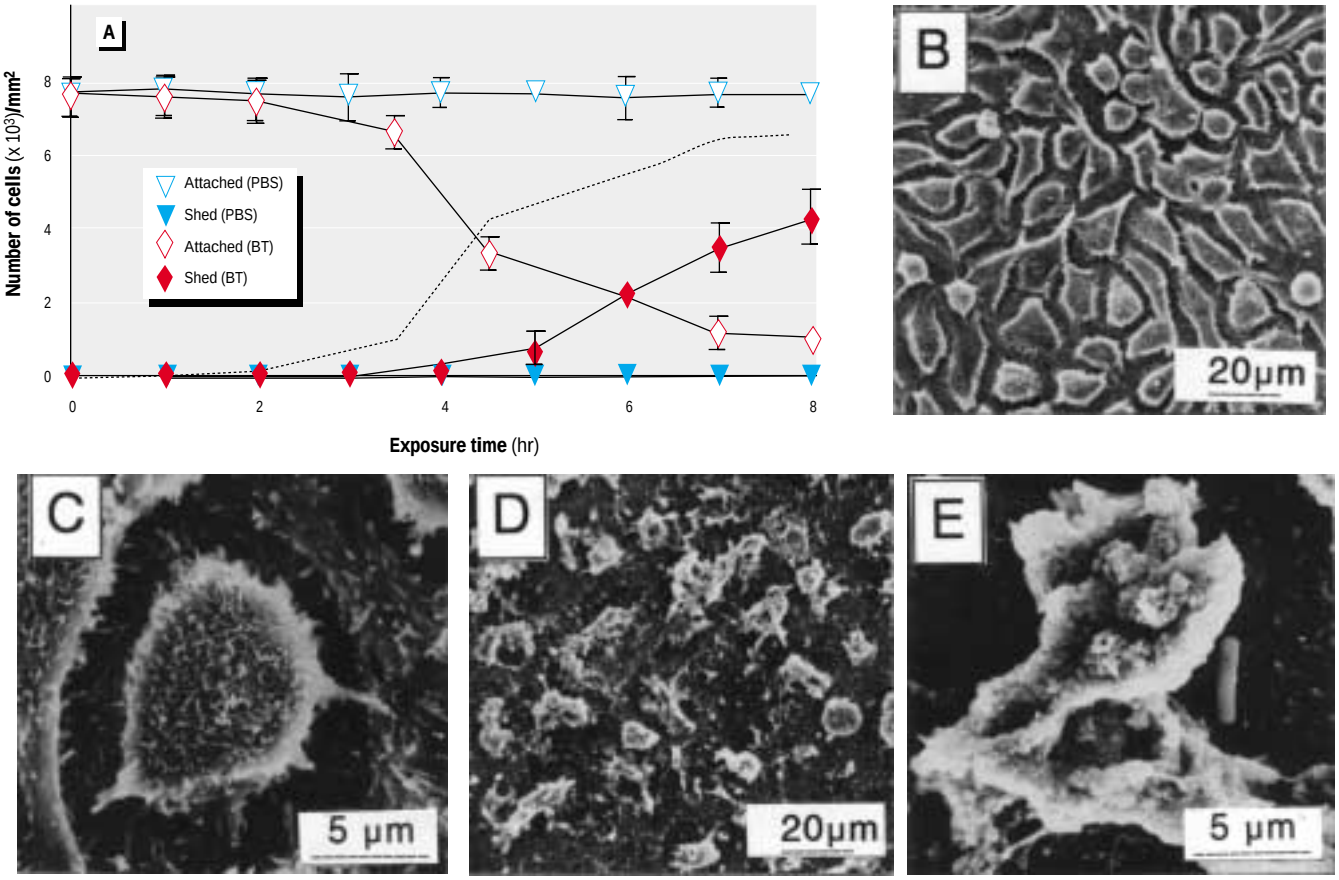

Figure 2. Changes in HT29 cell number and morphology during exposure to BT products. (*A*) The time course shows the number of attached and shed cells during exposure to PBS or 10^{-7}% BT product/target cell. The half-life (tLD_{50}), based on time required for 50% loss of the attached cells was 4.4 ± 0.3 hr. The dotted line represents the expected number of shed cells based on controls and remaining attached cells at each time point. (*B–E*) Scanning electron micrographs of HT29 cells exposed for 6 hr to either PBS (B, C) or BT product (D, E).

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 (67kDa 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 also contained spore-related products ~ 5–30 kDa and >200 kDa in size. The filtered supernatant of solubilized *Bti* fraction 3 (Figure 5C, lane 6) had both 22 and 27 kDa polypeptides, and the corresponding 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 100 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

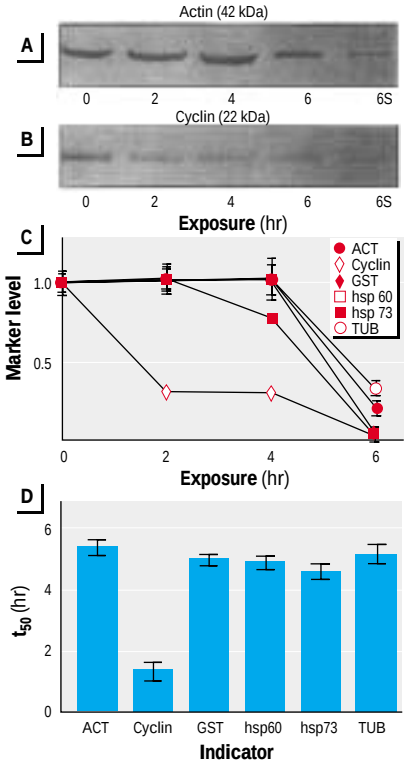

Figure 3. Changes in specific proteins during HT29 exposure to BT products. Example immunoblot assays for actin (*A*) and cyclin (*B*) after exposure to 10^{-7}% BT product/target cell (see "Materials and Methods"). Lane 6S shows only trace levels of actin and cyclin in shed cells at 6hr. (*C* and *D*)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

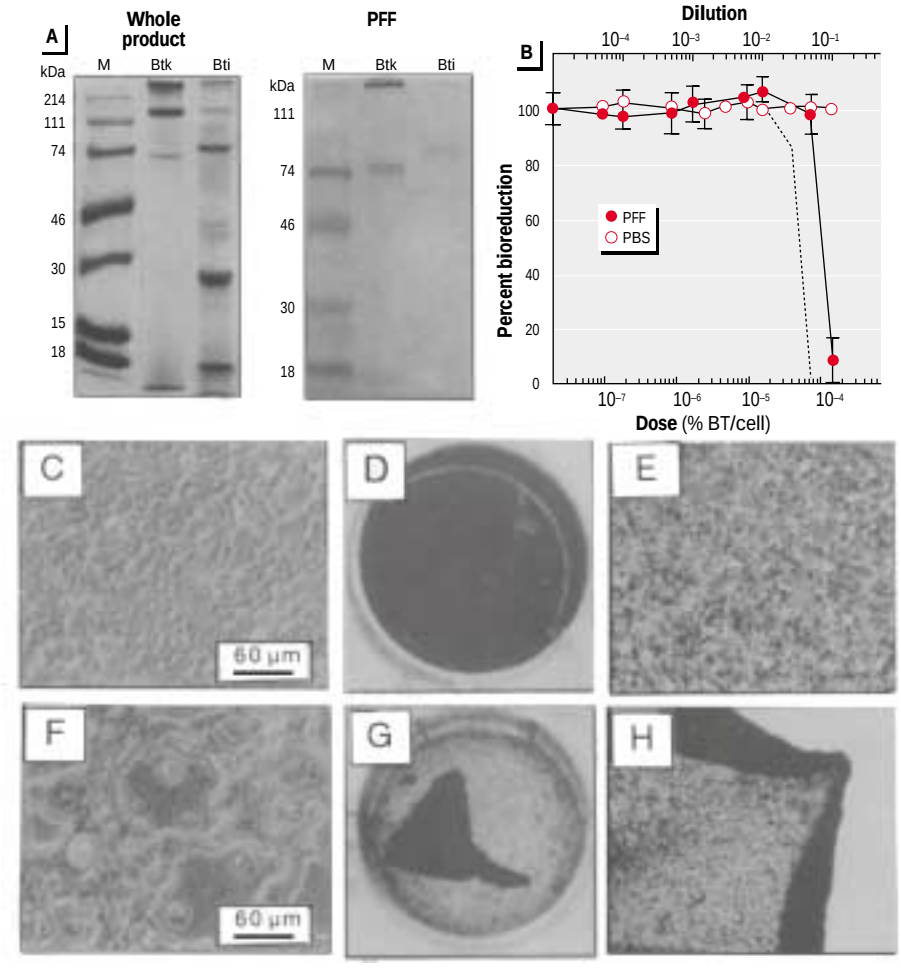

Figure 4. Protein content and toxicity of BT product particulate-free supernatant fractions. (*A*) Coomassie-stained SDS-PAGE gels of undiluted *Btk* and *Bti* BT products (4% final) and corresponding (PFFs concentrated to 10X (10 µL/lane). (*B*) Bioreduction assay of HT29 cells exposed 24 hr to PBS 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,E,F*) or *Bti* (*F,G,H*) products at a dose equivalent to 10^{-4}% BT product/target cell. Comparison of 1× (*D,G*) and 15× (*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 tLD_{50} values of 6–8 min (Figure 7E). Based on the number of vegetative cells present at 8

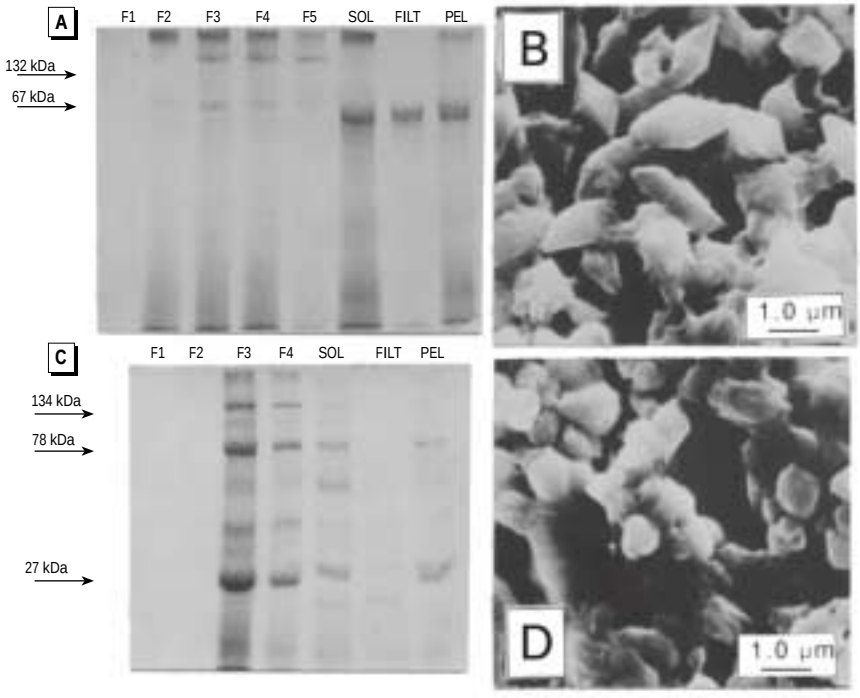

Figure 5. Properties of PIB enrichments and their solubilized derivatives. SDS-PAGE analysis of PIB enrichments obtained by sucrose gradient centrifugation using (*A*) *Btk* and resultant fractions (lanes F1–F5) and (*C*) *Bti* BT products and resultant fractions (lanes F1–F4). Fractions were trypsin 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.

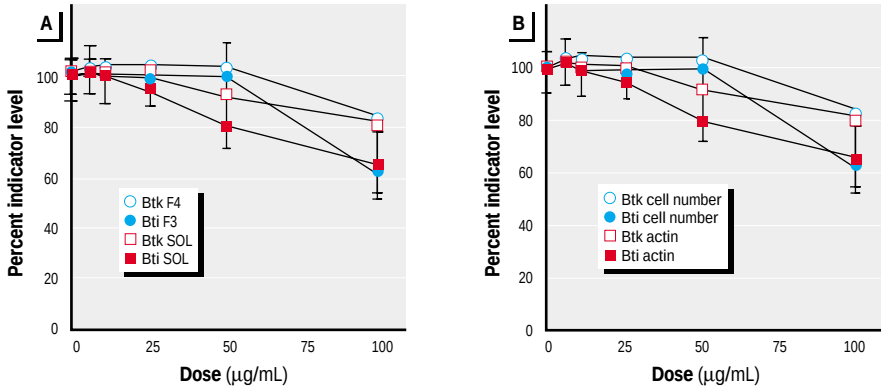

Figure 6. Cytotoxicity of PIBs and solubilized derivatives. (*A*) Changes in HT29 cell bioreduction activity after exposures to dilutions of unsolubilized fractions (F) and their trypsin-solubilized (SOL) filtrates. In all cases, the exposures were for 4 hr at 37°C with antibiotic added as a precaution 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 1.4×10^{-7}% BT product (spores)/target cell. In parallel assays with 0.5X VCP, the average tLD_{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 cytolysis and an inconsistent loss of protein indicators (Figure 7F). In all of these assays, *Bti* 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 1.4×10^{-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 tLD_{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 matricies (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") (*34*). Neither *Btk* nor *Bti* VCP activity was affected by repeated freeze–thaw cycles (five 10-min cycles of –80°C to 3°C) and storage up to 3 years at –80°C or –20°C. However, the half-life of VCP activity decreased from 40 hr to 24 hr when samples were pretreated at 23°C and 50°C, respectively, and to ≤ 5 min at 60°C. This inactivation was nonreversible and was similar to a pretreatment with a broad-spectrum protease (100 µg/mL proteinase K) for < 2 hr at 37°C. However, VCP activity was not affected by trypsin (0.025–0.25%, w/v), serine protease inhibitors, a chelating agent (0.1–10 mM EDTA), and agents such as cholesterol (60 µg/mL),

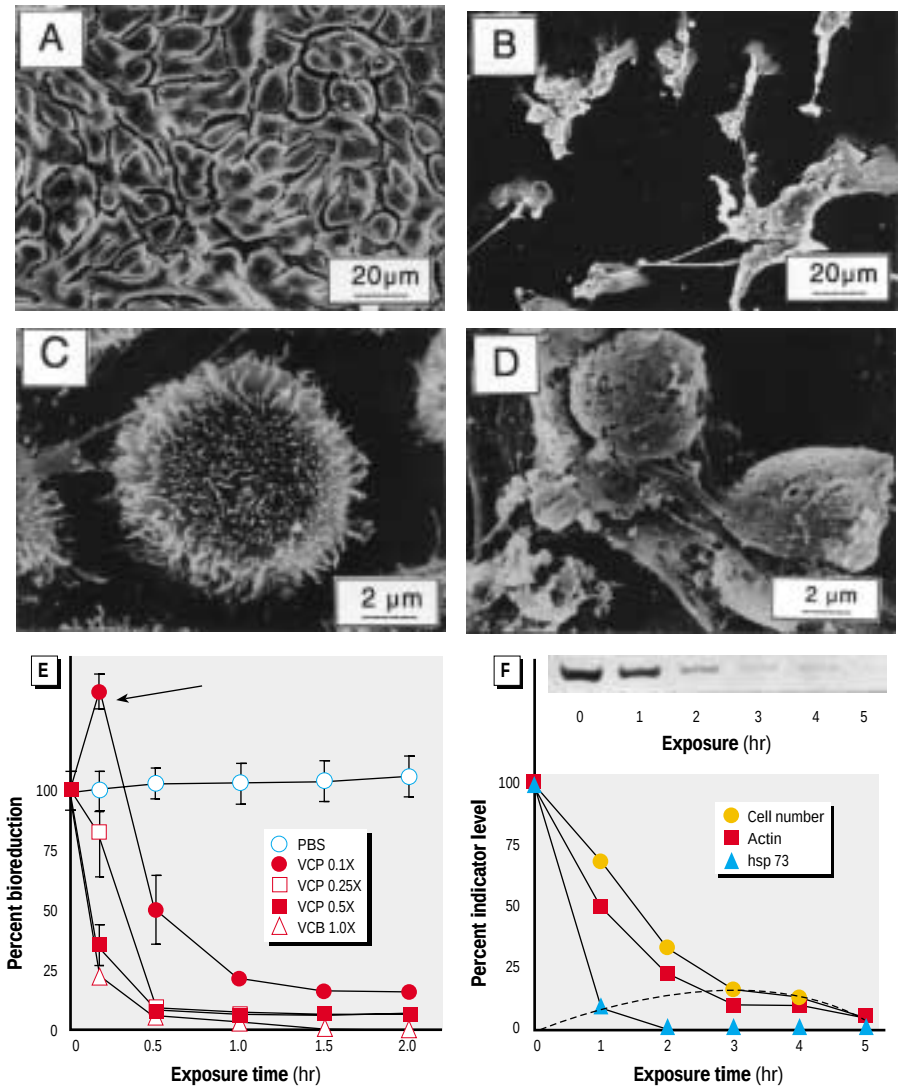

Figure 7. Cytotoxicity of VCP. HT29 cells were exposed 60 min to either PBS (*A,C*) or VCP (*B,D*) derived from a 6-hr outgrowth of *Btk* BT product (see "Materials and Methods"). The VCP concentration (1.0X) was equivalent to the output of one vegetative cell/target cell or approximately 3.5×10^{-7}% BT product/target cell. (*E*) Changes in bioreduction activity during the time-course exposure to PBS or VCP diluted to 0.1X, 0.25X, 0.5X, or 1.0X of the stock dose. These data were adjusted (subtracted) to take into account contributions from *Bt*-related exoreductase 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 hsp73 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 bioreduction 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 cell activity. After pooling and concentrating the VCPs from several experiments, we found that levels of some target cell bioindicators were greatly reduced, but only in the case of *B. cereus* VCP (Figure 9B, C).

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.

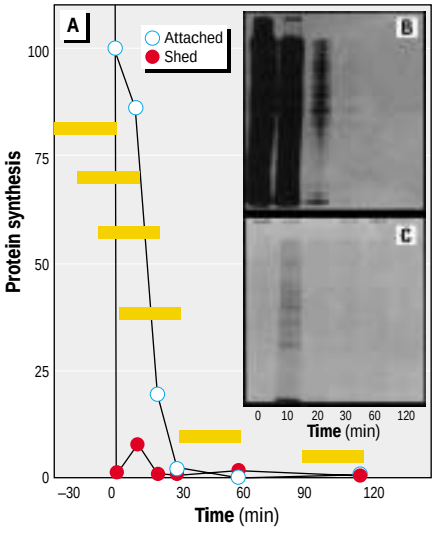

Figure 8. Effects of VCP exposure on ^{35}S-methionine-labeling of HT29 polypeptides. (*A*) Assays involved incubation with ^{35}S-methionine for 30-min intervals (indicated by shaded bars) before and during exposure to *Btk* VCP stock made equivalent by dilution to 3.5×10^{-7}% BT product/target cell. Total protein synthesized by cells that were attached (*B*) and shed (*C*) during VCP exposure was analysed by SDS-PAGE. Distribution of ^{35}S-label was detected by autoradiography (see "Materials and Methods").

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^{13}/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 septicaemia to take place as for 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 types 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, hardiness, 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 aerial 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}% BT product/cm^2 or 10^{-10}% 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 or the NOAEL was shifted upward by a factor of ≥ 10^9, which indicates that the most damage-causing agent(s) arises from viable spores within BT products. These results cannot be explained by contamination of these BT products with unrelated organisms (*5*,*7*).

The differential response between low and high doses of BT products (see summary Figure 1B) or of subcomponents with equivalent amounts of spores provides clues as to the mechanism of toxification at work. With low concentrations of spores, the lag time in toxic response per dose was similar to the regular shift (~ 40 min) in occurrence of early-to-middle vegetative cell production for each $\log_{10}$ dilution step of *Btk* or *Bti* BT product at 37°C (*2*). This indicates that at low doses (< 10^{-12}% BT product/target cell) the inherent number of spores, on conversion to vegetative cells, must produce several generations of cells in order to build up the critical amounts of toxic ingredient(s) necessary to kill ≥ 50% of the target cells present. However, with BT product doses in or above the optimized range used for aerial spray applications, the response times were essentially clustered (tLD_{50} = 3.9–4.8 hr), indicating that the number of spores is already near or may exceed the number of vegetative cells needed (on conversion) to generate sufficient cytotoxic product(s). Our rough estimates indicate that the minimum number of vegetative cells required to produce a cellular LD_{50} would be ≤ 10% of the target cell concentration or approximately 10^5 vegetative cells/mL of nutrient medium. In a nonmammalian environment, temperatures below the optimum growth range of 34–37°C would retard the rate of vegetative cell amplification (septicaemia) and buildup of lytic damage, thus significantly delaying uniformity of toxic response and death in insect target organisms (*2,7,13,38*).

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 cry1Ab 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 Dt of ≥ 10^{10}, 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*). Because of their actual concentration in spray droplets, these fermentation residues may play a role in the variation of product efficacy and potential health effects (*3,13*).

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 crystallike 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 pro-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 crystal-like 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 cytolysis 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 product (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 tLD_{50} of approximately 130–140 µg/mL. This dose corresponds to approximately 2×10^{-5}% BT product/target cell or ≥ 4×10^8

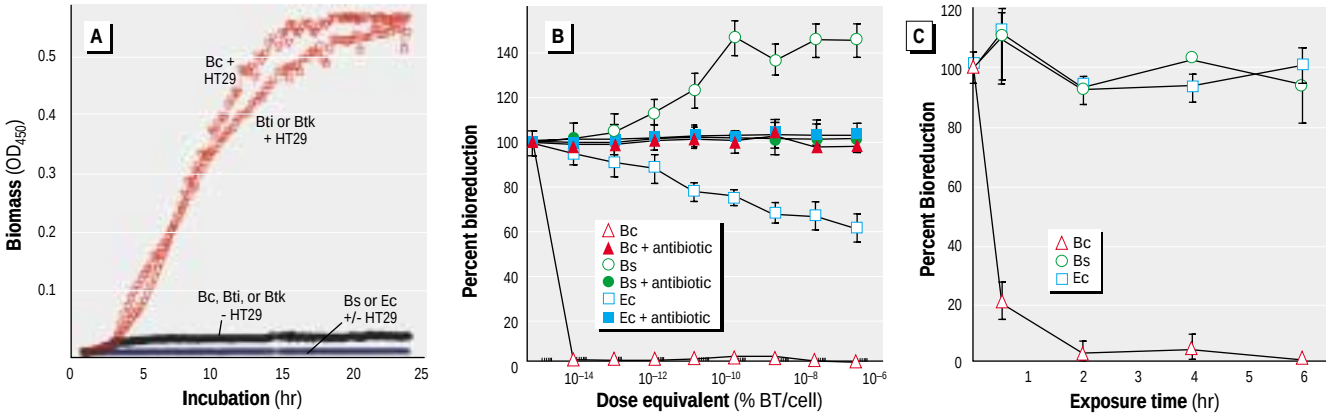

Figure 9. Toxic effects of *B. subtilis, E. coli,* and *B. cereus* and their VCPs. (*A*) Growth of *B. cereus* (Bc), *B. subtilis* (Bs), *E. coli* (Ec), and *B. thuringiensis* (*Btk* or *Bti*) incubated at 37°C in DMEM without phenol red and antibiotic and in the presence (+) or absence (-) of HT29 cells. Changes in optical density (OD; 450nm) caused by vegetative cell production (biomass) were automatically recorded every 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 *Bt* VCP at a dose of 3.5×10^{-7}% BT product/target cell, based on total protein concentration.

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 toxification 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 carry-over 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 (LD_{50} = 10–80 µg/mL in 30 min) and human erythrocytes (LD_{50} = 1–2 µg/mL in 60 min), but only if they were pretreated 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 LD_{50} 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 distantly 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 *Bti, 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 enterohemorragic (e.g., serogroup O157:H7), enteroinvasive (029:NM), or enterotoxigenic (C1845), 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 *Bacteriodes 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 McCoy 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 work, 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, phophatidylinositol-specific phospholipase C (PI-PLC), and vegetative insecticidal proteins (*52*). The ^{35}S-methionine experiments clearly show that an early step in toxification 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 flexnuri,* 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 ^{32}P-NAD as substrate (*35*), a characteristic of some ADP-ribosylating toxins. In other studies we tested *B. thuringiensis* PI-PLC 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 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 CryV (*58*) and Vip3A (*59*), which are proposed for use as novel insecticides.

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 JM, Tayabali AF. Quantitative bioreduction assays for calibrating spore content and viability of commercial *Bacillus thuringiensis* insecticides. J Ind Microbiol Biotechnol 18:370–378 (1997).
3. Bernstein IL, Bernstein JA, Miller M, Terzieva S, Bernstein DI, Lummus Z, Selgrade MJ, Doerfler D, Seligy VL. Immune responses in farm workers after exposure to *Bacillus thuringiensis* pesticides. Environ Health Perspect 107:575–582 (1999).
4. Tayabali AF, Seligy VL. Semiautomated quantification of cytotoxic damage induced in cultured insect cells exposed to commercial *Bacillus thuringiensis* biopesticides. J Appl Toxicol 15:365–373 (1995).
5. Seligy VL, Rancourt JM. Antibiotic MIC/MBC analysis of *Bacillus*-based commercial insecticides: use of bioreduction and DNA-based assays. J Ind Microbiol Biotechnol 22:565–574 (1999).
6. Cannon RJC. *Bacillus thuringiensis* use in agriculture: a molecular perspective. Biol Rev 71:561–636 (1996).
7. Tayabali AF, Seligy VL. Cell integrity markers for *in vitro* evaluation of cytotoxic responses to bacteria-containing commercial insecticides. Ecotoxicol Environ Saf 37:152–162 (1997).
8. Gill SS, Hornung JM. Cytolytic 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 CryIC δ-endotoxin from *Bacillus thuringiensis* subsp. *aizawai*. 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 60:181–197 (1983).
11. Vachon V, Paradis J, Marsolais M, Schwartz J-L, Laprade R. Ionic permeabilities induced by *Bacillus thuringiensis* in Sf9 cells. J Membr Biol 148:57–63 (1995).
12. Helgason E, Okstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, Hegna I, Kolstø AB. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl Environ Microbiol 66(6):2627–2630 (2000).
13. Seligy VL, Douglas GR, Rancourt JM, Tayabali AF, Otvos I, van Frankenhuyzen K, Dugal J, Rousseau G, Szabo AG. Comparative performance of conventional and molecular dosimetry methods in environmental biomonitoring: assessment using *Bacillus*-based commercial biopesticides as models. In. Rapid Methods for the Analysis of Biological Materials in the Environment (Stopa PJ, Bartoszcze MA, eds). NATO ASI Series. Dordrecht, Netherlands:Kluwer Academic Publishers, 2000;279–297.
14. Damgaard PH, Larsen HD, Hansen BW, Bresciani J, Jorgensen K. Enterotoxin-producing strains of *Bacillus thuringiensis* isolated from food. Lett Appl Microbiol 23:146–150 (1996).
15. Damgaard PH, Granum PE, Bresciani J, Torregrossa MV, Eilenberg J, Valentino L. Characterization of *Bacillus thuringiensis* isolated from infections in burn wounds. FEMS Immunol Med Microbiol 18:47– 53 (1997).
16. Green M, Heumann M, Sokolow R, Foster LR, Bryant R, Skeels M. Public health implications of the microbial pesticide *Bacillus thuringiensis*: an epidemiological study, Oregon, 1985–86. Am J Public Health 80:848–852 (1990).
17. Hernandez E, Ramisse F, Ducoureau JP, Cruel T, Cavallo JD. *Bacillus thuringiensis* subsp. *konkukian* (serotype H34) superinfection: case report and experimental evidence of pathogenicity in immunosuppressed mice. J Clin Microbiol 36:2138–2139 (1998).
18. Hernandez E, Ramisse F, Cruel T, le Vagueresse R, Cavallo JD. *Bacillus thuringiensis* serotype H34 isolated from human and insecticidal strains serotypes a, b and H14 can lead to death of immunocompetent mice after pulmonary infection. FEMS Immunol Med Microbiol 24:43–47 (1999).
19. Jackson SG. Rapid screening test for enterotoxin-producing *Bacillus cereus*. J Clin Microbiol 31:972–974 (1993).
20. Jackson SG, Goodbrand RB, Ahmed R, Kasatiya S. *Bacillus cereus* and *Bacillus thuringiensis* isolated in a gastroenteritis outbreak investigation. Lett Appl Microbiol 21:103–105 (1995).
21. Andersson A, Granum PE, Ronner U. The adhesion of *Bacillus cereus* spores to epithelial cells might be an additional virulence mechanism. Int J Food Microbiol 39:93–99 (1998).
22. Bernet-Camard MF, Coconnier MH, Hudault S, Servin AL. Differential expression of complement proteins and regulatory decay accelerating factor in relation to differentiation of cultured human colon adenocarcinoma cell lines. Gut 38:248–253 (1996).
23. Braun L, Ohayon H, Cossart P. The InIB protein of *Listeria monocytogenes* is sufficient to promote entry into mammalian cells. Mol Microbiol 27:1077–1087 (1998).
24. Cersini A, Salvia AM, Bernardini ML. Intracellular multiplication and virulence of *Shigella flexneri* auxotrophic mutants. Infect Immun 66:549–557 (1998).
25. Coconnier MH, Klaenhammer TR, Kerneis S, Bernet MF, Servin AL. Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell-lines in culture. Appl Environ Microbiol 58:2034–2039 (1992).
26. Gaillard J-L, Finlay BB. Effect of cell polarization and differentiation on entry of *Listeria monocytogenes* into the enterocyte-like Caco-2 cell line. Infect Immun 64:1299–1308 (1996).
27. Jung HC, Eckmann L, Yang S-K, Panja A, Fierer J, Morzycka-Wroblewska E, Kagnoff MF. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J Clin Invest 95:55–65 (1995).
28. Kerneis S, Chauviere G, Darfeuille-Michaud A, Aubel D, Coconnier MH, Joly B, Servin AL. Expression of receptors for enterotoxigenic *Escherichia coli* during enterocytic differentiation of human polarized intestinal epithelial cells in culture. Infect Immun 60:2572–2580 (1992).
29. Lu X, Walker T, MacManus JP, Seligy VL. Differentiation of HT-29 human colonic adenocarcinoma cells correlates with increased expression of mitochondrial RNA: effects of trehalose on cell growth and maturation. Cancer Res 52:3718–3725 (1992).
30. Lu X, Seligy VL. Mitochondrial RNA abundance in differentiating human colonic epithelial tumor cells estimated through use of a mitochondrial genome map. Gene 131:217–225 (1993).
31. Segal ED, Shon J, Tompkins LS. Characterization of *Helicobacter pylori* urease mutants. Infect Immun 60:1883–1889 (1992).
32. Seto NOL, Seligy VL. Genetically tagged putative differentiation intermediates derived undifferentiated HT29 human colon carcinoma cells. Cell Mol Biol 45:203–209 (1999).
33. Weikel CS, Grieco FD, Reuben J, Myers LL, Sack B. Human colonic epithelial cells, HT29/C1, treated with crude *Bacteriodes fragilis* enterotoxin dramatically alter their morphology. Infect Immun 60:321–327 (1992).
34. Whitehouse CA, Balbo PB, Pesci EC, Cottle DL, Mirabito PM, Pickett CL. *Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block. Infect Immun 66:1934–1940 (1998).
35. Tayabali AF, Seligy VL. Unpublished data.
36. Dubois NR. Laboratory batch production of *Bacillus thuringiensis* spores and crystals. Appl Microbiol 16:1098–1099 (1968).
37. Bryant JE, Yendol WG. Evaluation of the influence of droplet size and density of *Bacillus thuringiensis* against gypsy moth larvae (Lepdioptera: Lymantriidae). J Econ Entomol 81:130–134 (1988).
38. Payne NJ, van Frankenhuyzen K. Effect of spray droplet size and density on the efficacy of *Bacillus thuringiensis* Berliner against the spruce budworm, *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae). Can Entomologist 127:15–23 (1995).
39. Ang BJ, Nickerson KW. Purification of the protein crystal from *Bacillus thuringiensis* by zonal gradient centrifugation. Appl Environ Microbiol 36:625–626 (1978).
40. Czuba M, Tajbakhsh S, Walker T, Johnson BF, Seligy VL. Plaque assay and replication of *Tipula* iridescent virus in *Spodoptera frugiperda* ovarian cells. Res Virol 145:319–330 (1994).
41. Mahillon J, Delcour J. A convenient procedure for the preparation of highly purified parasporal inclusion bodies. J Microbiol Methods 3:69–76 (1984).
42. Sharpe ES, Herman AI, Toolan CS. Separation of spores and parasporal crystals of *Bacillus thuringiensis* by flotation. J Invertebr Pathol 34:315–316 (1979).
43. Zhu YS, Brooks A, Carlson K, Filner P. Separation of protein crystals from spores of *Bacillus thuringiensis* by Ludox gradient centrifugation. Appl Environ Microbiol 55:1279–1281 (1989).
44. Drobniewski FA, Ellar DJ. Purification and properties of a 28-kilodalton hemolytic and mosquitocidal protein toxin of *Bacillus thuringiensis* subsp. *darmstadiensis* 73-E10-2. J Bacteriol 171:3060–3067 (1989).
45. Nishiitsutsuji-Uwo J, Endo Y, Himeno M. Effects of *Bacillus thuringiensis* delta-endotoxin on insect and mammalian cells *in vitro*. Appl Entomol Zool 15:133–139 (1980).
46. Mizuki E, Ohba M, Akao T, Yamashita S, Saitoh H, Park YS. Unique activity associated with non-insecticidal *Bacillus thuringiensis* parasporal inclusions: *in vitro* cell

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. Toxin production by *Bacillus pumilus*. J Clin Pathol 44:455–458 (1991).
49. Steiner TS, Lima AA, Nataro JP, Guerrant RL. Enteroaggregative *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 J 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 JM, Seligy VL. PI-PLC-like activity in cultures of human and insect cells exposed to commercial *Bacillus thuringiensis*-based products. Cell Mol Biol 42:S63 (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 JT. 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 JR, 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).